Inorganic phosphate (Pi) is an essential nutrient required for cellular metabolism and skeletal mineralization. It is well established that both parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) regulate calcium homeostasis. It is also widely thought that molecules involved in calcium homeostasis mediate phosphate homeostasis (1-3). However, recent studies have demonstrated the existence of a novel Pi regulatory pathway that is independent of the classic mode of calcium regulation (1-3). A group of factors have been shown to function as major regulators of phosphate homeostasis, suggesting the existence of an elaborate network of humoral interactions and feedback loops that involve numerous organs including intestine, kidney and bone. Fibroblast growth factor 23 (FGF23) has been identified as a phosphaturic factor that is mainly produced in the bone (4, 5). FGF23 is known to mediate the phosphaturic actions of cadmium.

Fibroblast growth factor 23 mediates the phosphaturic actions of cadmium

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Abstract: Phosphaturia has been documented following cadmium (Cd) exposure in both humans and experimental animals. The fibroblast growth factor 23 (FGF23)/klotho axis serves as an essential phosphate homeostasis pathway in the bone-kidney axis. In the present study, we investigated the effects of Cd on phosphate (Pi) homeostasis in mice. Following Cd injection into WT mice, plasma FGF23 concentration was significantly increased. Urinary Pi excretion levels were significantly higher in Cd-injected WT mice than in control group. Plasma Pi concentration decreased only slightly compared with control group. No change was observed in plasma parathyroid hormone and 1,25-dihydroxy vitamin D3 in both group of mice. We observed a decrease in phosphate transport activity and also decrease in expression of renal phosphate transporter SLC34A3 [NaPi-IIc/NPT2c], but not SLC34A1 [NaPi-IIa/NPT2a]. Furthermore, we examined the effect of Cd on Npt2c in Npt2a-knockout (KO) mice which expresses Npt2c as a major NaPi co-transporter. Injecting Cd to Npt2aKO mice induced significant increase in plasma FGF23 concentration and urinary Pi excretion levels. Furthermore, we observed a decrease in phosphate transport activity and renal Npt2c expression in Cd-injected Npt2a KO mice. The present study suggests that hypophosphatemia induced by Cd may be closely associated with the FGF23/klotho axis. J. Med. Invest. 57: 95-108, February, 2010

Keywords: cadmium, FGF23, phosphate, transporter, proximal tubule

INTRODUCTION

Inorganic phosphate (Pi) is an essential nutrient required for cellular metabolism and skeletal mineralization. It is well established that both parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) regulate calcium homeostasis. It is also widely thought that molecules involved in calcium homeostasis mediate phosphate homeostasis (1-3). However, recent studies have demonstrated the existence of a novel Pi regulatory pathway that is independent of the classic mode of calcium regulation (1-3). A group of factors have been shown to function as major regulators of phosphate homeostasis, suggesting the existence of an elaborate network of humoral interactions and feedback loops that involve numerous organs including intestine, kidney and bone. Fibroblast growth factor 23 (FGF23) has been identified as a phosphaturic factor that is mainly produced in the bone (4, 5). FGF23 is known to mediate the phosphaturic actions of cadmium.
to promote renal Pi excretion by decreasing its re-absorption within the proximal tubules and simultaneously reducing plasma 1,25(OH)₂D₃ levels by decreasing its biosynthesis and increasing its metabolism (6). FGF23 requires the cofactor klotho to bind with high affinity and to signal efficiently through its cognate FGF receptor. Klotho and FGF receptor 1 (IIIc) form a heterodimeric receptor for FGF23 (7, 8). FGF23 is reported to also be regulated by the phosphate regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) and dentin matrix protein 1 (DMP-1) (8).

The kidney is a major regulator of phosphate homeostasis, and its phosphate reabsorption capacity has been shown to either increase or decrease in order to accommodate phosphate requirements (9, 10). The solute carriers of three distinct renal sodium dependent phosphate transporters known as SLC34A1 [NaPi-IIa/NPT2a], SLC34A3 [NaPi-IIc/NPT2c] and SLC20A2 [PiT-2] are specifically expressed on the brush border membrane of the renal proximal tubules in both the mouse and rat (9-11). Npt2a plays a major role in renal Pi reabsorption, while Npt2c is important for Pi reabsorption in weanling animals (12, 13). PiT-2 has also been shown to be expressed in the proximal tubule cells (14). Recently, several groups have demonstrated that a lack of functional Npt2c protein in patients demonstrating hereditary hypophosphatemic rickets with hypercalciuria (HHRH) causes severe renal Pi wasting and leads to hypophosphatemia (15-17). These findings indicated that Npt2c may play an important role in renal Pi re-absorption and bone mineralization, and may be a key determinant of plasma Pi concentration in human (15-17).

Npt2a knockout (KO) mice demonstrate lower levels of renal Pi transport activity by 60% of those of wild-type (WT) mice and hypophosphatemia, but not rickets/osteomalacia (18). We reported that in Npt2a KO mice, Npt2c plays a major NaPi co-transporter where it contributed to renal Pi re-absorption (19). In adult Npt2a KO mice, Pi abnormalities were largely rescued via the induction of renal Npt2c transporters (19). Thus, it is good model to examine the role of Npt2c in Pi homeostasis. Cadmium (Cd) is one of the most toxic metals observed in nature. Phosphaturia has been documented in both cadmium-exposed humans and experimental animals (20, 21). The toxic effects of cadmium on bone were first observed after the outbreak of severe Itai-itai disease (22, 23). Bone abnormalities associated with Itai-itai disease include osteomalacia and osteoporosis (24, 25). Accumulation of cadmium within the renal cortex has been proposed to indirectly and directly interfere with enzymes involved in 1,25(OH)₂D₃ production and with transporters involved in calcium and Pi homeostasis (26, 27). An increased prevalence of osteomalacia and nephrolithiasis observed in patients with advanced Cd-induced nephropathy is thought to be due to elevated fractional urinary excretion of calcium and Pi caused by impaired reabsorption of these ions (20). In regards to nephropathy, Cd accumulation induced defects in reabsorption at the level of the tubules (28). Clinical features associated with Cd induced nephropathy are similar to those exhibited during Fanconi’s syndrome. Most recently, several groups have demonstrated that iron-induced hypophosphatemic osteomalacia associated with marked FGF23 elevation (29, 30). Ion-induced hypophosphatemia has also been shown to be associated with impaired tubular Pi reabsorption and reduced levels of 1,25(OH)₂D₃ (29, 30). However, FGF23 expression is reduced in hypophosphatemia patients by Fanconi’s syndrome (31).

In the present study, we investigated whether FGF23 was involved in Cd induced nephropathy in wild type (WT) and Npt2a-KO mice.

METHODS

Animals

Female C57BL/6 mice were purchased from the Charles River Laboratories Japan (Yokohama, Japan), and male and female Npt2a⁻/⁻ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Crossing male and female Npt2a⁻/⁻ mice yielded Npt2a⁻/- mice. Npt2a⁻/- were used as WT control mice. Animals were housed in plastic cages and allowed free access to standard laboratory food and tap water. Nephrotoxicity was induced following a subcutaneous injection of CdCl₂ at a dose of 2 mg Cd/kg daily for up to 14 days (32). Control animals were injected with 0.9% sodium chloride (NaCl). Mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of the University of Tokushima Faculty of Medicine.

Determination of Cd concentration in liver, kidney and femur

The accumulation of Cd was measured using flame atomic absorption spectrometry (AA-6400,
Shimadzu, Tokyo, Japan). Samples were prepared for measurement as previously reported with some minor modifications (33). Briefly, each sample was first dried to eliminate water at 110°C for 36 h. The samples were then ashed at 250°C for 3 h, 350°C for 3 h and 550°C for 24 h. The ash samples were then wet-digested in 1% hydrochloric acid (HCl). Working concentrations of Cd were prepared from a standard solution of Cd containing 100 mg/ml Cd (Wako Japan).

Plasma and urine parameters

Two days prior to sacrifice, animals were individually placed in metabolic cages, and deprived of food and water for 24 h. The concentration of plasma and urinary inorganic calcium (Ca) and Pi were then determined using the Calcium-E test (Wako, Osaka, Japan) and the Phospha-C test (Wako), respectively (34). Urinary and plasma creatinine (Cr) concentration was determined using the LabAssay Creatinine (Wako, Osaka, Japan). The concentration of urine protein was determined using the BCATM Protein Assay kit (Thermo Scientific IL, USA). FGF23 protein concentration was determined using the FGF-23 enzyme-linked immunosorbent assay kit (ELISA, KAINOS Laboratories, Inc, Tokyo, Japan) (34). Blood urea nitrogen (BUN) was determined using the UN-B test (Wako, Osaka, Japan), and plasma 1,25(OH)2D3 was determined using the 1,25-(OH)2 -Vitamin D ELISA kit (Immundiagnostik AG, Bensheim, Germany) (35).

Preparation of brush border membrane vesicles and transport assay

Brush-border membrane vesicles (BBMV) were prepared from mouse kidney using the calcium (Ca2+) precipitation method as described previously (34, 36). BBMV 32P and Gly-Sar uptake was measured using the rapid filtration as described previously (37). Briefly, BBMV suspensions (20 μg/10 μl) were added to 90 μl of uptake solution containing 100 mM NaCl, 100 mM mannitol, 20 mM HEPES/TRIS, 0.1 mM KH2PO4 for 32P uptake or 100 mM NaCl, 100 mM mannitol, 20 mM HEPES/TRIS, 1 mM of unlabeled substrates, and [3H]Gly-Sar for peptide uptake. At specific times, the uptake process was quenched by adding ice-cold stop solution containing 150 mM NaCl and 9 mM KH2PO4/K2HPO4, (pH 7.4). BBMV suspensions were then immediately poured onto pre-wetted membrane filters (ADVANTEC, Tokyo, Japan) and washed three times with ice-cold stop solution. 3 ml of Aquasol 2 (Perkin Elmer, Inc, MA) was then added to the membrane filter and radioactivity counted using a liquid scintillation counter (ALOKA, Tokyo, Japan).

Immunoblotting Analysis

BBMV samples were firstly heated at 95°C for 5 min in sample buffer in either the presence or absence of 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were then transferred by electrophoresis to a polyvinylidene difluoride transfer membrane (Immobilon-P, Millipore Co., MA). The membranes were incubated with affinity purified anti-Npt2a (1 : 4000) or Npt2c (1 : 1000) or Megalin (1 : 10000) antibodies and then with horseradish peroxidase conjugated anti-rabbit IgG as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The signals were detected using the Immobilon Western chemiluminescent HRP substrate (Millipore Co.) (38). Anti-Megalin antibody was kindly provided by Dr.Saito (Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan).

Immunohistochemistry

Immunohistochemical analysis of the mouse kidney was performed as described previously with some minor modifications (12). Serial sections (5 μm) were incubated with affinity purified anti-Npt2a (1 : 3000) or Npt2c (1 : 1000) antibody overnight at 4°C. The sections were then treated with Envision (+) rabbit peroxidase (Dako) for 30 min. To detect immunoreactivity, the sections were treated with ImmPACTTM DAB Peroxidase Substrate (Funakoshi Co., Tokyo, Japan).

RNA extraction and quantitative PCR analysis

Total RNA was extracted from mouse tissues using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA synthesized using the Moloney murine leukemia virus reverse transcriptase (Superscript, Invitrogen, Carlsbad, CA), and oligo (dT)12-18 primer (37). The reaction mixture contained 10 μl of SYBR Premix Ex Taq (Perfect Real Time, Takara, Japan) and specific primers. PCR primer sequences for Npt2a and Npt2c (14), FGF23, DMP I and PHEX (39) and for 1,25(OH)2D3 (6) have been previously reported. The PCR conditions included denaturation at 95°C for 10 s, followed by amplification with 40 cycles at 95°C for 5 s, and annealing at 60°C for 34 s. Quantitative PCR was performed using the ABI Prism 7500 (Applied Biosystems).
Statistical Analysis

Data is presented as the mean± standard error (S.E.). Differences between experimental groups were determined by analysis of variance and $p$ values<0.05 were considered significant.

RESULTS

Accumulation of injected Cd in WT mice after 1 and 14 days

At 1, 3 and 14 days following Cd injection, the accumulation of Cd in various tissues was measured using flame atomic absorption spectrometry. Samples were ashed and dissolved in 1% HCl. Cd solution (Wako Japan) was used as a standard. At 1 day after injection, we observed a small accumulation of Cd in the kidney and liver (Fig. 1 a and b). At 14 days, significantly high concentrations of Cd were observed in the liver and kidney when compared to the control mice (Fig. 1 a and b). These results were consistent with those reported previously (40). The level of Cd accumulation in the bone was not significantly different to those observed between the Cd and control mice (Fig. 1 c).

Time course after injection of Cd

WT mice were subcutaneously injected with Cd every day for 14 days, and plasma and urine samples collected at 1, 3, 7 and 14 days after the injections. Plasma calcium levels did not demonstrate any differences following the Cd injection (data not shown), while plasma Pi and BUN were decreased 3 days after injection (Fig. 2 a and b). In contrast, plasma FGF23 levels in the WT mice were significantly increased at 3 and 14 days following Cd injection (Fig. 2 c). Serum Cr levels, a marker of renal function, were significant increase at 14 days after injection (Fig. 2 d).

![Graphs showing accumulation of Cd in liver, kidney, and bone of WT mice](image-url)

**Fig. 1** Accumulation of Cd in the liver, kidney and femur of WT mice
Accumulation of Cd was measured using flame atomic absorption spectrometry. a) Liver, b) Kidney and c) Femur. Cd-: non-injected mice Cd+: Cd-injected mice. $n=6-8$. *$p<0.05$ vs Cd-.
Plasma and urine analysis of WT mice injected with Cd for 14 days

The plasma and urine parameters of WT mice after Cd injection are presented in Table 1. Plasma and urine samples from WT mice were collected at 14 days. A urine sample was also collected 24 h before sacrifice. Mice injected with Cd showed a greater reduction in body weight when compared to control mice, even though differences in food intake were not observed. Urine volume was no difference between control group and Cd injected group (data not shown). Intoxicated mice demonstrated a significant increase in plasma Ca and a slight decrease in plasma Pi concentration. Plasma BUN, plasma Cr, plasma FGF23 and plasma glucose increased significantly, particularly 14 days after injection. The Tables provide a detailed list of biochemical measurements in WT mice injected with Cd for 14 days.

**Table 1.** Body weight, food intake, plasma and urine biochemical measurements in WT mice

<table>
<thead>
<tr>
<th></th>
<th>Cd-</th>
<th>Cd+</th>
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<tbody>
<tr>
<td>% reduction of body weight</td>
<td>5.3±1.36</td>
<td>18.9±1.88*</td>
</tr>
<tr>
<td>food intake (g/100 g body weight/day)</td>
<td>9.21±0.52</td>
<td>9.40±0.76</td>
</tr>
<tr>
<td>plasma Pi (mg/dL)</td>
<td>6.28±0.25</td>
<td>5.64±0.46</td>
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<tr>
<td>plasma Ca (mg/dL)</td>
<td>7.78±0.23</td>
<td>9.01±0.26*</td>
</tr>
<tr>
<td>plasma BUN (mg/ml)</td>
<td>25.0±0.88</td>
<td>46.2±8.47*</td>
</tr>
<tr>
<td>plasma Creatinine (mg/ml)</td>
<td>0.132±0.02</td>
<td>0.333±0.032*</td>
</tr>
<tr>
<td>plasma glucose (mg/dL)</td>
<td>210.9±7.61</td>
<td>154.7±6.42*</td>
</tr>
<tr>
<td>plasma 1,25(OH)2D3 (pg/ml)</td>
<td>43.65±0.14</td>
<td>43.08±8.09</td>
</tr>
<tr>
<td>plasma PTH (pg/ml)</td>
<td>33.3±3.52</td>
<td>42.4±5.7</td>
</tr>
<tr>
<td>plasma FGF23 (pg/ml)</td>
<td>66.9±4.34</td>
<td>171.5±40.9*</td>
</tr>
<tr>
<td>Pi/Creatinine</td>
<td>1.75±0.14</td>
<td>2.14±0.25*</td>
</tr>
<tr>
<td>Ca/Creatinine</td>
<td>0.13±0.01</td>
<td>0.24±0.05*</td>
</tr>
<tr>
<td>Protein/Creatinine</td>
<td>0.314±0.039</td>
<td>0.459±0.036*</td>
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</table>

n=6-8. Data represent the means± S.D. *p< 0.05 vs Cd-
concentrations were significantly increased in the Cd-injected mice when compared to those observed in the control mice. However plasma PTH and plasma 1,25(OH)₂D₃ indicated no change. These findings suggested that Cd treatment in the mice may cause low level renal dysfunction. In addition, Cd-injected mice showed higher levels of urine Pi and calcium, and a greater protein/creatinine ratio when compared to controls.

Effects of Cd on the phosphate transporters Npt2a and Npt2c in WT mice

To determine the effects of Cd on NaPi transporters, we carried out immunoblotting, immunohistochemistry and tracer studies on kidney BBMV. We found that the NaPi co-transporter activity was decreased by 59.6% in the BBMV isolated from Cd treated animals when compared to those from the non-injected mice (Fig. 3 a). In addition, the peptide transport activity did not change in the Cd-injected mice when compared to those in the control mouse group (Fig. 3 b). In order to investigate the mechanisms underlying the reductions in phosphate transport induced by Cd, we performed immunoblotting and immunostaining analyses. Based on the immunoblotting of BBMV, we found that the expression of Npt2c was significantly decreased when compared to the control mice (Fig. 3 c and e). In contrast, the protein levels of Npt2a and Megalin showed no differences between the Cd-injected and control mice (Fig. 3 c and d). A decrease in Npt2c protein without change in Npt2a protein expression was already seen at 3 days after Cd injection (Sup. Fig. 1 a-c). Real-time PCR revealed that the Npt2a

Sup. Fig. 1  Npt2a and Npt2c protein expression at day 3 of Cd injection in WT mice
Protein expression of Npt2a and Npt2c at day 3 of Cd injection was determined by Western blotting. a) Western blotting in the WT mice kidney BBMV in the presence of 2-mercaptoethanol. Left lane, Cd non-injected mouse (Cd-); Right lane, Cd-injected mouse (Cd+). Relative intensity of b) Npt2a and c) Npt2c against actin. n=5. *p<0.05 vs Cd-.
and Npt2c mRNA levels were significantly decreased following Cd exposure (Fig. 3 f and g). In addition, we also observed that Npt2a immunoreactivity was not altered between the Cd-injected and control mice, while the intensity of the Npt2c signals were markedly decreased (Fig. 4 a-h). These results were consisted with those reported for western blotting.

**Accumulation of injected Cd in the Npt2a-KO mice**

Accumulation of Cd in the Npt2a-KO mice injected with Cd showed similar results to the WT mice. That is, Cd was mainly accumulated in the liver and kidney of the Npt2a-KO mice, while only a small accumulation was observed in the bone (Fig. 5 a-c).

![Fig. 4 Npt2a and Npt2c expression in the kidney](image)

Expression of Npt2a and Npt2c in the kidney was analyzed by immunostaining. The kidney was fixed, embedded in paraffin, and 5-μm sections collected. The top panel (a and c) represents Npt2a staining. The lower panel (b and d) represents Npt2c staining. e, f, g and h show higher magnification micrographs of the highlighted areas in a-d. The scale bar represent 10 μm.

![Fig. 5 Accumulation of Cd in the liver, kidney and femur of Npt2a-KO mice](image)

Accumulation of Cd was measured using flame atomic absorption. The numbers below the graph represent days after Cd injection. Cd- : non-injected mice, Cd+ : Cd-injected mice. n=6-8. *p< 0.05 vs Cd-.
Time course study of Npt2a-KO mice injected with Cd

The plasma and urine parameters of Npt2a-KO mice after Cd injection are presented in Table 2. Plasma and urine samples were collected at 1, 3, 7 and 14 days post injection. Npt2a-KO mice demonstrated low plasma FGF23 levels, a result that has been reported previously (41). After injection of Cd, plasma Pi and BUN levels were significantly decreased in the Npt2a-KO mice (Fig. 6 a and b). We observed high plasma FGF23 concentrations at 3 and 14 days after injection of Cd in the WT mice. While in the Npt2a-KO mice, elevation of plasma FGF23 levels was observed at day 1 and day 14 (Fig. 6 c). In Npt2a-KO and WT mice, plasma Cr levels were significant increase in 14 days after injection (Fig. 6 d).

Table 2. Body weight, food intake, plasma and urine biochemical measurements in Npt2a-KO mice

<table>
<thead>
<tr>
<th></th>
<th>Cd-</th>
<th>Cd+</th>
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<tr>
<td>% reduction of body weight</td>
<td>9.6±1.67</td>
<td>20.6±3.02*</td>
</tr>
<tr>
<td>(g/100 g body weight/day)</td>
<td>10.32±0.1</td>
<td>11.43±0.68</td>
</tr>
<tr>
<td>plasma Pi (mg/dL)</td>
<td>4.43±0.36</td>
<td>4.56±0.40</td>
</tr>
<tr>
<td>plasma Ca (mg/dL)</td>
<td>7.56±0.32</td>
<td>9.16±0.57*</td>
</tr>
<tr>
<td>plasma BUN (mg/ml)</td>
<td>28.2±1.22</td>
<td>65.4±12.8*</td>
</tr>
<tr>
<td>plasma Creatinine (mg/ml)</td>
<td>0.182±0.012</td>
<td>0.345±0.02*</td>
</tr>
<tr>
<td>plasma glucose (mg/dl)</td>
<td>195.9±7.71</td>
<td>142.8±10.3*</td>
</tr>
<tr>
<td>plasma 1,25(OH)2D3 (pg/ml)</td>
<td>41.53±10.84</td>
<td>49.50±9.57</td>
</tr>
<tr>
<td>plasma PTH (pg/ml)</td>
<td>37.1±2.89</td>
<td>30.9±6.09</td>
</tr>
<tr>
<td>plasma FGF23 (pg/ml)</td>
<td>N.D.</td>
<td>136.4±8.48*</td>
</tr>
<tr>
<td>Pt/Creatinine</td>
<td>5.04±2.62</td>
<td>9.93±5.34*</td>
</tr>
<tr>
<td>Ca/Creatinine</td>
<td>0.32±0.03</td>
<td>0.40±0.14</td>
</tr>
<tr>
<td>Protein/Creatinine</td>
<td>0.198±0.018</td>
<td>0.259±0.032*</td>
</tr>
</tbody>
</table>

n=6-8. Data represent the means± S.D. N.D.: Not Detected *p<0.05 vs Cd-

Fig. 6 Time course study of Cd injection for 14 days in Npt2a-KO mice
Plasma parameters of Npt2a-KO mice are presented in the graphs. Mice were injected with Cd for up to 14 days. The open bars represent the non-injected mice and the closed bars represent the Cd-injected mice. a) plasma Pi, b) BUN, c) plasma FGF23 and d) plasma Cr. n=6-8. *p<0.05 vs Cd-.
Plasma and urine parameters in Npt2a-KO mice injected with Cd

The plasma and urine parameters of Npt2a-KO mice that were injected with Cd at days 14 and their controls are presented in Table 2. Similar to the WT mice, Npt2a-KO mice injected with Cd demonstrated reduced body weight, even though differences in food intake were not observed. Urine volume was no difference between control group and Cd injected group (data not shown). Npt2a-KO mice that received Cd injections demonstrated a significant increase in plasma Ca compared to the control mice. Cd treated animals did not show any changes in plasma Pi concentration when compared to the control mice. In addition, Cd injection also induced an increase in plasma BUN, plasma Cr, plasma FGF23 and plasma glucose levels. While plasma PTH and plasma 1,25(OH)2D3 did not show any change. As for the urine parameters, Pi, calcium and protein excretion shown in the creatinine ratio were also increased.

Effects of Cd on the Pi transporters Npt2a and Npt2c in Npt2a-KO mice

Similar to the WT mice, Npt2a-KO mice injected with Cd showed a significant decrease (30.1%) in NaPi co-transporter activity when compared to the controls (Fig. 7 a), but no change in peptide transport activity (Fig. 7 b). To determine the levels of Npt2c expression in the Npt2a-KO mice, we undertook immunoblotting and immunostaining. Npt2a-KO mice showed a reduction in renal Npt2c expression after injection of Cd (Fig. 7 c and d, and Fig. 8 a-d). Similar observations were also detected for Npt2c mRNA expression (Fig. 7 e).
in WT mice this situation was already seen at 3 days after Cd injection (Sup. Fig. 2 a-c).

**DISCUSSION**

In the present study, we investigated the effects of Cd on the Npt2/FGF23/klotho system in the kidney. The effects of Cd increased the levels of plasma FGF23 in WT mice. We observed a simultaneous decrease in renal phosphate transport activity. This was also associated with a decrease in protein and mRNA levels of the renal phosphate transporter Npt2c, but not Npt2a, in WT mice. We also used Npt2a-KO mice to further analyze the effects of Cd on Npt2c. Injection of Cd into Npt2a knockout mice resulted in severe hypophosphatemia and a significant increase in plasma FGF23 levels. The elevation of plasma FGF23 levels in the early stages after injection of Cd is also thought to be important for Cd-dependent hypophosphate expression.

The effects of Cd on Pi metabolism have been previously reported by Herak-Kramberger et al. (32). It is well established that Cd induces nephrotoxicity due to its accumulation within the renal tubules (42, 43). Previous animal models have demonstrated that subcutaneous injection of CdCl₂ into Wistar-strain rats for 2 weeks induced nephrotoxicity, a result that we have also shown in mice (32). The treatment regime of daily Cd injections for 2 weeks represents a model of long-term Cd-induced nephrotoxicity. Injection of Cd into rats resulted in the inhibition of protein and phosphate urine excretion, typical symptoms of Cd poisoning (32). These rats also demonstrated a decrease in Npt2a expression in the kidney following Cd injection. It was also demonstrated that expression of the water channel

**PHEX, DMP1, FGF23 mRNA levels in the bone**

As described above, Cd-injected mice demonstrated increased plasma FGF23 levels. To further investigate FGF23 production, we determined the levels of PHEX, DMP1 and FGF23 mRNA expression using real-time PCR. In both the WT and Npt2a-KO mice, Cd-injected mice showed no significant differences in gene expression when compared to control mice (Sup. Fig. 3-I a-c and Sup. Fig. 3-II a-c).

Sup. Fig. 2  Npt2c protein expression at day 3 of Cd injection in Npt2a-KO mice
Protein expression of Npt2a and Npt2c at day 3 of Cd injection was determined by Western blotting. a) Western blotting was performed on the Npt2a-KO mouse kidney BBMV in the presence of 2-mercaptoethanol. Left lane is Cd non-injected mice and right lane is Cd-injected mice. b) Relative intensity of Npt2c against actin. n=5. *p< 0.05 vs Cd-.

Sup. Fig. 3  mRNA expression of FGF23 related factors in WT and Npt2a-KO mice
mRNA expression was determined using quantitative real-time PCR. Relative intensity is shown against Cd- expressed in WT mice a) FGF23, b) DMP1, c) PHEX and for Npt2a-KO mice d) FGF23, e) DMP1, f) PHEX. n=6-8. *p< 0.05 vs Cd-.
PiT-2 on Pi reabsorption after Cd injection. Studies are required to precisely clarify the role of antibodies directed against murine PiT-2. Further mal tubule cells, as we could not obtain specific unable to investigate the levels of PiT-2 in the proximal tubule. In the present study, we were tion of Npt2a protein may result in a greater resistance to Cd toxicity. In the present study, we were not able to obtain specific antibodies directed against murine PiT-2. Further studies are required to precisely clarify the role of PiT-2 on Pi reabsorption after Cd injection.

An important finding in our study is that the biphasic peak of FGF23 elevation is observed in both WT and Npt2a-KO mice after Cd administration. In the early stage of Cd administration, plasma PTH, Cr and BUN levels were not changed. However in the later stage of Cd administration, plasma Cr and BUN levels were significantly increased. These results suggest that second peak of FGF23 elevation may be due to renal dysfunction as reported previously. In the analysis of the Npt2a and Npt2c expression, Cd administration resulted in a reduction of Npt2c protein in the renal proximal tubular cells. Even though there have been no reports suggesting that Cd accumulates within the kidney as early as 24 h after injection of Cd, we did observe low levels of accumulation at this time. Therefore, in the early stage of Cd administration the reduction of renal Npt2c protein expression may have occurred as a direct effect of increased plasma FGF23 levels. Although FGF23 is known to function as a negative regulator of 1,25(OH)2D3 production. In the WT and Npt2a-KO mice, plasma 1,25(OH)2D3 levels were not decreased following injection of Cd, while the plasma FGF23 concentration was significantly increased. Measurement of the enzymes related to 1,25(OH)2D3 production also resulted in no significant differences. One possibility for this result is that Cd directly disturbed FGF23-dependent vitamin D regulation. Further studies are required to define the precise role of Cd in vitamin D/Pi metabolism.

Although we analyzed the expression of PHEX, MEPE, DMP-1 and FGF23 mRNA levels in the bone, we did not observe any obvious changes in the expression of these genes. These findings suggested that the production of FGF23 in the bone was not stimulated by Cd administration. The mechanisms underlying the elevated plasma FGF23 levels are currently unknown. Recently, Shimizu et al. reported that saccharated ferric oxide induced up-regulation of FGF23 levels and resulted in hypophosphatemia. It has been hypothesized that saccharated ferric oxide caused proximal tubular dysfunction as the small size and neutral charge of the drug allowed it to be filtered through the glomeruli and be deposited in the proximal tubules. Thus, as Cd-injected mice also demonstrated elevated plasma FGF23 levels, nephrotoxicity induced by saccharated ferric oxide and Cd may demonstrate a similar molecular pathway.

The results of the present study suggest that the pathway through which Cd induces renal failure and bone abnormalities may be associated with the Npt2c/FGF23/Klotho system. Thus, the present
study may serve as a useful tool for furthering our understanding of the pathways governing Cd intoxication. In addition, these studies may also be helpful in the clarification of Npt2c/FGF23/Klotho system function.

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