Regulation of α-klotho Expression by Dietary Phosphate During Growth Periods

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Abstract

Inorganic phosphate (Pi) is an essential nutrient for maintaining various biological functions, particularly during growth periods. Excess intake of dietary Pi increases the secretion of fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) to maintain plasma Pi levels. FGF23 is a potent phosphaturic factor that binds to the α-klotho/FGFR complex in the kidney to promote excretion of Pi into the urine. In addition, excess intake of dietary Pi decreases renal α-klotho expression. Down-regulation or lack of α-klotho induces a premature aging-like phenotype, resulting from hyperphosphatemia, and leading to conditions such as ectopic calcification and osteoporosis. However, it remains unclear what effects dietary Pi has on α-klotho expression at different life stages, especially during growth periods. To investigate this, we used C57BL/6J mice in two life stages during growing period. Weaned (3 weeks old) and periadolescent (7 weeks old) were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. As a result, elevated plasma Pi and FGF23 levels and decreased renal α-klotho expression were observed in weaned mice fed with a high Pi diet. In addition, a high Pi diet clearly induced renal calcification in the weaned mice. However, in the periadolescent group, renal calcification was not observed, even in the 1.8% Pi diet group. The present study indicates that a high Pi diet in weaned mice has much greater adverse effects on renal α-klotho expression and pathogenesis of renal calcification compared with periadolescent mice.

Keywords

Dietary phosphate, Growth periods, α-Klotho, FGF23, Vitamin D metabolism, Kidney

Introduction

Inorganic phosphate (Pi) is an essential nutrient for maintaining various biological functions, particularly during growth periods [1, 2]. Pi deficiency can cause abnormal mineralization of bone and lead to metabolic bone disorders such as rickets and osteomalacia. On the other hand, an excessive intake of dietary Pi is known as to be a risk factor for impaired kidney function and cardiovascular diseases [3]. In recent years, excess intake of dietary Pi has been a concern in Japan as well as other developed countries [4, 5]. In particular, Pi intake from "food additives" used in various processed foods has increased significantly [6, 7]. According to the National Health and Nutrition Examination Survey in Japan, Pi intake for each age group is about 1000 mg [8]. On the other hand, total intake of Pi including food additives has been reported to be about two-fold [9].

Pi homeostasis is regulated by its absorption from the intestine, reabsorption, and excretion through the kidney, and mobilization from the bone. These processes are regulated by various Pi-regulating factors such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], and fibroblast growth factor 23 (FGF23) [10-12]. FGF23 is a potent phosphaturic hormone that is secreted from the bone in response to elevated serum Pi levels or increased dietary Pi intake. Secreted FGF23 can bind to FGF receptor with α-klotho, which is a co-receptor for FGF23 in the kidney, and suppress the expression of 25-hydroxyvitamin D-1 α-hydroxylase (CYP27B1) and

type II sodium-dependent Pi cotransporters (Npt2a and Npt2c) [13, 14]. Thus, the FGF23/α-klotho signaling system plays an important role in both Pi homeostasis and vitamin D metabolism.

α-klotho was originally identified as an aging-suppressor gene [15]. It is expressed mainly in the distal tubules of the kidney, and down-regulation or a lack of α-klotho induces a premature aging-like phenotype. Mutant mice which lack α-klotho show premature aging-like phenotypes such as shorter life-spans, arteriosclerosis, and ectopic calcification. These premature aging-like phenotypes are due to hyperphosphatemia and hypervitaminosis D [15-17]. α-klotho and Npt2a double-knockout mice did not show hyperphosphatemia or ectopic calcification [29]. On the other hand, double-knockout mice fed with high a Pi diet showed a similar phenotype to α-klotho knockout mice [18]. These results suggest that a high Pi diet may be responsible for the expression of a premature aging-like phenotype. According to several reports, α-klotho expression is suppressed under high dietary Pi intake, increased oxidative stress, and chronic kidney disease (CKD) [19-21].

α-klotho expression is decreased by a high Pi diet and increased by a low Pi diet in mature mice [19]. However, the effects of dietary Pi on α-klotho expression at different life stages, especially during growth periods, remain unclear. Therefore, we hypothesized that a high Pi diet during growth periods may suppress α-klotho expression and contribute to early-onset aging-related diseases. In this study, we examined the effects of dietary Pi on renal α-klotho expression;

phosphate, calcium, and vitamin D metabolism; and ectopic calcification during growth periods.

Materials and Methods

Animals and Diets

This study was approved by the Animal Experimentation Committee of Tokushima University. In this study, we chose to use male mice, because female mice have estrous cycle which affect bone and mineral metabolism. Male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan) at the ages of 2 and 6 weeks and housed in cages. All animals were kept on a 12-h: 12-h light-dark cycle with unlimited access to distilled water. Before mice were given the experimental diet, 2-week-old male mice were given breast milk and the normal diet (Oriental Yeast Co., ltd., Tokyo, Japan) containing 0.8% Pi and 1.0% calcium (Ca). 7-week-old male mice were given the normal diet only before experimental period. The experimental diets were based on the modified AIN-93G [22] the protein source of which was egg white and with a modified mineral mix without Ca and Pi to prepare a Pi deficient diet. $CaCO_3$ was added to each diet at 0.6% Ca, and KH_2PO_4 was added to prepare 0.02%, 0.3%, 0.6%, 0.9%, 1.2%, 1.5% and 1.8% Pi diets on the Pi deficient diet (Table 1). To compare the effects of dietary Pi intake on plasma Pi or other biochemical and patho-histological analyses between weaned and periadolescent mice, we conducted a “short-term study.” In this short-term study, weaned mice (3 weeks old) and periadolescent mice (7 weeks

old) were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Each mouse was given the experimental diet in accordance with a pair-feeding protocol. Therefore, daily food intake did not differ among the groups. To investigate the long-term effects of dietary Pi on growing mice, we conducted a "long-term study." In this long-term study, weaned mice were randomly divided into two experimental groups and fed with either a 0.6 or 1.8% Pi diet for 7, 14, or 21 days. At the end of the experimental period, all mice were euthanized under anesthesia and blood, urine, and kidney samples were collected for the following analyses.

Blood and urine parameters

Plasma and urine concentrations of Pi, Ca, and creatinine (Cre) were determined using the Phospha-C test (Wako, Osaka, Japan), the Calcium-E test (Wako), and the LabAssay™ Creatinine test (Wako). Concentrations of plasma intact-FGF23 and intact-PTH were determined using FGF23 ELISA kits (Kainos, Tokyo, Japan) and Mouse PTH 1-84 ELISA kits (Immutopics, San Clemente, CA). Plasma $1,25(OH)_2D$ levels were measured using a radioimmunoassay (RIA) kit (TFB, Tokyo, Japan) by SRL Co., Ltd. (Tachikawa, Japan).

Real-time PCR

Total RNA was isolated from homogenized kidneys using ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 2.5 μg of total RNA and primed with oligo (dT) using MMLV-reverse transcriptase (Invitrogen, San Diego, CA). Real-time quantitative polymerase chain reaction (RT-PCR) analysis was performed using StepOnePlus™ (Applied Biosystems, Forster City, CA, USA). The prepared first-strand cDNA was amplified by PCR using Fast SYBR®Green Master Mix (Applied Biosystems) in a 10 μl reaction volume, with 10 pmol of each primer. The primer sequences used for PCR amplification are described in Table 2. The amplification program was 95 °C for 20 s followed by 40 to 50 cycles of 95 °C for 3 s, 60 °C for 30 s, and 60 °C for 1 min. The PCR products were separated by electrophoresis using 1% agarose gels. The PCR products were quantified by fit-point analysis and the mRNA expression was normalized using β-actin as an internal control. The value from the periadolescent mice fed the 0.6% phosphate diet was considered to be 1.00.

Von Kossa staining

Von Kossa staining was performed to detect ectopic calcification. Harvested tissues were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and then dehydrated in an ascending ethanol series, embedded in paraffin, and sliced at 4 μm thickness. The tissue sections were treated with 5% silver nitrate solution under ultraviolet light for 1 h. The sections were then

127 washed with distilled water and immersed in 5% hypo solution (sodium thiosulfate). Sections
128 were counterstained with hematoxylin-eosin (HE).
129
130 **Statistical analysis**
131 Results are expressed as the mean ± SEM for each group. Statistical significance was
132 determined by the Tukey-Kramer post hoc test, after two-way ANOVA. A p value < 0.05 was
133 considered to be significant.
134
135 **Results**
136 **Effects of dietary Pi on biochemical parameters in plasma and urine**
137 To examine the effects of dietary Pi on biochemical parameters in plasma and urine, mice
138 were fed diets of different Pi content. As dietary Pi content increased, plasma Pi concentration
139 increased in the weaned group, but not in the periadolescent group (Fig. 1a). Plasma Ca
140 concentration did not change in either group except for mice on the 0.02% Pi diet (Fig. 1b). As
141 dietary Pi content increased, urinary Pi excretion increased in both groups (Fig. 1c). A high Pi diet
142 (1.5 and 1.8% Pi) did not increase urinary Ca excretion in the periadolescent group, but a high Pi
143 diet (1.5 and 1.8% Pi) did increase urinary Ca excretion compared to the 0.9% Pi diet (Fig. 1d).
144
145 **Effects of dietary Pi on phosphate-regulating factors in plasma**

146 To examine the effects of dietary Pi on phosphate-regulating factors in plasma, we measured
147 plasma FGF23, 1,25$(OH)_2$D, and PTH 1-84. The plasma FGF23 concentration was significantly
148 elevated in the weaned mice fed with a high Pi diet (1.5 and 1.8% Pi). In the periadolescent group,
149 a high Pi diet slightly, but significantly, increased plasma FGF23 levels (Fig. 2a). Plasma
150 1,25$(OH)_2$D concentration was significantly elevated in the low Pi diet group (0.02% Pi), and
151 tended to increase, as dietary Pi content increased, in both groups (Fig. 2b). A high Pi diet (1.5
152 and 1.8% Pi) increased plasma PTH 1-84 concentration compared with the 0.6% Pi diet in the
153 periadolescent group, but not in the weaned group (Fig. 2c).
154
155 **Effects of dietary Pi on mRNA expression of renal FGF23/α-klotho signal-related gene and**
156 **inflammatory cytokines**
157 To examine the effects of dietary Pi on the renal FGF23/α-klotho signaling pathway, we
158 measured renal α-klotho and FGFR1 mRNA expression levels using RT-PCR. In the
159 periadolescent group, renal α-klotho mRNA expression levels did not change significantly. On
160 the other hand, in the weaned group, α-klotho mRNA expression levels decreased significantly
161 following a high Pi diet (1.5% and 1.8% Pi) (Fig. 3a). Renal FGFR1 mRNA expression did not
162 change in either group. We hypothesized that the decrease in renal α-klotho expression in the
163 weaned group impaired the FGF23/α-klotho signal. Therefore, we examined renal Egr-1 mRNA

expression, which is a target gene of the FGF23/α-klotho signal [14]. As shown in Fig. 3c, renal Egr-1 mRNA expression was significantly elevated in the weaned fed with a high Pi diet (1.5 and 1.8% Pi), but unchanged in the periadolescent group. Furthermore, we examined the expression of one of the inflammatory cytokines, renal TNF-α mRNA. As shown in Fig. 3d, renal TNF-α mRNA expression was significantly elevated in weaned mice fed with a high Pi diet (1.5 and 1.8% Pi), but unchanged in the periadolescent group.

Effects of dietary Pi on renal mRNA expression of sodium-dependent Pi transporters, Ca transporter, and vitamin D metabolism-related genes

We also examined the effects of dietary Pi intake on renal mRNA expression for sodium-dependent Pi transporters (Npt2a and Npt2c) and vitamin D metabolism-related genes. Renal Npt2a mRNA expression was significantly decreased in the weaned mice fed with a high Pi diet (1.5 and 1.8% Pi) (Fig. 4a), and renal Npt2c mRNA expression also tended to decrease in the weaned mice fed with a high Pi diet (1.5 and 1.8% Pi) (Fig. 4b). Renal Cyp27b1 mRNA expression was significantly increased in the weaned mice fed with a low phosphate diet (0.02% Pi), and tended to increase in the weaned group as their dietary Pi content increased (Fig. 4c). Renal Cyp24a1 mRNA expression tended to increase in both groups as dietary Pi content increased (Fig. 4d). The expression of mRNA for the transient receptor potential vanilloid member

182 5 (TRPV5), which is a major calcium transporter in the apical membrane of renal distal tubules,
183 was significantly higher in the weaned mice fed with a high Pi diet (Fig. 4e).
184
185 **Effects of dietary Pi on renal calcification**
186 Recent reports have indicated that a high Pi diet can induce renal calcification [23, 24].
187 Therefore, we investigated the effects of dietary Pi on renal calcification using Von Kossa staining.
188 As shown in Fig. 5A, a high Pi diet (1.5 and 1.8% Pi) clearly induced renal calcification in the
189 weaned group (Fig. 5a). However, in the periadolescent group, renal calcification was not
190 observed, even in the 1.8% Pi diet group (Fig. 5b).
191
192 **Long-term effects of a high Pi diet on biochemical parameters in the plasma and urine of**
193 **weaned mice**
194 In the short-term study of the two life stages, it was clear that a high Pi diet had much greater
195 adverse effects on the kidneys of weaned mice compared with periadolescent mice. Moreover,
196 the FGF23/α-klotho signal was activated in the weaned mice fed with high a Pi diet, despite the
197 fact that renal α-klotho mRNA expression decreased. However, it was still unclear what long-
198 term effects a high Pi diet may have had on the FGF23/α-klotho signal in the weaned group.
199 Therefore, we examined the effects of long-term administration of a high Pi diet on the FGF23/α-

klotho signal in weaned mice. Weaned mice were randomly divided into two experimental groups and fed with either 0.6 or 1.8% Pi diets for 7, 14, or 21 days.

The plasma Pi concentration was significantly higher at 14 and 21 days in the 1.8% Pi diet group compared with the 0.6% Pi diet group (Fig. 6a). Plasma Ca concentration was significantly lower at 7 and 14 days in the 1.8% Pi diet group compared with the 0.6% Pi diet group, but no significant differences were observed after 21 days in the 1.8% Pi diet group compared with the 0.6% Pi diet group (Fig. 6b). Urinary Pi excretion was significantly higher in the 1.8% Pi diet group compared with the 0.6% Pi diet group. Interestingly, after 21 days of the 1.8% Pi diet group, urinary Pi excretion was significantly lower compared with days 7 and 14 (Fig. 6c). Urinary Ca excretion was significantly higher at 7 and 14 days of in the 1.8% Pi diet group compared with the 0.6% Pi diet group (Fig. 6d).

Long-term effects of a high Pi diet on the FGF23/α-klotho signal in weaned mice

To examine the effects of long-term administration of a high Pi diet on the FGF23/α-klotho signal in weaned mice, we measured plasma FGF23 concentration, and renal α-klotho and Egr-1 mRNA expression. The plasma FGF23 concentration of the 1.8% Pi diet group increased 60 times compared with that of 0.6% Pi diet group at 7 days, and this was sustained until 21 days (Fig. 7a). Renal α-klotho mRNA expression in the 1.8% Pi diet group was significantly decreased by about

20% compared with the 0.6% Pi diet group (Fig. 7b). Renal Egr-1 mRNA expression was significantly higher in the 1.8% Pi diet group compared with the 0.6% Pi diet group. Interestingly, this then significantly decreased at 21 days in the 1.8% Pi diet group compared with 7 and 14 days, although high plasma FGF23 levels were sustained (Fig. 7c).

Discussion

Here, we examined the effects of dietary Pi on Pi metabolism, renal calcification, and the FGF23/α-klotho signaling pathway under different doses of dietary Pi, at different life stages, and over different experimental periods in mice. Interestingly, a high Pi diet only decreased renal α-klotho mRNA expression and caused renal calcification in weaned mice. Previous reports showed that a high Pi diet decreased renal α-klotho expression and caused renal calcification in adult mice and rats [19, 24]. However, there have been no studies that have examined the effect of a high Pi diet on renal α-klotho mRNA expression and renal calcification at different life stages. Renal α-klotho mRNA expression is reported to have been suppressed in CKD patients [25], and TNF-α was increased by Pi overload in CKD rats [26-28]. In this study, renal TNF-α mRNA expression was significantly higher in the weaned mice fed with a high Pi diet. Thus, it is suggested that the decrease in renal α-klotho expression observed in the weaned mice fed with a high Pi diet could be related to impaired renal function and/or increased inflammatory cytokines such as TNF-α.

A high Pi diet suppressed expression of α-klotho mRNA and caused abnormal mineral metabolism in the weaned mice. Similar abnormal Pi and Ca metabolism have also been observed in kl/kl mice [16, 29]. Therefore, the abnormal Pi and Ca metabolism in the weaned mice fed with a high Pi diet could be due to the decrease in renal α-klotho expression. As shown by the results from the long-term administration of a high Pi diet in the weaned mice, plasma Pi levels increased significantly after 14 and 21 days in spite of a sustained increase in serum FGF23 levels. FGF23 decreases expression of sodium-dependent phosphate transporters Npt2a and Npt2c in the kidney, whose function is to increase urinary Pi excretion to maintain plasma Pi levels (22). However, urinary Pi excretion decreased at 14 and 21 days. At 21 days in particular, renal Egr-1 mRNA levels were decreased, suggesting that suppression of the α-klotho signaling pathway may be a cause of abnormal Pi metabolism under long-term administration of a high Pi diet. Thus, the suppression of α-klotho expression could be involved in a FGF23-resistant state.

On the other hand, kl/kl mice grow normally and are indistinguishable from their +/+ or kl/+ littermates up to 3 to 4 weeks of age [15]. The previous observation suggests that the effects of high Pi diet on weaned mice are not simply for the sake of decreased α-klotho expression. Although there are not enough results to support these hypotheses, high Pi intake in growing period must be harmful rather than that in adulthood.

On the other hand, urinary Ca excretion increased in the weaned mice fed with a high Pi diet;

254 urinary Ca excretion remained at the level of day 0, although it gradually decreased during the
255 growth period with a 0.6% Pi diet. Renal TRPV5 mRNA expression was significantly higher in
256 the weaned mice fed with a high Pi diet. Increased urinary Ca excretion and renal TRPV5
257 expression have also been reported in kl/kl mice [30]. This is consistent with increased Ca
258 excretion. In the kidney, Ca can be transported into cells across the apical membrane via TRPV5,
259 and can be exported to the interstitial space across the basolateral membrane via Na^+/Ca^{2+}
260 exchangers (NCX1) [31]. In kl/kl mice, renal TRPV5 mRNA expression increased; however,
261 NCX1 mRNA expression decreased [30]. It has been reported that TRPV5 cannot be retained at
262 the apical membrane in the absence of klotho [32]. Therefore, an increase in renal TRPV5 mRNA
263 expression is probably functionally insignificant, and this notion is further supported by the
264 decreased expression of NCX1 in kl/kl mice [30]. Therefore, urinary Ca excretion increased
265 despite the increased TRPV5 mRNA expression in our study, probably because the efflux of Ca
266 to the interstitial space was inhibited due to suppressed NCX1 expression. However, protein
267 expression or phosphorylation studies for TRPV5 are needed to clarify the details.
268 Furthermore, α-klotho is essential for the recruitment of Na^+/K^+-ATPase to the basolateral
269 membrane, which is important to reduce extracellular ionized Ca^{2+}, and it is suggested that
270 hypercalciuria in kl/kl mice resulted from abnormal Ca reabsorption caused by α-klotho
271 deficiency [33]. In this study, it is possible that a similar abnormality occurred, because renal α-

klotho expression decreased markedly in the weaned mice fed a high Pi diet.

Generally, a high Pi diet can increase plasma FGF23 and PTH concentration [34]. This study showed plasma FGF23 concentration increased in response to an increase in dietary Pi content. However, plasma PTH concentration in the weaned mice fed with a high Pi diet did not significantly increase. This might be due to high plasma FGF23 levels, because FGF23 can directly suppress the secretion of PTH [17]. Furthermore, secretion of PTH is also regulated by α-klotho dependent on Na^+/K^+-ATPase in the parathyroid glands [33]. The secretion of PTH was also suppressed in kl/kl mice compared to wild-type mice [33]. In this study, we did not examine parathyroid tissue to study PTH secretion. However, suppressed α-klotho expression in the parathyroid glands was also involved in the suppressed PTH secretion by a high Pi diet in the weaned mice. Therefore, it is suggested that the marked decrease in α-klotho expression caused by a high Pi diet induced abnormal Pi and Ca metabolism in the weaned mice.

In the short-term study, a high Pi diet increased FGF23/α-klotho in the weaned mice, despite decreased renal α-klotho expression. It is known that activation of the FGF23/α-klotho signal suppresses $1,25(OH)_2D$ production, by suppression of renal Cyp27b1 expression. However, plasma $1,25(OH)_2D$ concentration and renal Cyp27b1 expression tended to increase in the weaned mice fed with a high Pi diet. Recent reports have indicated that renal Cyp27b1 expression is induced by TNF-α [35-37]. In this study, renal TNF-α mRNA expression was significantly higher

290 in the weaned mice fed with a high Pi diet. Therefore, TNF-α may be an important factor behind
291 the increase in plasma $1,25(OH)_2D$ concentration. Unfortunately, we could not clarify the
292 mechanism at this time, further studies will be needed.

293 In addition, the activation of the FGF23/α-klotho signal also contributes to the maintenance
294 of Pi homeostasis; however, metabolic disorders of Pi such as increase in plasma Pi concentration
295 were caused in the weaned mice fed with a high Pi diet. Therefore, the activation of FGF23/α-
296 klotho signal would be not sufficient for the adaptation to the high dietary Pi intake in the weaned
297 mice due to the marked decrease in renal α-klotho expression.

298 This study has some limitation. We did not evaluate plasma circulating α-klotho and PTH
299 levels and analyzing mineralization and FGF23 expression in bone. Such data are important to
300 understand the effect of high Pi diet on bone phenotype such as osteomalacia, and regulation of
301 hormone secretion. However, we focused renal regulation of Pi metabolism, especially α-klotho
302 expression, which is the most important step for Pi homeostasis, and ectopic calcification which
303 is important phenotype in CKD and aging. In addition, long-term study was only performed using
304 weaned mice. Although the long-term effects of high Pi diet on adolescent or older mice is also
305 challenging question, our long-term study at this time is supportive data for short-term study. To
306 address those questions, further study will be needed.

307 The present study indicates that a high Pi diet has much greater adverse effects on renal α-

klotho expression and pathogenesis involving renal calcification in weaned mice compared with periadolescent mice. These results suggest that a high Pi intake during growth periods in juveniles must be more harmful than in periadolescent or later period. In addition, long-term administration of a high Pi diet may cause an FGF23-resistant state due to the suppression of renal α-klotho expression.

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Compliance with Ethical Standards

Conflict of interest Shiori Fukuda-Tatano, Hironori Yamamoto, Otoki Nakahashi, Ryouhei Yoshikawa, Mayu Hayashi, Maki Kishimoto, Yukiko Imi, Hisami Yamanaka-Okumura, Kohta Ohnishi, Masashi Masuda and Yutaka Taketani declare no conflicts of interest.

Human and Animal Rights and Informed Consent The present study was approved by the Animal Experimentation Committee of Tokushima University and was conducted in accordance with the guidelines for the management and handling of experimental animals.

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446 **Figure Captions**

447 **Fig. 1** Effects of dietary Pi on biochemical parameters in plasma and urine

448 Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven

449 experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. **a** plasma

450 Pi. **b** plasma Ca. **c** urine Pi/Cre. **d** urine Ca/Cre. Data are represented as means ± SEM (n = 6–9).

451 Different letters between groups show significant statistical differences with at least $p < 0.05$.

452 Significant effect ($p < 0.05$): D = effect of dietary Pi; A = effect of age; D*A = effect of interaction.

453

454 **Fig. 2** Effects of dietary Pi on phosphate-regulating factors in plasma

455 Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven

456 experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. **a** plasma

457 FGF23. **b** plasma $1,25(OH)_2D$. **c** plasma parathyroid hormone 1-84 (PTH 1-84). Data are

458 represented as means ± SEM (n = 6-9). Different letters between groups show significant

459 statistical differences with at least $p < 0.05$. Significant effect ($p < 0.05$): D = effect of dietary Pi;

460 A = effect of age; D*A = effect of interaction.

461

462 **Fig. 3** Effects of dietary Pi on mRNA expression of renal FGF23/α-klotho signal-related gene and

463 inflammatory cytokines

464 Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven
465 experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Total
466 mRNA was prepared from the kidney of each mouse, and gene expression was measured by
467 quantitative RT-PCR. **a** α-klotho mRNA expression. **b** FGF receptor 1 (FGFR1) mRNA
468 expression. **c** Egr-1 mRNA expression. **d** TNF-α mRNA expression. Data are represented as
469 means ± SEM (n = 6-9). Different letters between groups show significant statistical differences
470 with at least $p < 0.05$. Significant effect ($p < 0.05$): D = effect of dietary Pi; A = effect of age; D*A
471 = effect of interaction.
472
473 **Fig. 4** Effects of dietary Pi on mRNA expression for renal phosphate transporters, Ca transporter,
474 and related genes for vitamin D metabolism
475 Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven
476 experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Total
477 mRNA was prepared from the kidney of each mouse, and gene expression was measured by
478 quantitative RT-PCR. **a** Npt2a mRNA expression. **b** Npt2c mRNA expression. **c** Cyp27b1 mRNA
479 expression. **d** Cyp24a1 mRNA expression. **e** TRPV5 mRNA expression. Data are represented as
480 means ± SEM (n = 6-9). Different letters between groups show significant statistical differences
481 with at least $p < 0.05$. Significant effect ($p < 0.05$): D = effect of dietary Pi; A = effect of age; D*A

= effect of interaction.

Fig. 5 Effects of dietary Pi on renal calcification

Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Von Kossa staining was performed to detect renal calcification. **a** Renal calcification in the 0.6, 0.9, 1.2, 1.5 or 1.8% Pi diet groups in weaned mice kidneys. **b** Renal calcification in the 1.8% Pi diet groups in weaned and periadolescent mice kidneys. Scale bars = 100 μm

Fig. 6 Effects of a long-term high Pi diet in weaned mice on biochemical parameters in their plasma and urine

Weaned (3 weeks old) mice were randomly divided into two experimental groups and fed with either 0.6 % (○) or 1.8% (■) Pi diet for 7, 14, or 21 days. **a** plasma Pi. **b** plasma Ca. **c** urine Pi/Cre. **d** urine Ca/Cre. Data are represented as means ± SEM (n = 6-9). $^{*}p < 0.05$ vs 0.6% Pi diet group at the same time point. $^{\#}p < 0.05$ vs 0 day, $^{¶}p < 0.05$ vs 1.8% Pi diet for the 21-day group. $^{§}p < 0.05$ vs 0.6% Pi diet group for 7-day group.

Fig. 7 Effects of a long-term high Pi diet in weaned mice on the FGF23/α-klotho signal

500 Weaned (3 weeks old) mice were randomly divided into two experimental groups and fed with
501 either 0.6 % (○) or 1.8% (■) Pi diet for 7, 14, or 21 days. **a** plasma FGF23. Total mRNA was
502 prepared from the kidney of each mouse, and gene expression was measured by quantitative RT-
503 PCR. **b** α-klotho mRNA expression. **c** Egr-1 mRNA expression. Data are represented as means ±
504 SEM (n = 6-9). $^{*}p < 0.05$ vs 0.6% Pi diet group at the same time point. ¶$p < 0.05$ vs 1.8% Pi diet
505 for 21-day group.
506

Table 1 Composition of experimental diets

Ingredient (g)	Pi						
	0.02%	0.3%	0.6%	0.9%	1.2%	1.5%	1.8%
Egg white	20.0	20.0	20.0	20.0	20.0	20.0	20.0
L-Cysteine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cornstarch	39.7	39.7	39.7	39.7	39.7	39.7	39.7
α-Cornstarch	13.2	13.2	13.2	13.2	13.2	13.2	13.2
Sugar	10.31	9.08	7.77	6.45	5.13	3.81	2.49
Soybean oil	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Tert-butylhydroquinone	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$CaCO_3$	1.4980	1.4980	1.4980	1.4980	1.4980	1.4980	1.4980
KH_2PO_4	0.0879	1.3183	2.6366	3.9548	5.2731	6.5914	7.9097
Mineral mix changed	1.5645	1.5645	1.5645	1.5645	1.5645	1.5645	1.5645

The mineral mix did not contain $CaCO_3$ or KH_2PO_4.

Table 2 Sequence of oligonucleotide primers for quantitative RT-PCR analysis

Gene Name	Sense Primer (5' to 3')	Antisense Primer (5' to 3')	Accession Number
α-klotho	CAAAAGCTGATAGAGGACAATGGC	GGCAGAGAAATCAACACAGTAAGG	NM_013823
FGFR1	CCAGTGCATCCATGAACTCTGGGGTTCTCC	GGTCACACGGTTGGGTTTGTCCTTATCCAG	NM_010206
Egr-1	AGCGAACAACCCTATGAGCA	TCGTTTGGCTGGGATAACTC	NM_007913
TNF-α	AGCCTGTAGCCCACGTCGTA	TCTTTGAGATCCATGCCGTTG	NM_013693
Npt2a	AGAGCCCTTCACAAGACTCATCAT	TACCCTGGACATAGAAGTGGAAGC	NM _011392
Npt2c	TGAAGAACGCTGACCAACTGA	AGCAGAGCTGAGGATGTCCAG	NM _080854
Cyp27b1	ATGGTGAAGAATGGCAGAGG	TAGTCGTCGCACAAGGTCAC	NM _010009
Cyp24a1	TGCCATTCACAACTCGGACCCT	TCAAGCCAGCGTTCGGGTCTAA	NM _009996
TRPV5	CAGCACGTGGATCAGCTACA	CTCTTTGCCGGAAGTCACAG	NM_001007572
β-actin	CTGACCCTGAAGTACCCCATTGAACA	CTGGGGTGTTGAAGGTCTCAAACATG	NM_007393

Fig. 1

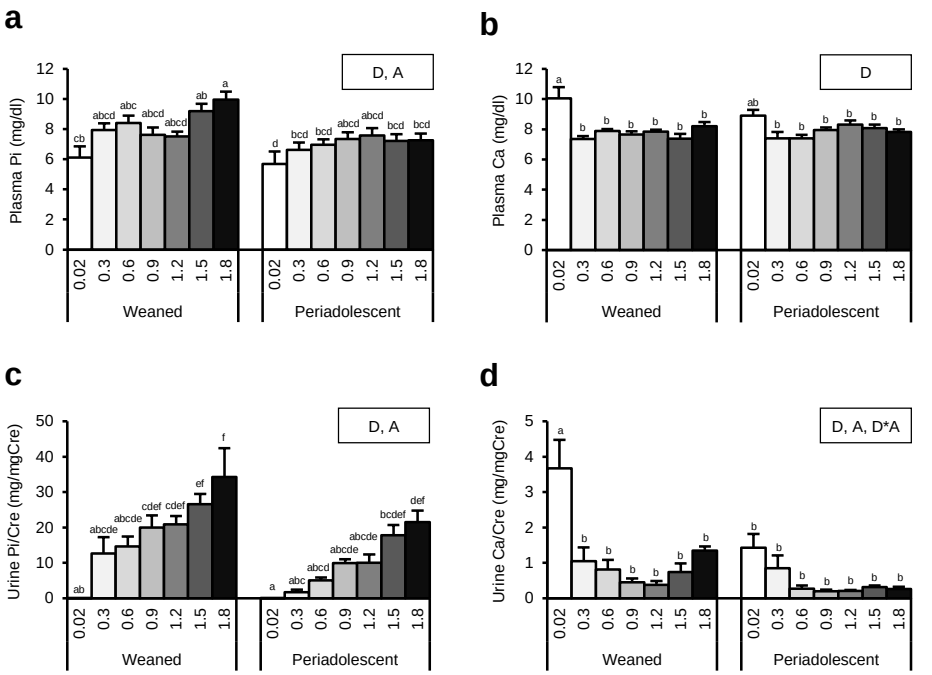

a
Plasma Pi (mg/dl)
D, A
cb
abcd
abc
abcd
abcd
ab
a
d
bcd
bcd
abcd
abcd
bcd
bcd
0.02
0.3
0.6
0.9
1.2
1.5
1.8
Weaned
Periadolescent
b
Plasma Ca (mg/dl)
D
a
b
ab
c
Urine Pi/Cre (mg/mgCre)
D, A
ab
abcde
abcde
cdef
cdef
ef
f
a
abc
abcd
abcde
abcde
bcdef
def
d
Urine Ca/Cre (mg/mgCre)
D, A, D*A

Fig. 2

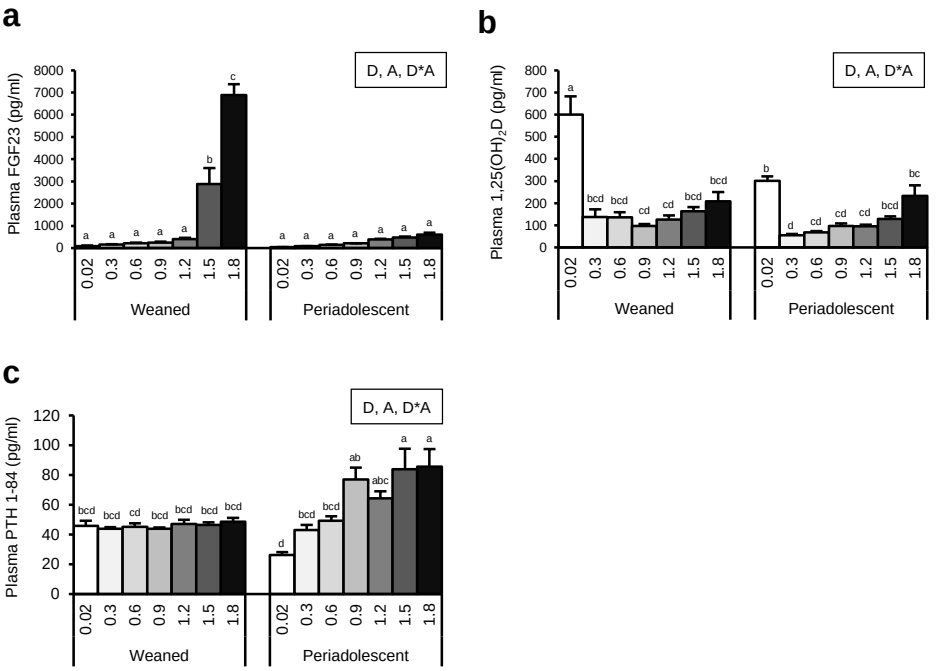

a
b
c
D, A, D*A
Plasma FGF23 (pg/ml)
Plasma 1,25(OH)2D (pg/ml)
Plasma PTH 1-84 (pg/ml)
Weaned
Periadolescent
0.02
0.3
0.6
0.9
1.2
1.5
1.8

Fig. 3

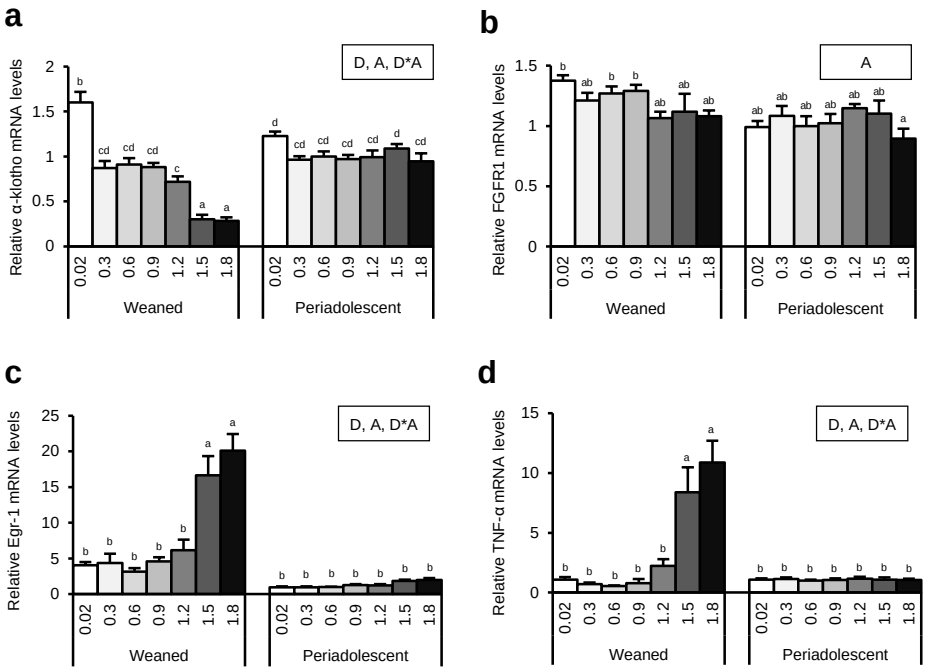

a
Relative α-klotho mRNA levels
D, A, D*A
Weaned
Periadolescent
b
Relative FGFR1 mRNA levels
A
c
Relative Egr-1 mRNA levels
d
Relative TNF-α mRNA levels

Fig. 4

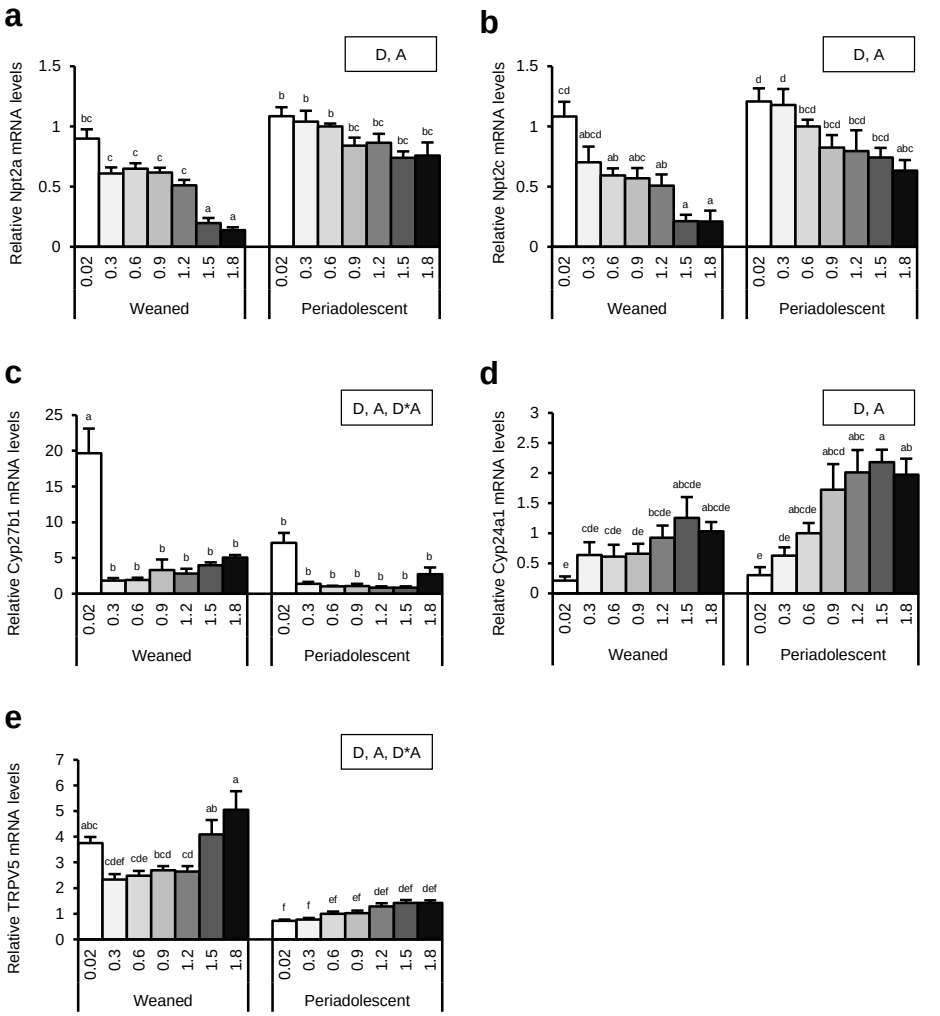
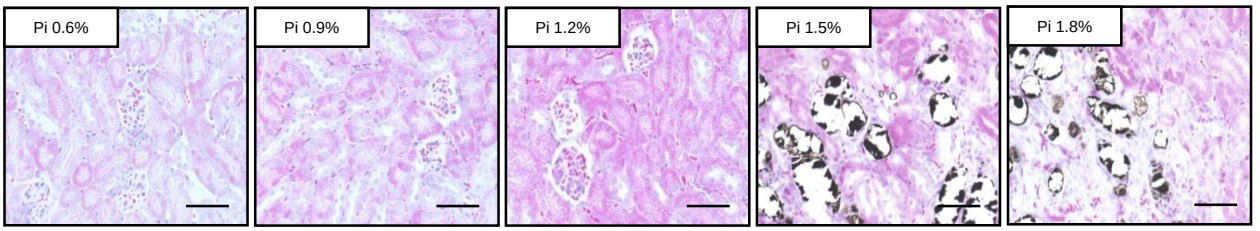

a
D, A
Relative Npt2a mRNA levels
1.5
1
0.5
0
bc c c c c a a b b b bc bc bc bc
0.02 0.3 0.6 0.9 1.2 1.5 1.8 0.02 0.3 0.6 0.9 1.2 1.5 1.8
Weaned
Periadolescent
b
D, A
Relative Npt2c mRNA levels
cd abcd ab abc ab a a d d bcd bcd bcd bcd abc
c
D, A, D*A
Relative Cyp27b1 mRNA levels
25
20
15
10
5
0
a b b b b b b b b b b b b b
d
D, A
Relative Cyp24a1 mRNA levels
3
2.5
2
1.5
1
0.5
0
e cde cde de bcde abcde abcde e de abcde abcd abc a ab
e
D, A, D*A
Relative TRPV5 mRNA levels
7
6
5
4
3
2
1
0
abc cdef cde bcd cd ab a f f ef ef def def def

Fig. 5

a



b

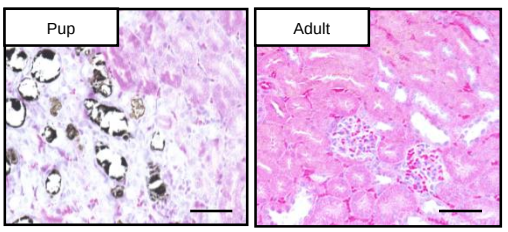


Fig. 6

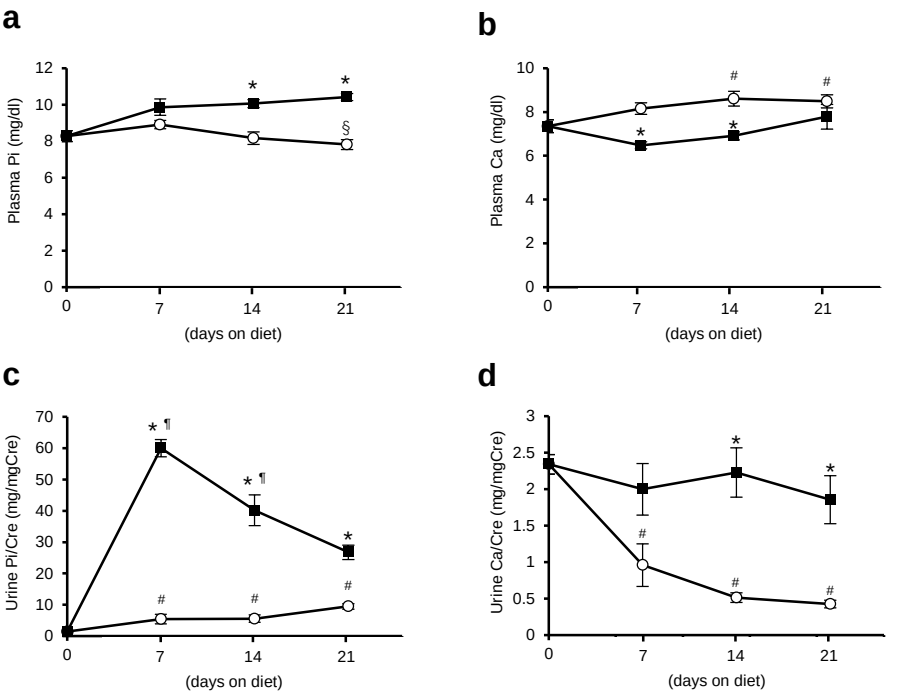
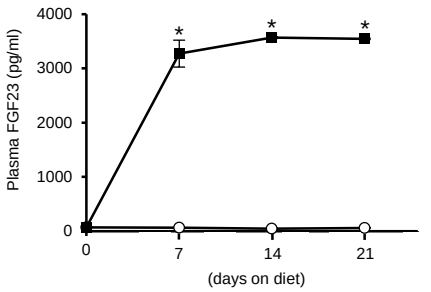
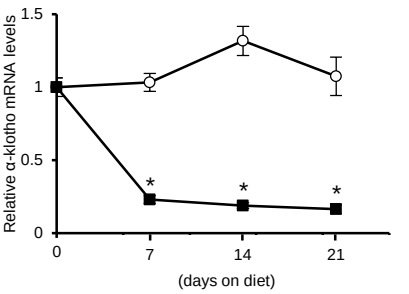

a
Plasma Pi (mg/dl)
12
10
8
6
4
2
0
0
7
14
21
(days on diet)
*
*
§
b
Plasma Ca (mg/dl)
10
8
6
4
2
0
0
7
14
21
(days on diet)
#
#
*
*
c
Urine Pi/Cre (mg/mgCre)
70
60
50
40
30
20
10
0
0
7
14
21
(days on diet)
* ¶
* ¶
*
#
#
#
d
Urine Ca/Cre (mg/mgCre)
3
2.5
2
1.5
1
0.5
0
0
7
14
21
(days on diet)
*
*
#
#
#

Fig. 7

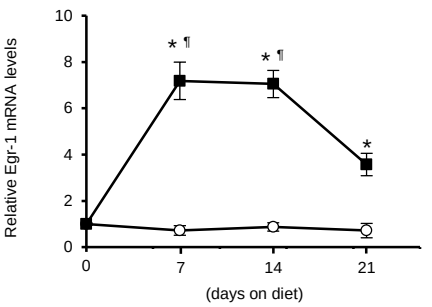
a


Plasma FGF23 (pg/ml)
4000
3000
2000
1000
0
0
7
14
21
(days on diet)
*
*
*

b


Relative α-klotho mRNA levels
1.5
1
0.5
0
0
7
14
21
(days on diet)
*
*
*

c


Relative Egr-1 mRNA levels
10
8
6
4
2
0
0
7
14
21
(days on diet)
* ¶
* ¶
*