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Kidney fibroblast growth factor 23 does not contribute to elevation of its circulating levels in uremia

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Fibroblast growth factor 23 (FGF23) secreted by osteocytes is a circulating factor essential for phosphate homeostasis. High plasma FGF23 levels are associated with cardiovascular complications and mortality. Increases of plasma FGF23 in uremia antedate high levels of phosphate, suggesting a disrupted feedback regulatory loop or an extra-skeletal source of this phosphatonin. Since induction of FGF23 expression in injured organs has been reported, we decided to examine the regulation of FGF23 gene and protein expressions in the kidney and whether kidney-derived FGF23 contributes to the high plasma levels of FGF23 in uremia. FGF23 mRNA was not detected in normal kidneys, but was clearly demonstrated in injured kidneys, already after four hours in obstructive nephropathy and at 8 weeks in the remnant kidney of 5/6 nephrectomized rats. No renal extraction was found in uremic rats in contrast to normal rats. Removal of the remnant kidney had no effect on plasma FGF23 levels. Well-known regulators of FGF23 expression in bone, such as parathyroid hormone, calcitriol, and inhibition of the FGF receptor by PD173074, had no impact on kidney expression of FGF23. Thus, the only direct contribution of the injured kidney to circulating FGF23 levels in uremia appears to be reduced renal extraction of bone-derived FGF23. Kidney-derived FGF23 does not generate high plasma FGF23 levels in uremia and is regulated differently than the corresponding regulation of FGF23 gene expression in bone.


KEYWORDS: FGF23; hyperparathyroidism; mineral metabolism; phosphate; uremia

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Patients with chronic kidney disease (CKD) develop very high plasma levels of fibroblast growth factor 23 (FGF23), an early and important component of their disturbed mineral and bone disorder (MBD).¹⁻³ A large increase in FGF23 is an important factor associated with high risk of morbidity and mortality.¹⁻² Still, the mechanisms initiating and maintaining the high plasma levels of FGF23 in CKD are poorly understood,³⁻¹¹ and excessive secretion of FGF23 by the injured kidney has been suggested.¹²,¹³

FGF23 is a 32-kDa glycoprotein that activates the fibroblast growth factor receptor (FGFR) in the presence of the required coreceptor αKlotho.¹¹,¹⁴ FGF23 decreases the type II sodium-dependent phosphate (P) cotransporters NaPi2a and NaPi2c in the proximal tubule of the kidney and thereby inhibits P resorption.¹⁵⁻¹⁸ Furthermore, FGF23 reduces 1,25-dihydroxy vitamin D (1,25vitD) levels by inhibition of the 25-(OH)vitD 1-α-hydroxylase Cyp27b1 and stimulation of the 1,25vitD 24-hydroxylase Cyp24a1.¹⁹⁻²¹ FGF23 is part of the complex endocrine network maintaining Ca²⁺ and P homeostasis, and classical negative feedback loops have been demonstrated between FGF23, 1,25vitD, and parathyroid hormone (PTH).¹⁹,²⁰,²²⁻²₃ FGF23 is also regulated by FGFR1-dependent signaling and local factors in bone.²⁴⁻²⁷

It is currently believed that osteoblasts and osteocytes are the major source of circulating FGF23.²⁸⁻³⁰ However, recent evidence has partially challenged this view because conditional ablation of FGF23 in bone cells resulted in moderate reduction in circulating FGF23 levels; therefore, other cellular sources may contribute to circulating FGF23.³¹,³² Furthermore, the FGF23 increase in uremia antedate high plasma levels of P, suggesting a disrupted feedback regulatory loop or an extraskeletal source of this phosphatonin.¹¹ The kidney has been proposed as an extraskeletal source of FGF23, because induced expression of FGF23 has recently been demonstrated in injured kidney models of CKD.¹²,¹³,³³

The purpose of the present experimental study was to examine whether kidney-derived FGF23 contributes to the high plasma levels of FGF23 in CKD. This was undertaken by studying the expression of FGF23 induced in the kidney rudiment of the 5/6 nephrectomy CKD model and in the kidney of a short-term obstructive nephropathy model. The impact of known regulators of FGF23 expression in bone, such as calcitriol, PTH, dietary P load, and FGFR signaling...
was also examined for potential impact on the regulation of FGF23 expression levels in the injured kidney.

RESULTS

Group characteristics
Long-term uremia was induced by 5/6 nephrectomy, and the uremic rats were examined after 8 weeks and compared with age-matched control rats. A subgroup of the uremic rats had their parathyroid glands removed (PTX-uremic). Biochemistry of all groups is shown in Table 1.

Induction and regulation of FGF23 expression in the kidney in uremia
The kidney rudiment of the 5/6 nephrectomy model was examined after 8 weeks of uremia. FGF23 gene expression was not detected in kidneys of control rats with normal kidney function, including control rats given calcitriol (Figure 1a). On the contrary, FGF23 mRNA was clearly detected in kidneys of all uremic rats fed a standard or high-phosphate (HP) diet. The presence of FGF23 protein was confirmed by Western blot, with significantly higher protein levels in uremic HP rats ($P < 0.05$) (Figure 1b and c). The injured kidney’s FGF23 expression was not further stimulated by calcitriol (Figure 1a). However, calcitriol affected the endogenous 1,25vitD regulation by a significant downregulation of the synthesis enzyme Cyp27b1 and upregulation of the degradation enzyme Cyp24a1 ($P < 0.05$) (Figure 2h and i). An HP diet strongly stimulated the kidney’s FGF23 expression in uremic rats ($P < 0.01$) (Figure 1a). It should, however, be noted that the HP diet also caused more severe kidney disease (Table 1). Uremic HP-diet rats had lower expression levels of the genes coding for the proteins constituting the FGF23 receptor complex FGFR1 IIIc and αKlotho and the phosphate cotransporters NaPi2a and NaPi2c ($P < 0.01$) compared with uremic standard-diet rats and control rats (Figure 2a–g and j). There was no difference in expression of these genes in uremic standard-diet rats and control rats ($P < 0.05$ calcitriol versus noncalcitriol).

Importance of PTH in the regulation of FGF23 gene expression in the kidney and bone in uremia
The effect of PTH signaling on the regulation of FGF23 gene expression in kidney and bone was examined in rats that underwent combined parathyroidectomy and 5/6 nephrectomy (PTX-uremic).35 Expression of FGF23 was induced in the kidney rudiment of PTX-uremic rats to a similar extent as in uremic rats (Figure 3a). Expression of FGF23 was found in all bone samples, where its relative expression was higher than in the injured kidney. The uremic rats had higher bone FGF23 mRNA and plasma FGF23 levels compared with levels in PTX-uremic rats ($P < 0.05$) (Figure 3b, Table 1).
coreceptor Klotho was similarly downregulated in both uremic groups (Supplementary Figure S1) but was not detected in bone (data not shown).

Plasma levels of intact FGF23 in the renal artery and vein
In order to examine whether kidney-derived FGF23 contributes to the high circulating level of FGF23 in uremia, the concentration of intact FGF23 (iFGF23) was measured in the renal artery and vein. Control rats had a renal iFGF23 extraction ratio of 0.31 \pm 0.04 (Figure 4a). No renal extraction of iFGF23 was found in uremic rats, and we did not find higher iFGF23 levels in the renal vein of uremic rats compared with the renal artery (Figure 4a). To study the importance of the kidney in FGF23 metabolism, acute unilateral nephrectomy (UNX) was performed in normal rats, resulting in an immediate, large iFGF23 rise \((P < 0.05)\). Excision of the kidney rudiment of uremic rats had no effect on the high plasma iFGF23 levels, especially no decrease in plasma iFGF23 after nephrectomy was found (Figure 4b).

FGF receptor signaling
The influence of FGFR signaling was examined using a high dose of the FGFR inhibitor (FGFRi) PD173074, which within 5 hours gradually decreased plasma iFGF23 levels to approximately one-third of baseline in both uremic and normal rats \((P < 0.01)\) (Figure 5a). Despite stable plasma Ca\(^{2+}\) and P in all groups during the study (Supplementary Table S1), FGFRi caused a large increase in PTH levels in both control and uremic rats \((P < 0.05\) and \(P < 0.01)\) (Figure 5b). FGFRi resulted in not only a suppression of plasma iFGF23, but also a downregulation of the bone’s FGF23 expression in both control and uremic rats \((P < 0.001)\) (Figure 5c). FGF23 expression in the kidney remained similar in both FGFRi- and vehicle-treated uremic rats (Figure 5d). The acute administration of FGFRi increased the expression levels of Klotho mRNA and protein (Figure 6a–c) and concomitantly increased NaPi2a and NaPi2c mRNA in normal rats \((P < 0.05)\), whereas it had no effect on these genes in uremic rats (Figure 6g–i). The protein expression of NaPi2a was not affected by FGFRi (Figure 6h and i). This might be due to the short observation time or to the rapid phosphaturic effect of PTH. Lower plasma levels of Klotho were found in uremic rats. Plasma Klotho was not affected by FGFRi (Figure 6d). FGFRi affected the enzymes involved in 1,25vitD metabolism in both normal and uremic rats, increasing Cyp27 and decreasing Cyp24 mRNA (Figure 6e and f).

Kidney FGF23 correlates to the degree of fibrosis
The kidney FGF23 mRNA levels were negatively associated with gene expressions of the kidney protective markers Klotho and BMP7, and positively associated with the
Figure 2 | The effect of chronic uremia, calcitriol, and a high-phosphate (HP) diet on kidney expression of the fibroblast growth factor 23 (FGF23) receptor complex and FGF23 target genes. (a) The same expression levels of Klotho mRNA were found in control and uremic standard-diet (SD) rats, whereas they were reduced in the uremic HP rats (**P < 0.01 vs. sham and uremic SD). (b) Kidney Klotho protein shown on a representative Western blot with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference protein, and (c) a quantitative analysis of the Western blot of kidney Klotho protein. Similar to the lower Klotho mRNA levels, the protein levels were reduced in the uremic HP rats (control n = 5, uremic n = 6; *P < 0.05). (d) The sodium-dependent phosphate cotransporter NaPi2a gene was expressed at the same level in control and uremic SD rats but significantly decreased in uremic HP rats (**P < 0.01 vs. control and uremic). (e) NaPi2a protein is shown on a representative Western blot with GAPDH as a reference protein. (f) A quantitative analysis of the Western blot shows a significant (Continued)
fibrosis markers periostin and TGF1b (Supplementary Figures S2 and S3).

Expression of FGF23 detected in obstructive nephropathy
In order to examine whether FGF23 was expressed in the injured kidney tissue in the absence of severe mineral deprevations and systemic effects of chronic kidney failure, unilateral ureter obstruction (UUO) was performed. Interestingly, minor levels of FGF23 mRNA were already detected after 2 hours of obstruction, with a further significant increase at 4 and 6 hours (Figure 7a). The increased levels persisted for 10 days in the obstructed kidney. FGF23 mRNA was not detected in the contralateral kidney or in the remaining kidney after UNX (Figure 8). Plasma levels of iFGF23 increased significantly after 2 hours of UUO (P < 0.05) (Figure 7) and remained at a higher level for 10 days of observation time (Figure 8). A similar plasma FGF23 increase was found after UNX (Figure 8b). Bone FGF23 mRNA levels were similar among all groups (Figure 8c). There was no change in the expression levels of Aktlotho and phosphate transporters for up to 6-hour UUO, but these became downregulated after 1 day of obstruction (Figure 7b–d).

Localization of renal FGF23 mRNA by in situ hybridization
In situ hybridization was performed in order to illustrate which cells were expressing FGF23 in the kidney. In both the obstructed kidneys and the kidney rudiments of long-term uremic rats, cells within the interstitial space were expressing FGF23 mRNA. In 6-hour UUO, positive FGF23 cells were found in the cortex, medulla, and papilla (Figure 9), which supported the high expression levels found by polymerase chain reaction (Figure 7). The results of immunohistochemistry are discussed in the Supplementary Figure S5.

DISCUSSION
In the present study we demonstrated significant expression of FGF23 in the injured kidney tissue of the 5/6 nephrectomy CKD model and in obstructive nephropathy in rats. Measurements of iFGF23 in the renal artery and vein demonstrated that kidney-derived FGF23 is not generating the high plasma levels of iFGF23 in uremia. The relative gene expression of FGF23 was lower in the kidney compared with bone, supporting the notion of bone being the primary source of FGF23 in CKD. The well-known stimulators of FGF23 expression in bone such as 1,25vitD and PTH did not affect the regulation of FGF23 gene expression in the injured kidney. Inhibition of the FGF receptor resulted in a downregulation of bone FGF23, but it had no effect on the kidney’s FGF23 gene expression. A high dietary P load was, however, associated with significantly increased kidney FGF23 expression, although these rats also became more uremic and had further increased markers of fibrosis.

We have previously demonstrated a significant extraction of FGF23 in the normal kidney and a short half-life of FGF23 of 4 minutes in normal rats.10 In the kidney rudiment of the uremic rat no extraction of FGF23 was found, supporting our earlier findings of a significant prolonged half-life of FGF23 after bilateral nephrectomy and illustrating an
FGF23 levels as control rats. Uremic rats had ten-fold higher plasma FGF23, and removal of the remnant kidney tissue did not result in a drop in FGF23 plasma. Collectively, these results illustrate that kidney-derived FGF23 is not contributing significantly to the high plasma levels of FGF23 in uremia.

Although significant FGF23 expression in injured kidney has been reported by different investigators, the intrarenal localization of FGF23 is still controversial. In the present study, in situ hybridization clearly demonstrated that the main sites of FGF23 mRNA were exclusively in cells within the interstitial space of both the obstructed kidney and kidney rudiment of uremic rats. This is in contrast to other investigations reporting that FGF23 protein was predominantly expressed in tubular cells by immunostaining. Zanchi et al. found focal expression of FGF23 in both proximal and distal tubulus cells in a model of diabetic nephropathy. Expression of FGF23 was detected when the rats had developed proteinuria and early kidney injury but did not coincide with higher plasma levels of FGF23. Spichtig et al. described expression of FGF23 in the cells lining the cysts in a mouse model and in a rat model of polycystic kidney disease before a rise in plasma creatinine and urea. This was associated with an increase in plasma FGF23, while FGF23 mRNA levels in bone remained unchanged. We demonstrated FGF23 protein using Western blot and found significant bands in the kidney tissue of uremic rats and no or minimal protein detected in control rats. The latter could be due to FGF23 protein in capillaries and bound in receptors. We also demonstrated a very early expression of kidney FGF23 gene at 4 and 6 hours of obstructive nephropathy. Collectively, all studies illustrate that the injured kidney expresses FGF23 regardless of the kidney disease model. Further investigations are needed to better characterize these cells localized in the interstitial space expressing FGF23.

Similar expression of FGF23 was found in the injured kidneys of PTX-uremic rats and uremic rats with intact parathyroids. There was no increase in iFGF23 plasma levels and bone expression in PTX-uremic rats. Lavi-Moshayoff et al. have previously demonstrated an important impact of hyperparathyroidism on the development of the high levels of FGF23 in uremia. In the present investigation we found no impact of PTH on the kidney’s FGF23 expression. 1,25vitD is a strong stimulus for FGF23 expression in bone. In the present study, no effect of calcitriol was found on the kidney’s FGF23 expression, although it cannot be excluded that calcitriol administration might affect kidney FGF23 via its impact on endogenous 1,25vitD metabolism. As such, our results demonstrate that kidney FGF23 is expressed independently of the classical regulatory feedback loops.

FGFR1 signaling has emerged as an important pathway by which FGF23 is regulated in bone. In the present investigation, inhibition of the FGF receptor resulted already after 5 hours in a suppression of FGF23 expression in bone in both control and uremic rats, along with decreased plasma levels. In contrast to this effect on bone, FGFRi had no effect
on the kidney’s expression of FGF23, demonstrating again that the kidney’s expression of FGF23 is not regulated in a manner similar to the corresponding expression in bone.

It was shown that FGFRi, despite stable Ca\textsuperscript{2+} and P, triggered a large increase in PTH in both control and uremic rats. This could be explained by abolition of a suppressive tonus of FGF23 on the parathyroids\textsuperscript{36}, although this would be in contrast to the notion of parathyroid resistance to FGF23 in uremia\textsuperscript{37–39}. Our results demonstrate a relative renal resistance to FGF23 in uremia, as FGFRi did not increase αKlotho, NaPi2a, and NaPi2c expression in uremic rats in contrast to the increase seen in normal rats. FGFR1 and perhaps FGFR4 have key roles in FGF23 bioactivity in the kidney.\textsuperscript{14} Thus, the lack of response of NaPi2a and NaPi2c expression to FGFRi in uremic rats might be due to the fact that FGFR1 expression was already decreased in advance, as shown in our results.

The demonstration in the present study of an increased expression of αKlotho in the kidney of FGFRi rats is novel. In another investigation of transgenic mice overexpressing FGF23, renal αKlotho was the most decreased transcript, which together with our results indicates an impact of the FGF23-FGFR pathway on Klotho regulation in the kidney.\textsuperscript{40} This is in accordance to conditions described in the parathyroid, where FGF23 was found to inhibit αKlotho mRNA.\textsuperscript{36}

A high phosphate load stimulated FGF23 expression in the injured kidney, plausibly an adaptive mechanism in response to higher phosphate filtration in the functioning nephrons. We were, however, unable to differentiate between a possible adaptive response and a cytotoxic effect of phosphate overload, because the uremic HP rats suffered from more severe uremia with significant downregulation of the FGF23 receptor complex and target genes compared with uremic SD rats. Present results clearly showed expression of the FGF23 gene...
Figure 6 | Effect of fibroblast growth factor receptor inhibition (FGFRi) on the expression of fibroblast growth factor 23 (FGF23) coreceptor Klotho and FGF23 target genes in kidneys of normal and uremic rats. The FGFR inhibitor (FGFRi) PD173074 or the vehicle was administered to control and uremic rats. (a) The uremic rats had lower expression of αKlotho versus control rats (*P < 0.05). FGFRi increased the expression levels of αKlotho in normal rats (*P < 0.05) but had no effect on αKlotho levels in uremic rats. (b) Western blot showing kidney Klotho protein expression in control rats given vehicle or FGFRi. A quantitative analysis of the Western blot showed that FGFRi increased the expression levels of Klotho protein in normal rats (n = 5 vehicle, n = 6 FGFRi; *P < 0.05). (c) Western blot showing kidney Klotho protein expression in uremic rats given vehicle or FGFRi. A quantitative analysis showed that FGFRi had no effect on levels of Klotho protein in uremic rats (n = 5 vehicle, n = 6 FGFRi). (d) Plasma Klotho levels were significantly lower (*P < 0.05) in uremic rats but were not affected by FGFRi in these short-duration experiments. (e,f) FGFRi upregulated Cyp27b1 gene expression in both control and uremic rats (**P < 0.01), whereas Cyp24a1 gene expression was significantly downregulated in both uremic and control rat kidneys (*P < 0.05; **P < 0.01). (g,j) The sodium-dependent phosphate cotransporter NaPi2a and NaPi2c genes were downregulated in uremic rats compared with the control group (*P < 0.05). FGFRi increased the expression levels of NaPi2a and NaPi2c in normal rats but had no effect on these genes in uremic rats. (h,i) In contrast to the gene expression, the protein levels in both control and uremic rat kidneys were not affected by FGFRi. All mRNA levels are shown as the ratio to the mean of reference genes B2M, Arbp, and EEF1A1. Control vehicle and FGFRi n = 6, uremic vehicle n = 8, and uremic FGFRi n = 10. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
early in the obstructive nephropathy model, but this increase was not associated with any change in NaPi2a and NaPi2c, and therefore these data do not point toward a direct effect of renal FGF23 on phosphate excretion. Because FGF23-expressing cells were located in the interstitium, this further supports no direct relation to FGF23-dependent enzyme and transporters. Furthermore, regulators of renal phosphate excretion and calcitriol homeostasis such as PTH, FGF23, and calcitriol did not regulate renal FGF23 expression. Collectively, these results do not support the notion of renal FGF23 having a direct effect on phosphate transporters or calcitriol-regulating enzymes. The kidney’s expression level of FGF23 correlated with the degree of fibrosis based on expression of peristin, and correlated negatively with the kidney protecting factors BMP7 and αKlotho. It therefore seems likely that the kidney’s expression of FGF23 increases with progression of renal fibrosis, but whether FGF23 plays a role in regeneration or in a profibrotic process remains to be established.

Induction of FGF23 expression might take place in other injured organs as well as in tumors. Ischemia of the heart resulted in an increased expression of FGF23 in cardiomyocytes and cardiac fibroblast cells. Expression of FGF23 in injured liver tissue coincided with higher plasma FGF23. FGF23 is expressed in both heart and liver during embryogenesis up to week 8. FGF9 and FGF20 play an important role in nephrogenesis, whereas FGF23 expression was not found in the developing zebrafish kidney. FGF23 belongs to the family of fibroblast growth factors, in which the majority of proteins regulate embryogenesis, growth, and angiogenesis and play an important role in organ regeneration and repair. Whether kidney-derived FGF23 has an autocrine or paracrine function in the renal repair system merits further study. Inflammatory activation of macrophages

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**Figure 7** | Expression of kidney FGF23, αKlotho, NaPi2a, and NaPi2c genes and plasma fibroblast growth factor 23 (FGF23) levels in early obstructive nephropathy. A unilateral ureter obstruction (UUO) model was used. (a) Normal kidney tissue had no detectable FGF23 gene expression. Minor FGF23 expression was detected after 2-hour (h) UUO, and significant levels were detected at 4 h of UUO, with a further increase at 6 h (*P < 0.001). (b–d) The expressions of kidney αKlotho, NaPi2a, and NaPi2c genes remained unchanged at 2, 4, and 6 h of UUO but decreased significantly (*P < 0.01) after 24 h of obstruction. (e) Plasma FGF23 increased significantly (*P < 0.05) after 2-h UUO to the level we have previously shown in a single-kidney rat model. Kidney FGF23 mRNA is shown as the ratio to reference genes actin, B2M, and TBP. Kidney αKlotho, NaPi2a, and NaPi2c are shown as the ratio to baseline (set to 1); n = 6 in each group.
and bone marrow dendritic cells induce expression of FGF23, and FGF23 has been shown to stimulate expression of tumor necrosis factor alpha in a macrophage cell line.\textsuperscript{54} The mechanisms inducing FGF23 expression and its function in these cells and injured tissues are unknown. Even though the phosphaturic and 1,25vitD regulatory functions of FGF23 are clearly established, additional functions of the protein in the inflammatory process are possible.\textsuperscript{54–56}

In conclusion, FGF23 mRNA is not expressed in the normal kidney but is rapidly induced in injured kidneys. The regulation of kidney FGF23 mRNA expression is different from that of its corresponding expression in bone. Kidney-derived FGF23 is not generating the high FGF23 plasma levels in uremia.

**METHODS**

**Animals**

Adult male Wistar rats (Taconic A/S, Ejby, Denmark) were used in this study. They were housed in a temperature-controlled environment with a 12-hour light-dark cycle and free access to water and food. The standard diet contained 0.9% Ca, 0.7% P, and 600 IU vitamin D3 per kg food. The HP diet contained 0.9% Ca, 1.4% P, and 600 IU vitamin D3 per kg food (Altromin, Lage, Germany). The biochemistry of rats given calcitriol and HP has been described previously.\textsuperscript{37} In long-term studies, all uremic rats were fed an HP diet.

**Study approval**

The experimental protocols were approved by the Danish Animal Experiments Inspectorate (2012-DY-2934-00023/BES) and performed in accordance with national guidelines.

**Experimental protocols**

CKD was induced by 1-step 5/6 nephrectomy.\textsuperscript{5,57} Duration of uremia was 8 weeks (renal artery and vein sampling were performed after 14 weeks of uremia). UUO was performed by ligation of the left ureter. UNX was performed by ligation of the renal vessel and removal of the kidney.\textsuperscript{5} A group of rats had their parathyroid glands removed by excision under stereomicroscope.\textsuperscript{9,35,58} Rats were anesthetized with fentanyl/fluanisone and midazolam for the surgical procedures and received carprofen as pain relief. Pento-barbital was used at killing.\textsuperscript{5}

The renal artery was punctured close to its junction with the aorta. The renal vein was punctured after prior ligation of the adrenal and testicular veins and clamping of the vena cava inferior.\textsuperscript{10} The aorta was punctured 15 minutes after UNX or NX.

A single 200-ng dose of calcitriol (Leo Pharma, Ballerup, Denmark) was given i.p., and the kidney was harvested after 24 hours.\textsuperscript{37} The impact of FGFR signaling was studied using a single 20-mg dose of the pan-FGFR inhibitor PD173074 (LC Laboratories, Woburn, MA) dissolved in 200\textsuperscript{a}ml 96% ethanol administered by oral gavage.

**Biochemistry**

Plasma Ca\textsuperscript{2+} was measured at actual pH by ABL 505 (Radiometer, Brønshøj, Denmark). Plasma P, creatinine, and urea were analyzed by

levels are shown as the ratio to the mean of reference genes B2M and EEF1A1 (in all groups, 1- to 4-day UUO n = 6, 10-day UUO n = 7, and 10-day UNX n = 5).
Vitros 250 (Ortho-Clinical Diagnostics, Raritan, NJ). PTH levels were measured using rat bioactive intact PTH enzyme-linked immunosorbent assay (Immutopics, San Diego, CA). FGF23 was measured using an intact FGF23 enzyme-linked immunosorbent assay (Kainos Laboratories, Tokyo, Japan). Klotho plasma levels were measured by the O’Brien Kidney Research Core Centre (University of Texas Southwestern, Dallas, TX) using a synthetic Klotho antibody.

**Western blot**
Protein was extracted using T-PER (Thermo Fisher Scientific, Waltham, MA), and 50 µg of it was loaded on stain-free precast Bio-Rad 4% to 15% gels, transferred onto nitrocellulose Bio-Rad (Hercules, CA) membrane, and blocked in 5% bovine serum albumin in Tris-buffered saline with Tween 20 0.1% (Bio-Rad). Primary FGF23 and Klotho antibody (AB56326, AB154163; Abcam, Cambridge, UK) were diluted in 3% bovine serum albumin 1:1000. NaPi2a (NBP2-13328; Novus Biologicals, Littleton, CO) 1:250 and glyceraldehyde-3-phosphate dehydrogenase antibody (MAB374; Merck Millipore, Billerica, MA) were diluted in milk 1:500. Secondary antibodies were anti-goat, anti-rabbit and anti-mouse (P0160, P0448, and P0447; Dako, Santa Clara, CA). Recombinant FGF23 (PeproTech, Rocky Hill, NJ) was used as a positive control. Quantitative analysis was performed using AlphaEaseFC v.6 (Alpha Innotech, San Leandro, CA).

**Quantitative polymerase chain reaction**
The kidney and femur (after removal of bone marrow) total RNA were extracted using the EZNA RNA isolation kit (Omega Bio-tek, Oss, Netherlands).
Norcross, GA). Synthesis of cDNA was performed using Superscript III (Invitrogen Corp, Waltham, MA). Roche LightCycler 480 (Roche, Basel, Switzerland) was used with a temperature profile of 94°C for 2 minutes, 45 cycles of 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 90 seconds. The mRNA levels were normalized to the mean of reference genes. Reference gene stability was confirmed using geNorm.62 Primers are listed in Supplementary Table S2.

**In situ hybridization**

Kidneys were fixed in 10% neutral buffered formalin and embedded in blocks of paraffin. *In situ* hybridization was carried out on 5-μm thick kidney sections using the RNAscope 2.5 HD RED assay (Advanced Cell Diagnostics, Hayward, CA) according to the manufacturer's instructions. FGF23 was detected with a rat-specific probe against the sequence 9-652 of the mRNA (catalog no. 484501, accession no. NM_130754.1). A probe against the housekeeping gene peptidylprolyl isomerase B (catalog no. 313921, accession no. NM_022536.2) was used as a positive control to verify mRNA quality and assay procedures. A probe targeting the bacterial gene dapB (catalog no. 310043, accession no. EF191515) was used as a negative control.

**Statistical analyses**

Data are expressed as means ± SEM or range. Statistical significance was tested using a 2-tailed t-test for data with normal distribution and the Mann-Whitney U test as a nonparametric test. Linear regression and curve fitting was calculated in GraphPad Prism 4.0. The significance level was set at P ≤ 0.05.

**DISCLOSURE**

All the authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

**Table S1.** Plasma calcium2+ and phosphate levels at baseline and 5 hours after administration of FGF receptor inhibitor (FGFRi) or vehicle in normal and uremic rats.

**Table S2.** Primers.

**Figure S1.** Expression of *ζKlotho* mRNA in kidneys of uremic and parathyroidectomized-uremic (PTX-uremic) rats compared with control rats. The expression of *ζKlotho* was significantly reduced by one-half in uremic rats (*P < 0.05). Similar levels of *ζKlotho* mRNA were found in uremic and PTX-uremic rats, despite a significant difference in circulating plasma levels of intact fibroblast growth factor 23 (FGF23) (Table 1). Furthermore, this reduction of *ζKlotho* expression in the kidney rudiment of the 5/6 nephrectomy model was found despite increased expression of *Cyp27b1* (Figure 2). *ζKlotho* mRNA is normalized to the mean of reference genes *actin, B2M, Arbp, EEF1A1,* and *GAPDH,* and is shown as the relative expression to control group. Control and uremic rats n = 6; PTX-uremic rats n = 3.

**Figure S2.** The expression levels of kidney fibroblast growth factor 23 (FGF23) mRNA are plotted against the expression levels of renal protective factors *ζKlotho* and BMP7, and of fibrosis markers peristin and TGFβ1. (A,B) The kidneys' FGF23 expression levels were negatively associated with the protective markers *ζKlotho* and BMP7. (C,D) The kidneys' FGF23 expression levels were positively associated with the fibrosis markers peristin and TGFβ1. The curve is fitted to the data. All uremic rats treated with calcitriol or vehicle and fed a standard or high-phosphate diet were pooled in the figure (n = 18).

**Figure S3.** Linear correlations of kidney fibroblast growth factor 23 (FGF23) mRNA expression and gene expression of renal protective markers *ζKlotho* and BMP7 and the fibrosis marker peristin in uremic rats. (A,B) The kidneys' FGF23 expression was significantly negatively correlated to *ζKlotho* (P < 0.01) and BMP7 (P < 0.05). (C) The kidneys' FGF23 expression was positively correlated to the fibrotic marker peristin (P < 0.05). Uremic fibroblast growth factor receptor inhibitor and uremic vehicle were pooled in the analysis (n = 18).

**Figure S4.** In situ hybridization with probes against fibroblast growth factor 23 (FGF23) and the bacterial gene *dapB* (negative control) on adjacent kidney sections. The panel shows representative images from the cortical region of the obstructed kidney of unilateral ureteral obstruction (UUO) and the kidney rudiment of 5/6 nephrectomized uremic rats (PNX). FGF23 mRNA (arrowheads) was located in between tubuli structures and sometimes lining the luminal side of vessels. No *in situ* hybridization signal was detected with the *dapB* probe, indicating that the assay was specific.

**Figure S5.** Fibroblast growth factor 23 (FGF23) immunostaining with MyBioSource (San Diego, CA) MB5854462 antibody. Immunostaining by the MyBioSource MB5854462 rabbit polyclonal antibody diluted 1:300 on kidney tissue from a normal control rat and the obstructed kidney from unilateral ureteral obstruction (UUO) rats, after 6 hours of ureter obstruction. (A–C) Proteinase K pre-treatment was used. (A) No immunostaining was observed in kidney tissue obtained from normal control rats. (B) No immunostaining was observed in the cortex, (C) while immunoreactivity was observed in the collecting ducts of the medulla in the obstructed kidney. However, when using a citrate buffer and pH 6 as pre-treatment, only weak immunoreactivity was observed in the distal tubuli (no brush border) in (D) the cortex and in (E) the endothelium of vessel, (F) and no immunoreactivity was observed in collecting ducts of the medulla. Immunostaining with the Santa Cruz Biotechnology (Dallas, TX) sc-50291 rabbit polyclonal antibody and Abcam (Cambridge, UK) ab56326 goat polyclonal antibody did not result in any specific immunoreactivity (data not shown). The observed immunostaining might therefore be considered as a potential fault.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

**REFERENCES**


