



# Effect of Continuous Intravenous Calcium Loading on Fibroblast Growth Factor 23 in Normal and Uremic Rats

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## Abstract

Fibroblast growth factor 23 (FGF23) is associated with mortality in patients with CKD. However, the mechanisms underlying stimulation of FGF23 remain to be investigated. We examined whether hypercalcemia induced by continuous intravenous (CIV) calcium (Ca) infusion regulates FGF23 levels in normal rats (Normal) and 5/6 nephrectomized uremic rats (Nx). Microinfusion pumps were implanted in the Normal and Nx rats for CIV Ca infusion, and blood, urine, kidney, and tibia were collected. The results showed an increase in serum Ca-stimulated FGF23 independently of serum phosphate (P) and creatinine levels in Normal and Nx rats. FGF23 mRNA from the tibia was also increased by the Ca infusion. Despite high FGF23 levels after Ca infusion, urinary P excretion was decreased. Renal  $\alpha$ -Klotho expression was significantly reduced by Ca infusion. These results suggest that intravenous Ca loading might stimulate FGF23 production from bone in normal and uremic rats. Reduction of renal P excretion suggests that the bioactivity of FGF23 is inhibited, and the decrease in renal  $\alpha$ -Klotho expression might have a role in this pathological process. In conclusion, CIV Ca loading increased FGF23 in normal and uremic rats, and renal  $\alpha$ -Klotho is necessary to maintain the bioactivity of FGF23 as a phosphaturic factor.

**Keywords** FGF23 · Calcium · Klotho · Intravenous loading

## Introduction

Calcium (Ca) and phosphate (P) homeostasis are interrelated and share common regulatory hormonal pathways that involve parathyroid hormone (PTH) and 1,25-dihydroxyvitamin-D (1,25D). Fibroblast growth factor-23 (FGF23) has been identified as the cause of autosomal dominant hypophosphatemic rickets [1]. FGF23 is mainly secreted by osteocytes in the bone in response to P loading [1, 2], but the

molecular pathway underlying this FGF23 secretion remains to be investigated. High dietary P upregulates FGF23 production in humans [3, 4], and we have found that chronic intravenous P loading increases bioactive FGF23, indicating that an alternative pathway for FGF23 regulation may be present, in addition to the dietary route [5]. Recently, it was shown that FGF23 contributes directly to the development of left ventricular hypertrophy, suggesting a plausible biologic basis for the observed association between elevated FGF23 and mortality [6–9].

Several groups have investigated regulation of FGF23. It is well known that 1,25D upregulates FGF23 [10], and that this upregulation occurs in a vitamin D receptor (VDR)-dependent manner [11–14]. PTH also upregulates FGF23 [15] and is involved in modulation of FGF23 production in bone, since FGF23 mRNA levels are increased by direct addition of PTH to osteoblasts [16]. However, regulation of FGF23 synthesis and secretion are still incompletely understood.

Ca has also recently been shown to be a regulator of FGF23. Dietary Ca intake increases FGF23 in mice [17, 18], but intravenous Ca infusion does not increase FGF23 in mice

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[19] or humans [20]. However, the effect of chronic intravenous infusion of Ca on FGF23 has not been examined. Therefore, the aim of this study was to examine whether continuous intravenous (CIV) Ca loading affects levels of FGF23 in normal and uremic rats.

## Materials and Methods

### Experimental Protocol

All studies were approved by the Showa University Animal Studies Committee in accordance with federal regulations. Adult male Sprague–Dawley rats weighing 225–250 g and of age 8 weeks were housed in a temperature-controlled environment. Renal insufficiency was induced by 5/6 nephrectomy (Nx) in a group of rats. Nx involves ligation of several branches of the left renal artery and excision of the right kidney. All rats were fed a regular (P 0.8%, Ca 1.0%, and vitamin D 3200 IU/kg) diet. Experiments were started 1 week after Nx to allow time for recovery. At this time, an infusion mini-pump (iPRECIO®, Primtech Corp., Tokyo, Japan) was implanted into normal rats (Normal) and uremic rats (Nx), after which saline was first infused for 7 days and then Ca solution was infused for another 7 days through a catheter placed in the right jugular vein. The Ca solution was infused as 50 or 10% CaCl<sub>2</sub>. All solutions were administered continuously at 20 µl/h to give a daily amount of Ca of about 250 mg (50% CaCl<sub>2</sub>) or 50 mg (10% CaCl<sub>2</sub>) and replenished in the infusion mini-pumps percutaneously every 24 h. Thus, six groups were established: Normal-control, Normal-IV-50%, Normal-IV-10%, Nx-control, Nx-IV-50%, and Nx-IV-10% (number of rats in each group are Normal-control: 14, Normal-IV-10%: 6, Normal-IV-50%: 6, Nx-control: 11, Nx-IV-10%: 6, Nx-IV-50%: 7). On the first and last day of the CIV Ca loading period, rats were placed in metabolic cages and 24-h urine samples were collected. 1 day after the start of the CIV Ca loading, about 1 ml of blood was collected from the tail vein after cutting the tip of the tail. After 7 days of the CIV Ca loading, all rats were sacrificed by exsanguination via the dorsal aorta. About 1 ml of blood was collected from the dorsal aorta at the time of sacrifice, and the kidney and tibia were removed for analysis.

### Analytical Determinants

Urine and serum levels of albumin, creatinine (Cr), Ca, and P were measured with commercial kits. Serum PTH and FGF23 were determined using a Rat Bioactive Intact PTH ELISA Kit (Immutopics, Inc. San Clemente, CA, USA) and an Intact FGF23 Assay Kit (Kainos, Tokyo, Japan), respectively. Ionized calcium (iCa) levels were measured by using an i-STAT 1 analyzer (Fuso, Osaka, Japan).

### Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After extraction from the tibia and kidney using TRIzol (Invitrogen, Carlsbad, CA), total RNA (1 µg) was reverse-transcribed to first-strand cDNA using Superscript II reverse transcriptase (Invitrogen). The synthesized cDNA was amplified using a standard polymerase chain reaction (PCR) protocol using a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA) with Universal SYBR Green Supermix (BioRad) and rat-specific primers for FGF23,  $\alpha$ -Klotho, NaPi2a, CYP24A1 and CYP27B1. Parallel amplification was performed with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sets for FGF23,  $\alpha$ -Klotho, NaPi2a, CYP24A1 and CYP27B1 and GAPDH were purchased from Qiagen K.K. (Tokyo, Japan; assay ID for FGF23: Rn\_Fgf23\_1\_SG,  $\alpha$ -Klotho: Rn\_Kl\_1\_SG, NaPi2a: Rn\_Slc34a1\_1\_SG CYP24A1: Rn\_Cyp24a1\_1\_SG, CYP27B1: Rn\_Cyp27b1\_1\_SG, and GAPDH: Rn\_Gapd\_1\_SG). The amounts of  $\alpha$ -Klotho, NaPi2a, CYP24A1, and CYP27B1 were normalized to the amount of GAPDH mRNA in each sample. All measurements were performed in duplicate.

### Histological Examination

The kidney tissue was fixed in 10% formalin overnight, embedded in paraffin, cut into 4-µm-thick sections, and stained with hematoxylin and eosin (HE) for assessment of tubulointerstitial damage, which was semi-quantified using a previously reported method [21]. Immunohistochemical staining for  $\alpha$ -Klotho was performed using rabbit anti- $\alpha$ -Klotho antibody (Bioss antibodies, Boston, MA, USA). The sections were deparaffinized, rehydrated, and microwaved in 0.01 mol/l citrate buffer (pH 6.0) for 10 min to retrieve the antigens. The sections were then treated with 0.6% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase and subsequently blocked with 10% preimmune goat serum for 30 min at room temperature. The primary  $\alpha$ -Klotho antibody (1:200 dilution) or preimmune IgG was added, followed by incubation at room temperature for 2 h. The biotinylated secondary antibody was applied, followed by a streptavidin-HRP conjugate. The immune complexes were visualized with 3-amino 9-ethylcarbazole substrate-chromagen. Finally, all sections were counterstained with hematoxylin. Areas that were immunohistochemically positive for  $\alpha$ -Klotho were semi-quantified. Five images of the cortical area obtained at a high magnification ( $\times 200$ ) were selected randomly from each section. The optical density

per section of tissue was calculated by dividing the sum of the integrated optical density by the sum area.

## Statistical Analysis

Data for each group are expressed as mean  $\pm$  SEM. Analysis of variance with post hoc pairwise comparisons of group means using a Tukey–Kramer honestly significant difference test was performed. Since the distribution of serum PTH and FGF23 levels was biased, we used a Kruskal–Wallis test for these parameters. Spearman rank correlation (non-parametric) analysis was used to test associations between two parameters. Log-transformation was used for FGF23 mRNA expression. Differences were considered significant at  $p < 0.05$ .

## Results

### Effects of Intravenous Ca Loading on Serum Chemistry

Serum chemistry for normal rats is shown in Table 1. Serum Cr and P levels were similar in all three groups throughout the study. iCa significantly increased on days 1 and 7 in the Normal-IV-50% group, but was unchanged at all time points in the Normal-IV-10% group. Serum PTH was significantly decreased in the Normal-IV-50% group only on day 1 and in the Normal-IV-50% and Normal-IV-10% groups on day 7, compared with the Normal-control group at each time point. Urine Ca excretion was significantly higher in the Normal-IV-50% group, but not in the Normal-IV-10% group, compared to the Normal-control group.

**Table 1** Serum chemistry of normal rats on days 1 and 7

Normal rats ( $n=26$ )	Cr (mg/dl)	iCa (mmol/l)	P (mg/dl)	PTH (pg/ml)	U-Ca (mg/day)
Day 1					
Normal-control ( $n=14$ )	0.2 $\pm$ 0.0	1.36 $\pm$ 0.0	6.6 $\pm$ 0.2	65 $\pm$ 22	0.6 $\pm$ 0.3
Normal-IV-50% ( $n=6$ )	0.2 $\pm$ 0.0	1.44 $\pm$ 0.1**	6.0 $\pm$ 0.2	18 $\pm$ 1*	1.2 $\pm$ 0.6
Normal-IV-10% ( $n=6$ )	0.2 $\pm$ 0.4	1.41 $\pm$ 0.0	7.6 $\pm$ 0.5	61 $\pm$ 23	0.6 $\pm$ 0.3
Day 7					
Normal-control	0.3 $\pm$ 0.0	1.35 $\pm$ 0.0	6.1 $\pm$ 0.2	62 $\pm$ 45	0.7 $\pm$ 0.1
Normal-IV-50%	0.3 $\pm$ 0.0	1.64 $\pm$ 0.1**	6.0 $\pm$ 0.2	3 $\pm$ 1**	11.0 $\pm$ 3.5**
Normal-IV-10%	0.3 $\pm$ 0.0	1.41 $\pm$ 0.1	6.0 $\pm$ 0.2	10 $\pm$ 3*	2.1 $\pm$ 0.6

Data are means  $\pm$  s.e. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Normal-IV-10%: Normal intravenous 10% calcium chloride-loaded rats

Cr serum creatinine, iCa ionized calcium, P serum phosphate, PTH parathyroid hormone, U-Ca urine calcium excretion

\* $p < 0.05$  or \*\* $p < 0.01$  vs. Normal-control on days 1 and 7, respectively

Serum chemistry for Nx rats is shown in Table 2. Similarly to Normal rats, serum Cr and P levels were similar in all three groups on days 1 and 7. iCa also showed similar results in Nx rats, with significant increases on days 1 and 7 in the Nx-IV-50% group only. Serum PTH was significantly decreased on days 1 and 7 in the Nx-IV-50% and Nx-IV-10% groups, compared with the Nx-control group at each time point. Urine Ca excretion was significantly increased on days 1 and 7 in the Nx-IV-50% group only, compared to the Nx-control group.

### FGF23 and Urinary P Excretion

Serum FGF23 levels in Normal and Nx rats are shown in Fig. 1. In Normal rats, FGF23 was significantly higher on day 7 in the Normal-IV-50% group only, compared to the Normal-control group. In Nx rats, FGF23 was significantly higher only on day 7 in the Nx-IV-50% group, compared to the Nx-control group. Urinary P excretion is shown in Fig. 2. In Normal rats, urinary P excretion significantly decreased on days 1 and 7 in the Normal-IV-50% and Normal-IV-10% groups, compared to the Normal-control group. In Nx rats, urinary P excretion in the Nx-IV-50% group was significantly decreased on days 1 and 7.

### FGF23 mRNA Expression in the Bone

FGF23 mRNA levels in tibia in Normal-IV-50%, Normal-control, Nx-IV-50%, and Nx-control rats on day 7 are shown in Fig. 3. FGF23 mRNA was significantly higher in the Normal-IV-50% group compared with the Normal-control group. In the Nx-IV-50% group, FGF23 mRNA expression

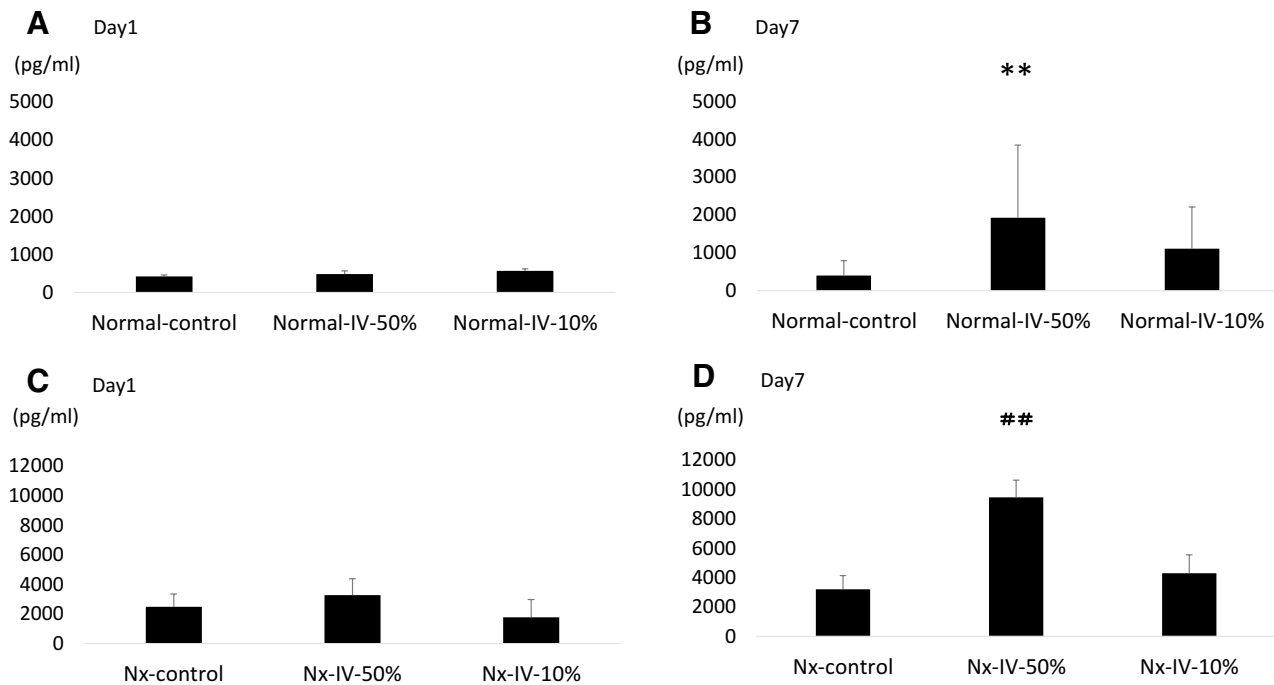
**Table 2** Serum chemistry of uremic rats on days 1 and 7

Uremic rats ( <i>n</i> = 24)	Cr (mg/dl)	iCa (mmol/l)	P (mg/dl)	PTH (pg/ml)	U-Ca (mg/day)
Day 1					
Nx-control ( <i>n</i> = 11)	1.0 ± 0.1	1.32 ± 0.0	7.3 ± 0.3	292 ± 120	0.5 ± 0.0
Nx-IV-50% ( <i>n</i> = 7)	0.9 ± 0.1	1.56 ± 0.0 <sup>##</sup>	7.6 ± 0.3	24 ± 9 <sup>##</sup>	2.4 ± 0.7 <sup>##</sup>
Nx-IV-10% ( <i>n</i> = 6)	1.2 ± 0.4	1.41 ± 0.0	7.6 ± 0.5	61 ± 23 <sup>#</sup>	0.8 ± 0.2
Day 7					
Nx-control	1.3 ± 0.2	1.31 ± 0.0	7.5 ± 0.5	330 ± 98	0.8 ± 0.1
Nx-IV-50%	1.7 ± 0.5	2.15 ± 0.6 <sup>##</sup>	6.0 ± 0.4	9 ± 5 <sup>##</sup>	13.7 ± 5.0 <sup>##</sup>
Nx-IV-10%	1.2 ± 0.5	1.41 ± 0.1	7.5 ± 0.4	61 ± 28 <sup>#</sup>	1.5 ± 0.3

Data are means ± s.e. Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats, Nx-IV-10%: uremic intravenous 10% calcium chloride-loaded rats

Cr serum creatinine, iCa ionized calcium, P serum phosphate, PTH parathyroid hormone, U-Ca urine calcium excretion

<sup>#</sup>*p* < 0.05 or <sup>##</sup>*p* < 0.01 vs. Nx-control on days 1 and 7, respectively



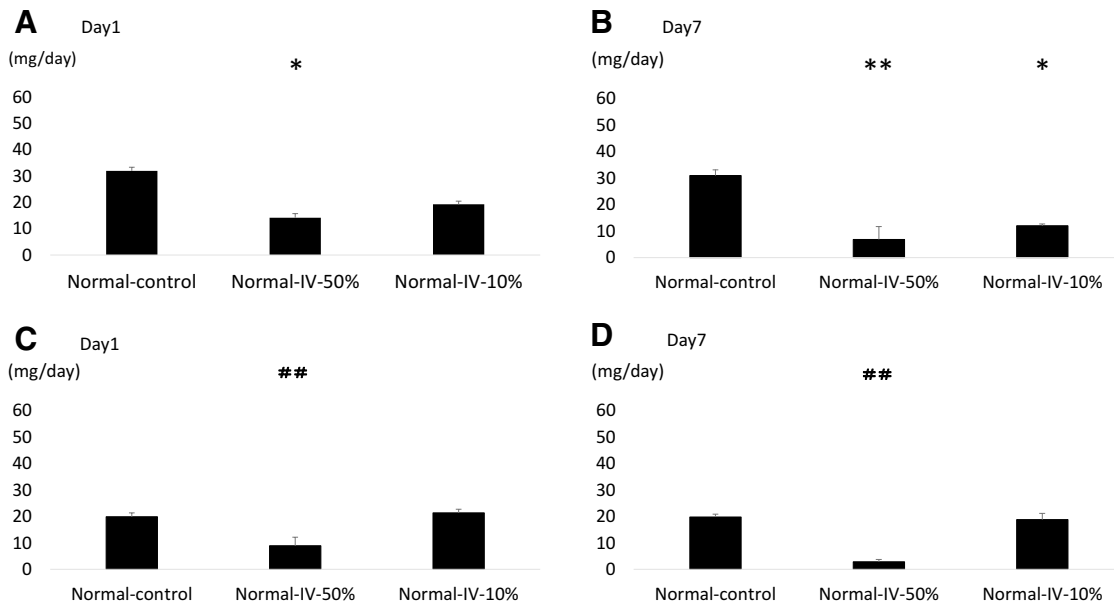
**Fig. 1** Serum FGF23 levels and urinary phosphate excretion. Serum FGF23 levels in the Normal-control, Normal-IV-50%, and Normal-IV-10% groups on day 1 (**A**) and day 7 (**B**), and in the Nx-control, Nx-IV-50%, and Nx-IV-10% groups on day 1 (**C**) and day 7 (**D**). *N* = 6–14. Data are shown as mean ± s.e. \**p* < 0.05 or \*\**p* < 0.01 vs. Normal-control and <sup>#</sup>*p* < 0.05 or <sup>##</sup>*p* < 0.01 vs. Nx-control on days 1 and 7, respectively. FGF23 fibroblast growth factor 23, CaCl<sub>2</sub> cal-

cium chloride. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Normal-IV-10%: Normal intravenous 10% calcium chloride-loaded rats, Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats, Nx-IV-10%: uremic intravenous 10% calcium chloride-loaded rats

was also significantly increased compared with the Nx-control group.

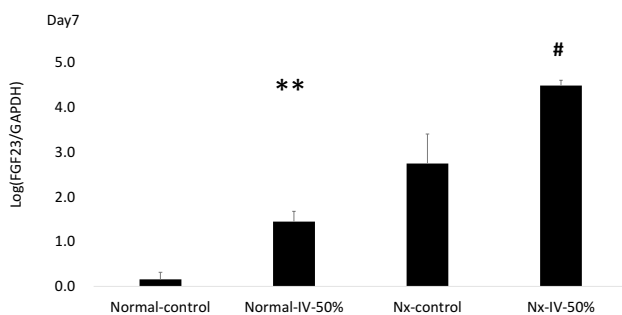
### α-Klotho mRNA Expression in the Kidney

Renal α-Klotho mRNA levels in Normal-IV-50%, Normal-control, Nx-IV-50%, and Nx-control rats at day 7 are shown

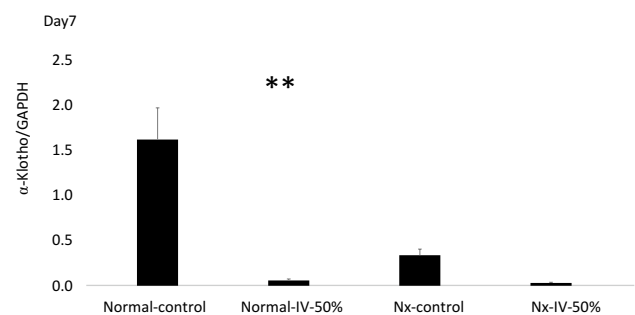


**Fig. 2** Urinary phosphate excretion. Urinary phosphate levels in the Normal-control, Normal-IV-50%, and Normal-IV-10% groups on day 1 (**A**) and day 7 (**B**), and in the Nx-control, Nx-IV-50%, and Nx-IV-10% groups on day 1 (**C**) and day 7 (**D**).  $N=6-11$ . Data are shown as mean  $\pm$  s.e. \* $p < 0.05$  or \*\* $p < 0.01$  vs. Normal-control and # $p < 0.05$  or ## $p < 0.01$  vs. Nx-control on days 1 and 7, respectively.  $CaCl_2$  cal-

cium chloride. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Normal-IV-10%: Normal intravenous 10% calcium chloride-loaded rats, Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats, Nx-IV-10%: uremic intravenous 10% calcium chloride-loaded rats



**Fig. 3** FGF23 mRNA expression in the bone. Data are means  $\pm$  s.e. ( $n=3$  each). \*\* $p < 0.01$  vs. Normal-control and # $p < 0.05$  vs. Nx-control. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats



**Fig. 4**  $\alpha$ -Klotho mRNA expression in the kidney. Data are means  $\pm$  s.e. ( $n=4$  each). \*\* $p < 0.01$  vs. Normal-control. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats

in Fig. 4.  $\alpha$ -Klotho mRNA expression was significantly lower in Normal-IV-50% rats compared to Normal-control rats. A tendency for suppression of  $\alpha$ -Klotho mRNA was observed in Nx-IV-50% rats, but the change was not significant.

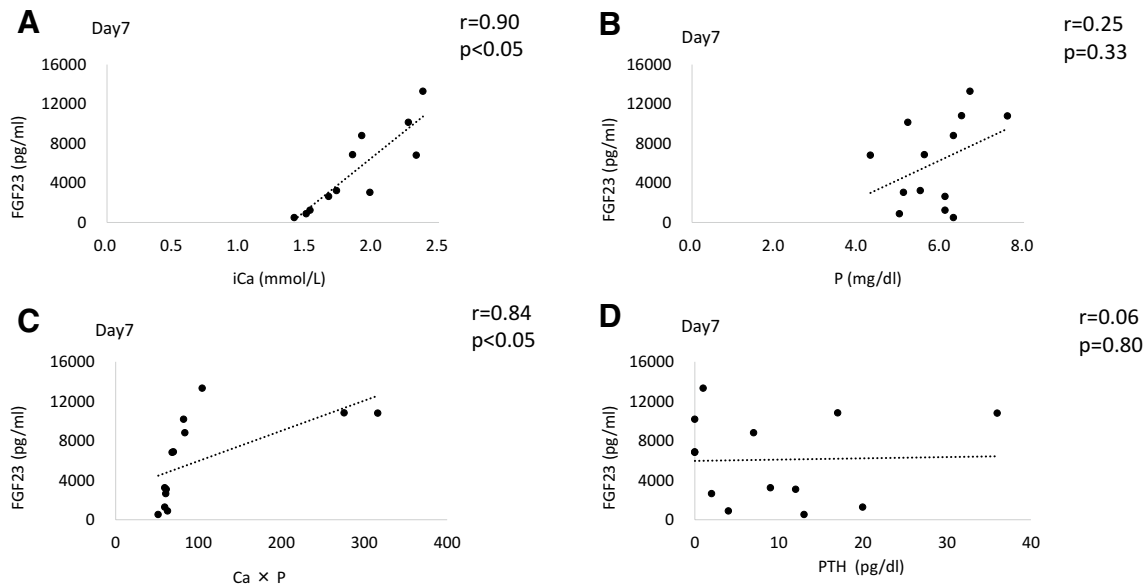
### NaPi2a, CYP24A1, and CYP27B1 mRNA Expression in the Kidney

Renal NaPi2a, CYP24A1, and CYP27B1 mRNA levels in Normal-control, Normal-IV-50%, Nx-control, and

Nx-IV-50% rats are shown in Supplemental Fig. 1A–C. None of these mRNA levels were altered in Normal-IV-50% rats compared with Normal-control rats. Similar results were observed in Nx rats.

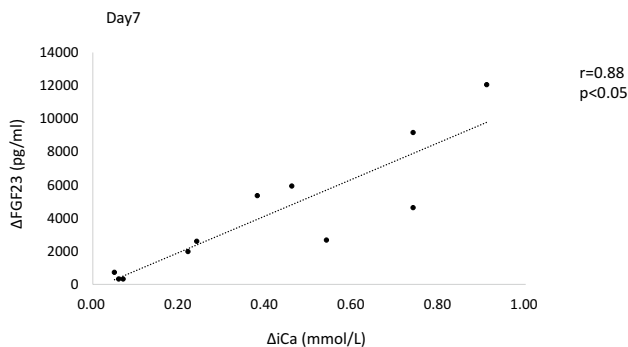
### Correlations of FGF23 with Ionized Ca, P, Ca $\times$ P, and PTH

Relationships of FGF23 with iCa, P, Ca  $\times$  P, and PTH in Normal-IV-50% and Nx-IV-50% rats on day 7 are shown in Fig. 5. FGF23 was positively correlated with iCa ( $r=0.90$ ,



**Fig. 5** Correlations of FGF23 with ionized-Ca, P, and Ca×P. Serum FGF23 concentrations plotted as a function of serum ionized-Ca (**A**), serum P (**B**), Ca×P (**C**), and PTH (**D**) in Normal-IV-50% rats and

Nx-IV-50% rats on day 7. Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats



**Fig. 6** Correlation between  $\Delta$ FGF23 and  $\Delta$ ionized-Ca. Serum  $\Delta$ FGF23 plotted as a function of serum  $\Delta$ ionized-Ca in Normal-IV-50% rats and Nx-IV-50% rats on day 7. Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats

$p < 0.05$ ), and Ca×P ( $r = 0.84$ ,  $p < 0.05$ ), but was not correlated with P and PTH. There was also a significant correlation between  $\Delta$ FGF23 and  $\Delta$ iCa ( $r = 0.88$ ,  $p < 0.05$ ) (Fig. 6).

### $\alpha$ -Klotho Protein Expression in the Kidney

Representative microphotographs of kidney cortex sections stained for  $\alpha$ -Klotho expression from Normal-control, Normal-IV-50%, Nx-control, and Nx-IV-50% rats are shown in Fig. 7A–D. A semi-quantification of the  $\alpha$ -Klotho positive area is shown in Fig. 7E. This area was significantly lower in Normal-IV-50% rats compared with Normal-control rats,

and was also significantly decreased in Nx-IV-50% rats compared with Nx-control rats.

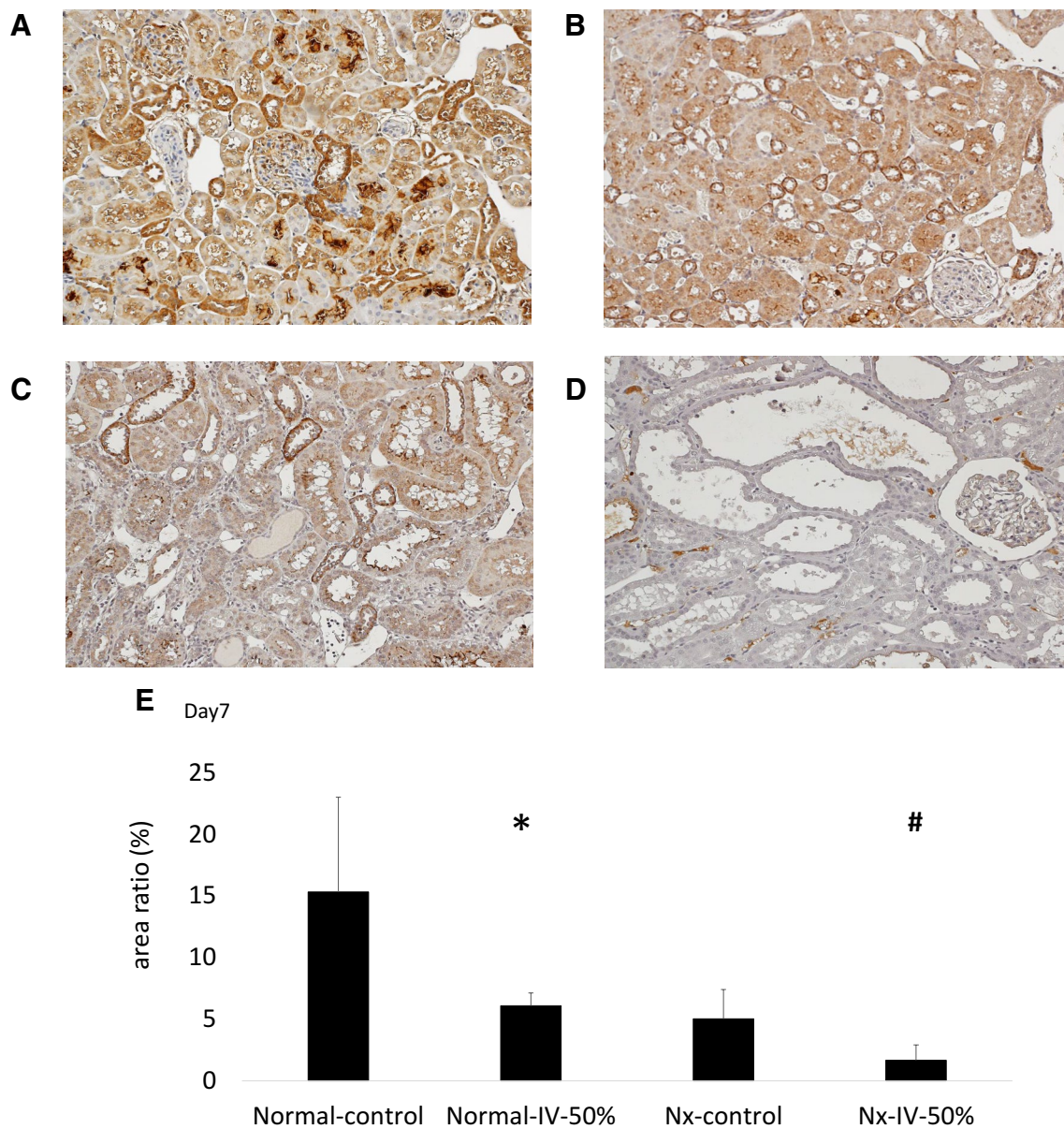
### Tubulointerstitial Damage

Representative microphotographs of HE stained kidney cortex sections of Normal-control Normal-IV-50%, Nx-control, and Nx-IV-50% rats are shown in Supplemental Fig. 2A–D. A semi-quantification of the histologic damage score is shown in Supplemental Fig. 2E. There was no significant difference in this score in Normal-control rats and Normal-IV-50% rats. Although the damage was exacerbated, a similar trend was found for Nx rats.

### Discussion

In this study, we found an increase in serum Ca-stimulated FGF23 independently of serum P and Cr levels in both Normal and Nx rats. These results suggest that CIV Ca loading stimulated FGF23. We also found that urinary P excretion was decreased, suggesting that the bioactivity of FGF23 was inhibited by CIV Ca loading, despite high FGF23 levels during Ca infusion. Renal  $\alpha$ -Klotho expression was also significantly reduced by Ca infusion; therefore, urinary P excretion might be decreased.

Elevated FGF23 is independently associated with increased mortality in patients with renal disease [6] and is an important mineral metabolism marker in the uremic milieu. However, the mechanisms through which FGF23 is



**Fig. 7**  $\alpha$ -Klotho expression in renal tubules. Representative sections for  $\alpha$ -Klotho expression in renal tubules ( $\times 200$ ) in Normal-control rats (**A**), Normal-IV-50% rats (**B**), Nx-control rats (**C**), and Nx-IV-50% rats (**D**) on day 7, and semi-quantification of  $\alpha$ -Klotho expression in renal tubules (**E**). Data are means  $\pm$  s.e. ( $n=4$  each). \* $p < 0.05$

vs. Normal-control and # $p < 0.05$  vs. Nx-control. Normal-control: Normal control rats, Normal-IV-50%: Normal intravenous 50% calcium chloride-loaded rats, Nx-control: uremic control rats, Nx-IV-50%: uremic intravenous 50% calcium chloride-loaded rats

regulated remain unclear. Dietary and intravenous P loading up-regulate production of FGF23 [3–5], but FGF23 is also controlled by other factors. Several recent studies have shown that Ca is a direct regulator of FGF23: in VDR-null mice with almost undetectable levels of FGF23, high dietary Ca induced marked elevation of FGF23 at both the mRNA and protein levels [17]; in rats fed a diet deficient in Ca and vitamin D, serum FGF23 was extremely low despite very high PTH, which should stimulate FGF23 production [18]. After parathyroidectomy (PTx), rats showed elevation of

serum Ca after an acute (6-h) Ca infusion or after chronic oral administration (10 days) of a high Ca diet produced an increase in serum FGF23 [18]. In humans, a positive correlation between Ca and FGF23 in patients on hemodialysis was found in a cross-sectional study [22], and a similarly designed study showed that dietary Ca intake is significantly associated with higher levels of FGF23 in non-CKD patients [23]. These data suggest that dietary Ca loading is a modulator of the FGF23 level. In contrast to dietary Ca, intravenous Ca loading resulted in no detectable changes in FGF23 in

60 min of hypocalcemic and hypercalcemic clamps in normal rats, and acute intravenous infusion of Ca in PTx rats also had no effect on serum FGF23 [19]. There were also no changes in FGF23 after an acute change (120 min) after intravenous Ca infusion in healthy humans and in patients with CKD [20]. However, the observation period may have been too short to detect changes in FGF23 in these studies. We hypothesized that Ca loading in blood may influence the FGF23 level, and we found that CIV Ca loading resulted in an increase in serum Ca and FGF23 in NC and Nx rats, which was independent of serum P and Cr levels. The increase in FGF23 after Ca infusion was observed on day 7, but not on day 1, which suggests that regulation of FGF23 by Ca infusion requires some time. This result is consistent with a previous report showing that the effect of Ca on FGF23 is not rapid [20]. In Normal-IV-50% and Nx-IV-50% rats on day 7, there were positive correlations between iCa and FGF23 levels, and between  $\Delta$ FGF23 and  $\Delta$ iCa. These results suggest that CIV Ca loading was closely related to FGF23 elevation. This loading increased FGF23, indicating that an alternative pathway for FGF23 regulation by Ca might be present, in addition to the dietary route. Serum PTH was significantly decreased in Normal-IV-50% and Nx-IV-50% rats on day 7. Since PTH has been shown to promote secretion of FGF23 [24, 25], there seems to have been only a minor impact of a decrease in PTH after CIV Ca loading on elevation of FGF23. Furthermore, a previous examination of the role of the calcium-sensing receptor (CaSR) in regulation of FGF23 by Ca suggested that the stimulatory effect of Ca on FGF23 was independent of both PTH and CaSR [26]. This study suggests that Ca stimulates FGF23 secretion regardless of PTH, and our results are consistent with this finding.

A noteworthy finding in the present study was that urinary P excretion was decreased despite the high FGF23 level induced by CIV Ca loading, even in rats with normal kidney function. This suggests that the bioactivity of FGF23 was inhibited by CIV Ca loading. Unlike other FGFs, FGF23 has extremely low affinity for the FGF receptor (FGFR), and thus rarely binds to FGFR. In contrast, FGF23 has an extremely high affinity for the FGFR- $\alpha$ -Klotho complex [27, 28]. FGF23 probably targets the kidney due to the limited renal expression of  $\alpha$ -Klotho [29], and  $\alpha$ -Klotho expression in the kidney was significantly reduced by CIV Ca loading in the present study. This might contribute to the decrease in urinary P excretion (blockade of FGF23 bioactivity), consistent with the decrease in urinary P excretion in Klotho KO mice [30]. We also examined expression of mRNA for NaPi2a, CYP27B1, and CYP24A1 in the kidney to evaluate FGF23 bioactivity. The mRNA levels for these markers were not altered by CIV Ca loading, suggesting that FGF23 bioactivity was inhibited. These results might be associated with the reduction of renal  $\alpha$ -Klotho expression.

Reduction of PTH might also contribute to reduction of urinary P excretion. Since PTH provides strong phosphorus diuresis [31], it is possible that low PTH, in addition to reduction of renal  $\alpha$ -Klotho expression, provoked low urinary P excretion. PTH reduction by cinacalcet in XLH [32] and TIO [33] results in an increase in Tmp/GFR, which indicates that PTH is necessary for FGF23 to be a phosphaturic factor. Further studies are needed to clarify the effect of PTH on urinary P excretion under the condition of a high FGF23 level.

We also examined FGF23 mRNA in bone and made a comparison between Normal-control and Normal-IV-50% rats, and between Nx-control and Nx-IV-50% rats. Normal-IV-10% and Nx-IV-10% rats were not considered because they did not exhibit a significant increase in serum Ca. FGF23 is produced mainly by osteocytes and osteoblasts in bone [1, 2], but lower expression is also detected in other tissues, including the heart, thymus, muscle, brain, and liver [2]. FGF23 mRNA expression both in Normal-IV-50% rats and in Nx-IV-50% rats was significantly higher than that in each control rats. This suggests that Ca has a crucial role in FGF23 secretion from bone, but further studies are needed to determine whether Ca loading directly induces FGF23 production in bone.

Another new finding in this study was that CIV Ca loading resulted in decreased  $\alpha$ -Klotho expression in the kidney. We evaluated renal  $\alpha$ -Klotho expression by immunohistochemistry and tubulointerstitial damage.  $\alpha$ -Klotho expression was significantly reduced in Normal-IV-50% rats compared with Normal-control rats, while there was no significant difference in tubulointerstitial damage between these rats. Similar results were found in comparison of Nx-control rats and Nx-IV-50% rats. These results suggest that  $\alpha$ -Klotho reduction in rats after CIV Ca loading was independent of tubulointerstitial damage. The relationship between Ca loading and  $\alpha$ -Klotho reduction in the kidney remains to be determined. The precise mechanism is unclear, but two possibilities are conceivable. First, hypercalcemia may decrease  $\alpha$ -Klotho expression in the kidney.  $\alpha$ -Klotho has not been reported to be controlled by Ca; however, some studies have shown a relationship between Ca and  $\alpha$ -Klotho. In a cross-sectional study, serum Ca levels showed a negative correlation with serum soluble  $\alpha$ -Klotho levels in healthy humans [34] and hemodialysis patients [35]. Soluble Klotho is not the same as renal  $\alpha$ -Klotho, but a recent study showed a parallel reduction in renal and soluble Klotho under the condition of a high FGF23 level in patients with glomerulonephritis [36]. Therefore, Ca may be one of the factors regulating renal  $\alpha$ -Klotho expression. The second possible mechanism is that FGF23 produced by Ca infusion might down-regulate renal  $\alpha$ -Klotho expression. The effects of FGF23 on renal and soluble Klotho need to be clarified. In vivo, FGF23 rapidly increases renal  $\alpha$ -Klotho



expression and the serum soluble Klotho level in 3 h [37], but this result does not match our finding. However, FGF23 did not increase both renal  $\alpha$ -Klotho expression and serum soluble Klotho in 24 h. The negative correlation between the serum soluble Klotho and FGF23 levels in healthy humans [34] and CKD patients [38], and a recent report showing that the kidney is the major source of soluble Klotho [39], suggesting that elevation of FGF23 could down-regulate renal  $\alpha$ -Klotho expression. Conversely, the possibility that renal  $\alpha$ -Klotho reduction might contribute to FGF23 elevation was not ruled out. In this study, kidney damage was not exacerbated by CIV Ca loading in normal and Nx rats, indicating that renal  $\alpha$ -Klotho reduction was independent of kidney damage. Since renal  $\alpha$ -Klotho reduction might contribute to FGF23 elevation, the interaction between FGF23 elevation and renal  $\alpha$ -Klotho reduction is an important issue that requires clarification.

This study has several limitations. First, we did not examine the direct effect of Ca loading on FGF23 production from bone. In vitro experiments are needed for confirmation of a direct action. Second, the effect of PTH reduction on urinary P excretion was not examined. Third, serum 1,25(OH)<sub>2</sub>D levels were not measured. However, as shown in Supplemental Fig. 1B, C, mRNA levels of CYP27B1 and CYP24A1 in the kidney were not altered in Normal-IV-50% and Nx-IV-50% rats, which suggests that calcitriol production was not affected by CIV Ca loading in these models.

In conclusion, this is the first study to show that continuous and intravenous Ca loading increases FGF23 in normal and uremic rats. Renal  $\alpha$ -Klotho is necessary to maintain the bioactivity of FGF23 as a phosphaturic factor.

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## Compliance with Ethical Standards

**Conflict of interest** Yasuto Shikida, Masahide Mizobuchi, Takashi Inoue, Toma Hamada, Hiroaki Ogata, Fumihiko Koiwa, and Takanori Shibata have no competing interests.

**Human and Animal Rights and Informed Consent** All animal experiments were carried out in accordance with the Guide for Use and Care of Laboratory Animals and were approved by the Showa University Animal Studies Committee. Human informed consent statements are not applicable since this is an animal study.

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