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ORIGINAL RESEARCH

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 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

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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 twofold [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

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

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 washed with distilled water and immersed in 5% hypo solution (sodium thiosulfate). Sections were counterstained with hematoxylin–eosin (HE).

Statistical Analysis

Results are expressed as the mean ± SEM for each group. Statistical significance was determined by the Tukey–Kramer post hoc test, after two-way ANOVA. A p value < 0.05 was considered to be significant.

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

Gene name	Sense primer (5′–3′)	Antisense primer (5′–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

Results

Effects of Dietary Pi on Biochemical Parameters in Plasma and Urine

To examine the effects of dietary Pi on biochemical parameters in plasma and urine, mice were fed diets of different Pi content. As dietary Pi content increased, plasma Pi concentration increased in the weaned group, but not in the periadolescent group (Fig. 1a). Plasma Ca concentration did not change in either group except for mice on the 0.02% Pi diet (Fig. 1b). As dietary Pi content increased, urinary Pi excretion increased in both groups (Fig. 1c). A high Pi diet (1.5 and 1.8% Pi) did not increase urinary Ca excretion in the periadolescent group, but a high Pi diet (1.5 and 1.8% Pi) did increase urinary Ca excretion compared to the 0.9% Pi diet (Fig. 1d).

Effects of Dietary Pi on Phosphate-Regulating Factors in Plasma

To examine the effects of dietary Pi on phosphate-regulating factors in plasma, we measured plasma FGF23, $1,25(OH)_2D$, and PTH 1-84. The plasma FGF23 concentration was significantly elevated in the weaned mice fed with a high Pi diet (1.5 and 1.8% Pi). In the periadolescent group, a high Pi diet slightly, but significantly, increased plasma FGF23 levels (Fig. 2a). Plasma $1,25(OH)_2D$ concentration was significantly elevated in the low Pi diet group (0.02% Pi), and tended to increase, as dietary Pi content increased, in both groups (Fig. 2b). A high Pi diet (1.5 and 1.8% Pi) increased plasma PTH 1-84 concentration compared with the 0.6% Pi diet in the periadolescent group, but not in the weaned group (Fig. 2c).

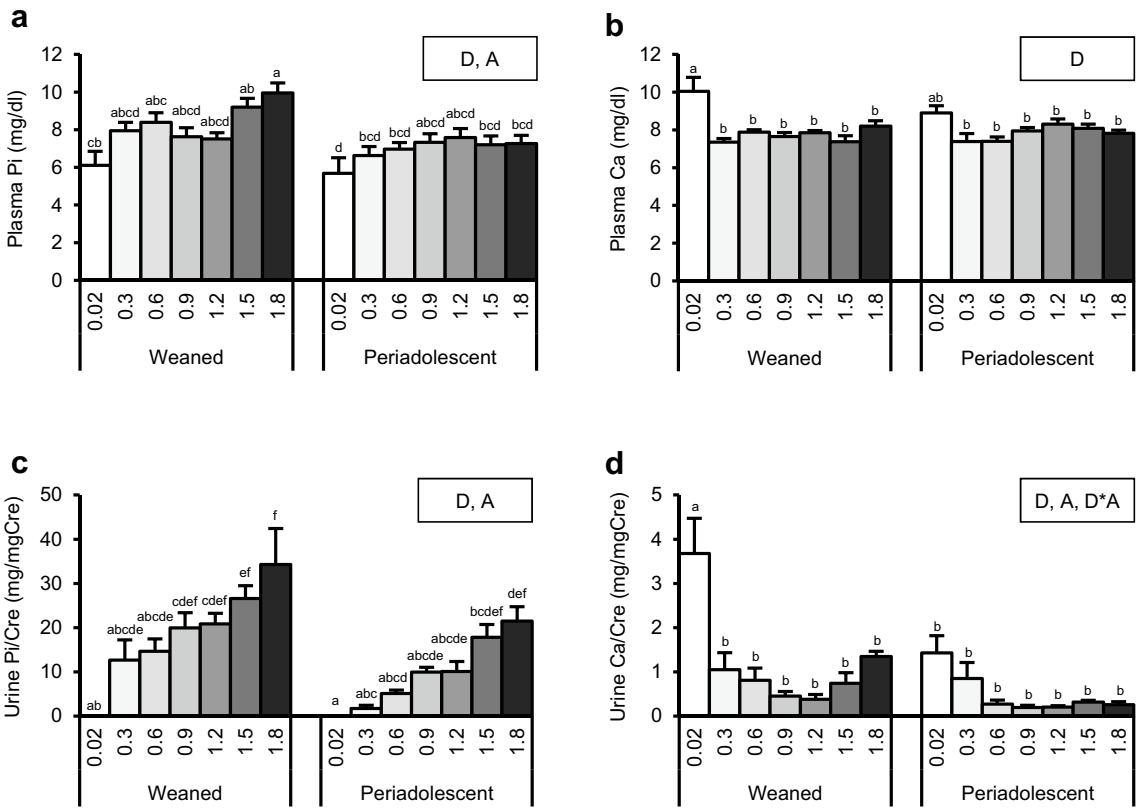

Fig. 1 Effects of dietary Pi on biochemical parameters in plasma and urine. Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. **a** Plasma Pi. **b** Plasma Ca. **c** Urine Pi/Cre. **d** Urine Ca/Cre. Data are represented as means ± SEM ($n = 6$–9). Different letters between groups show significant statistical differences 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

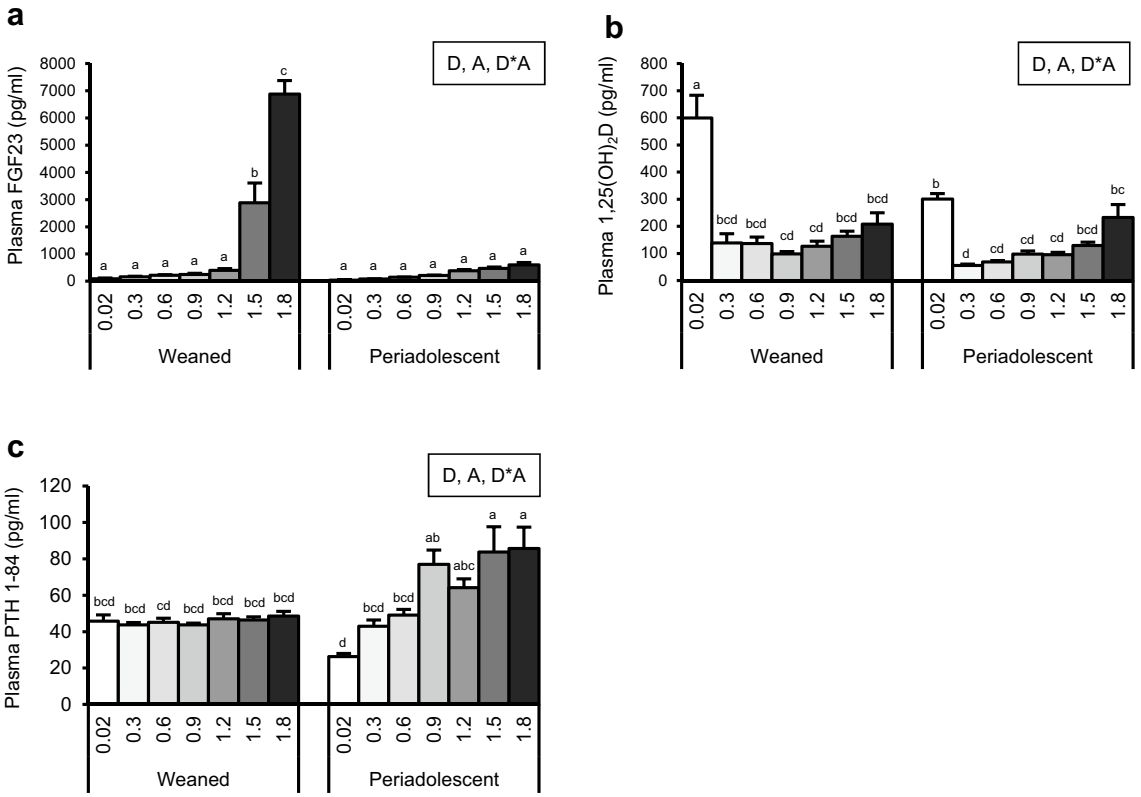

Fig. 2 Effects of dietary Pi on phosphate-regulating factors in plasma. Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. **a** Plasma FGF23. **b** Plasma $1,25(OH)_2D$. **c** Plasma parathyroid hormone 1-84 (PTH 1-84). Data are represented as means ± SEM ($n = 6$–9). Different letters between groups show significant statistical differences 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

Effects of Dietary Pi on mRNA Expression of Renal FGF23/α-klotho Signal-Related Gene and Inflammatory Cytokines

To examine the effects of dietary Pi on the renal FGF23/α-klotho signaling pathway, we measured renal α-klotho and FGFR1 mRNA expression levels using RT-PCR. In the periadolescent group, renal α-klotho mRNA expression levels did not change significantly. On the other hand, in the weaned group, α-klotho mRNA expression levels decreased significantly following a high Pi diet (1.5% and 1.8% Pi) (Fig. 3a). Renal FGFR1 mRNA expression did not change in either group. We hypothesized that the decrease in renal α-klotho expression in the 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

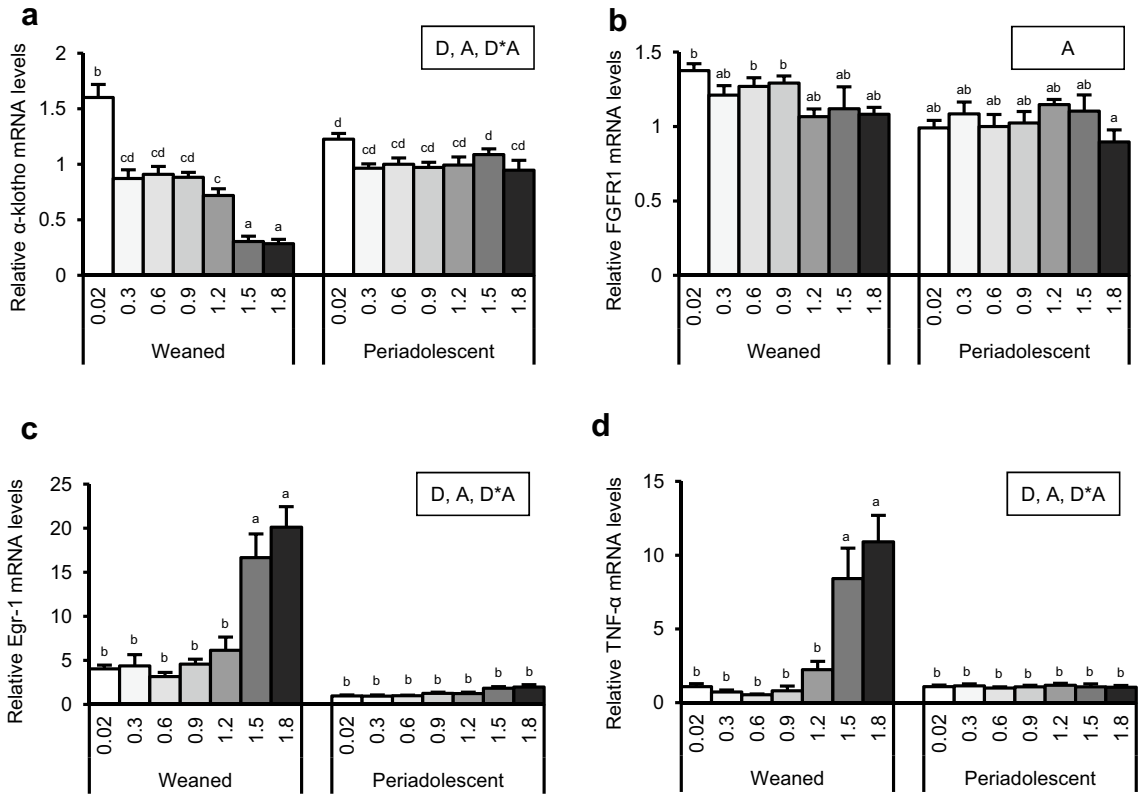

Fig. 3 Effects of dietary Pi on mRNA expression of renal FGF23/α-klotho signal-related gene and inflammatory cytokines. Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Total mRNA was prepared from the kidney of each mouse, and gene expression was measured by quantitative RT-PCR. **a** α-Klotho mRNA expression. **b** FGF receptor 1 (FGFR1) mRNA expression. **c** Egr-1 mRNA expression. **d** TNF-α mRNA expression. Data are represented as means ± SEM ($n = 6$–9). Different letters between groups show significant statistical differences 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

mRNA for the transient receptor potential vanilloid member 5 (TRPV5), which is a major calcium transporter in the apical membrane of renal distal tubules, was significantly higher in the weaned mice fed with a high Pi diet (Fig. 4e).

Effects of Dietary Pi on Renal Calcification

Recent reports have indicated that a high Pi diet can induce renal calcification [23, 24]. Therefore, we investigated the effects of dietary Pi on renal calcification using Von Kossa staining. As shown in Fig. 5a, a high Pi diet (1.5 and 1.8% Pi) clearly induced renal calcification in the weaned group (Fig. 5a). However, in the periadolescent group, renal calcification was not observed, even in the 1.8% Pi diet group (Fig. 5b).

Long-Term Effects of a High Pi Diet on Biochemical Parameters in the Plasma and Urine of Weaned Mice

In the short-term study of the two life stages, it was clear that a high Pi diet had much greater adverse effects on the kidneys of weaned mice compared with periadolescent mice. Moreover, the FGF23/α-klotho signal was activated in the weaned mice fed with high a Pi diet, despite the fact that renal α-klotho mRNA expression decreased. However, it was still unclear what long-term effects a high Pi diet may have had on the FGF23/α-klotho signal in the weaned group. 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

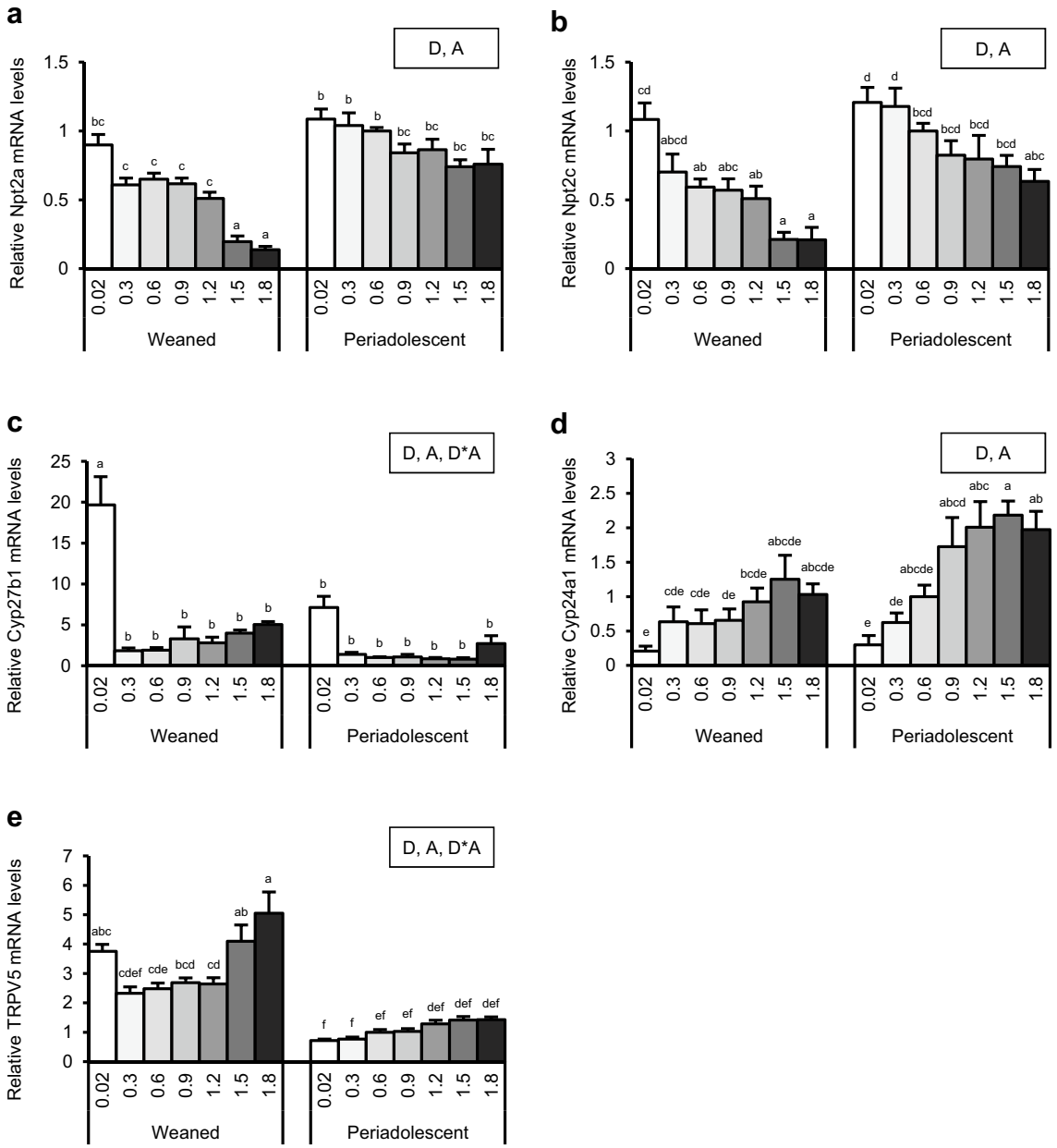

Fig. 4 Effects of dietary Pi on mRNA expression for renal phosphate transporters, Ca transporter, and related genes for vitamin D metabolism. Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Total mRNA was prepared from the kidney of each mouse, and gene expression was measured by quantitative RT-PCR. **a** Npt2a mRNA expression. **b** Npt2c mRNA expression. **c** Cyp27b1 mRNA expression. **d** Cyp24a1 mRNA expression. **e** TRPV5 mRNA expression. Data are represented as means ± SEM ($n = 6$–9). Different letters between groups show significant statistical differences 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

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

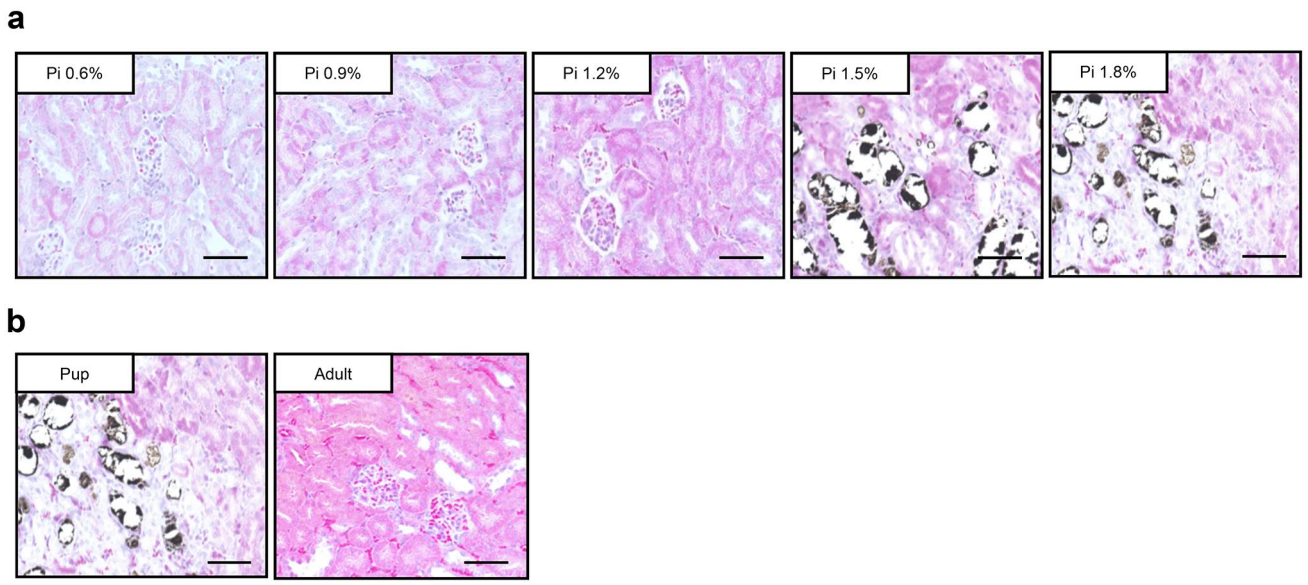

Fig. 5 Effects of dietary Pi on renal calcification. Weaned (3 weeks old) and periadolescent (7 weeks old) mice were randomly divided into seven experimental groups and fed with 0.02, 0.3, 0.6, 0.9, 1.2, 1.5, or 1.8% Pi diets for 7 days. Von Kossa staining was performed to detect renal calcification. **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

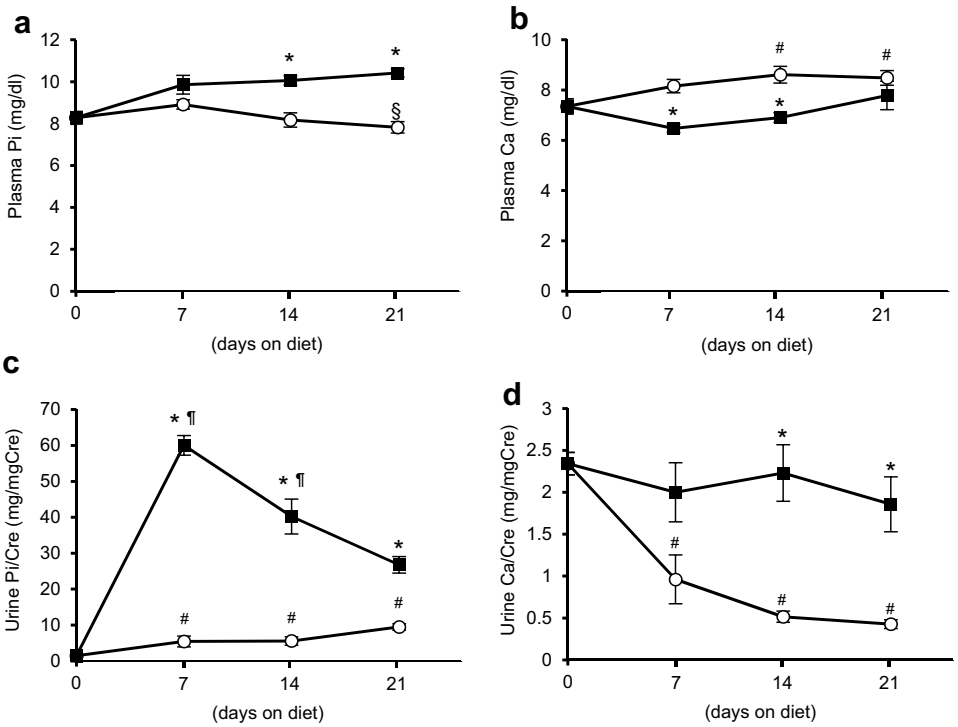

Fig. 6 Effects of a long-term high Pi diet in weaned mice on biochemical parameters in their plasma and urine. Weaned (3 weeks old) mice were randomly divided into two experimental groups and fed with either 0.6% (open circle) or 1.8% (filled square) Pi diet for 7, 14, or 21 days. **a** Plasma Pi. **b** Plasma Ca. **c** Urine Pi/Cre. **d** Urine Ca/Cre. Data are represented as means ± SEM ($n = 6$–9). *$p < 0.05$ versus 0.6% Pi diet group at the same time point. $^{\#}p < 0.05$ versus 0 day, $^{¶}p < 0.05$ versus 1.8% Pi diet for the 21-day group. $^{§}p < 0.05$ versus 0.6% Pi diet group for 7-day group

and Egr-1 mRNA expression levels. 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

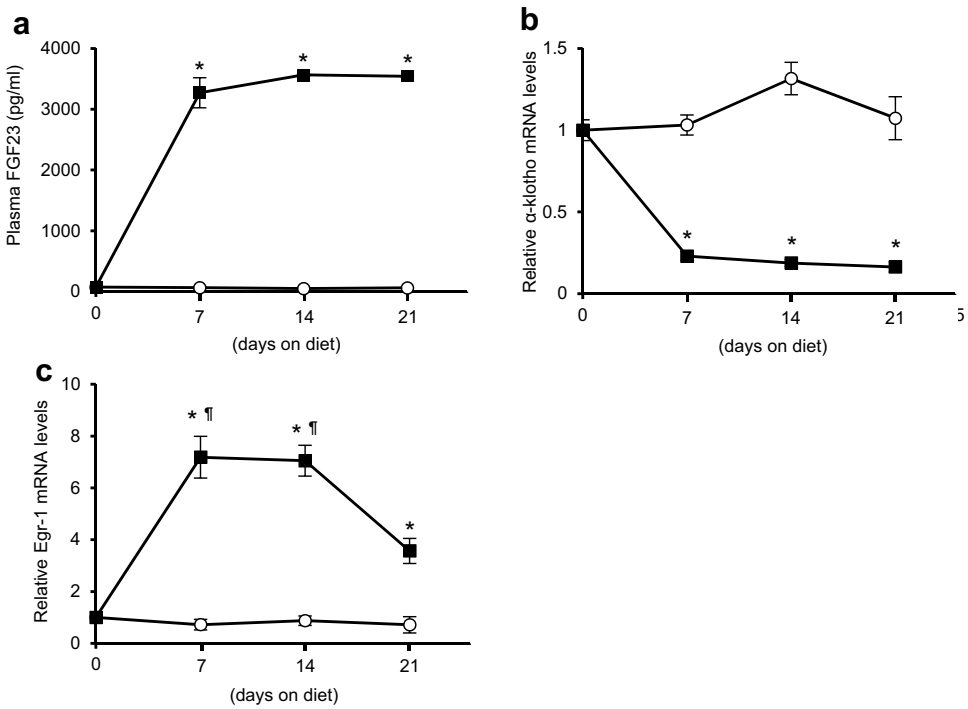

Fig. 7 Effects of a long-term high Pi diet in weaned mice on the FGF23/α-klotho signal. Weaned (3 weeks old) mice were randomly divided into two experimental groups and fed with either 0.6% (open circle) or 1.8% (filled square) Pi diet for 7, 14, or 21 days. **a** Plasma FGF23. Total mRNA was prepared from the kidney of each mouse, and gene expression was measured by quantitative RT-PCR. **b** α-klotho mRNA expression. **c** Egr-1 mRNA expression. Data are represented as means ± SEM ($n = 6$–9). *$p < 0.05$ versus 0.6% Pi diet group at the same time point. ¶$p < 0.05$ versus 1.8% Pi diet for 21-day group

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 3–4 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; urinary Ca excretion remained at the level of day 0, although it gradually decreased during the growth period with a 0.6% Pi diet. Renal TRPV5 mRNA expression was significantly higher in the weaned mice fed with a high Pi diet. Increased urinary

Ca excretion and renal TRPV5 expression have also been reported in kl/kl mice [30]. This is consistent with increased Ca excretion. In the kidney, Ca can be transported into cells across the apical membrane via TRPV5, and can be exported to the interstitial space across the basolateral membrane via Na^+/Ca^{2+} exchangers (NCX1) [31]. In kl/kl mice, renal TRPV5 mRNA expression increased; however, NCX1 mRNA expression decreased [30]. It has been reported that TRPV5 cannot be retained at the apical membrane in the absence of klotho [32]. Therefore, an increase in renal TRPV5 mRNA expression is probably functionally insignificant, and this notion is further supported by the decreased expression of NCX1 in kl/kl mice [30]. Therefore, urinary Ca excretion increased despite the increased TRPV5 mRNA expression in our study, probably because the efflux of Ca to the interstitial space was inhibited due to suppressed NCX1 expression. However, protein expression or phosphorylation studies for TRPV5 are needed to clarify the details. Furthermore, α-klotho is essential for the recruitment of Na^+/K^+-ATPase to the basolateral membrane, which is important to reduce extracellular ionized Ca^{2+}, and it is suggested that hypercalciuria in kl/kl mice resulted from abnormal Ca reabsorption caused by α-klotho 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 in the weaned mice fed with a high Pi diet. Therefore, TNF-α may be an important factor behind the increase in plasma $1,25(OH)_2D$ concentration. Unfortunately, we could not clarify the mechanism at this time, further studies will be needed.

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

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

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.

References

1. Takeda E, Yamamoto H, Nashiki K, Sato T, Arai H, Taketani Y (2004) Inorganic phosphate homeostasis and the role of dietary phosphorus. J Cell Mol Med 8:191–200
2. Murer H, Hernando N, Forster I, Biber J (2000) Proximal tubular phosphate reabsorption: molecular mechanisms. Physiol Rev 80:1373–1409
3. Takeda E, Yamamoto H, Yamanaka-Okumura H, Taketani Y (2014) Increasing dietary phosphorus intake from food additives: potential for negative impact on bone health. Adv Nutr 5:92–97
4. Kalantar-Zadeh K, Gutekunst L, Mehrotra R, Kovesdy CP, Bross R, Shinaberger CS, Noori N, Hirschberg R, Benner D, Nissenson AR, Kopple JD (2010) Understanding sources of dietary phosphorus in the treatment of patients with chronic kidney disease. Clin J Am Soc Nephrol 5:519–530
5. Calvo MS, Park YK (1996) Changing phosphorus content of the U.S. diet: potential for adverse effects on bone. J Nutr 126:1168S–1180S
6. Benini O, D'Alessandro C, Gianfaldoni D, Cupisti A (2011) Extra-phosphate load from food additives in commonly eaten foods: a real and insidious danger for renal patients. J Ren Nutr 21:303–308
7. Sherman RA, Mehta O (2009) Phosphorus and potassium content of enhanced meat and poultry products: implications for patients who receive dialysis. Clin J Am Soc Nephrol 4:1370–1373
8. Japan MoHLi (2015) The National Health and Nutrition Survey in Japanese
9. Japan MoHLi (2014) Fiscal food additives daily intake overhaul survey in Japanese
10. Miyamoto K, Segawa H, Ito M, Kuwahata M (2004) Physiological regulation of renal sodium-dependent phosphate cotransporters. Jpn J Physiol 54:93–102
11. Wöhrle S, Bonny O, Beluch N, Gaulis S, Stamm C, Scheibler M, Müller M, Kinzel B, Thuery A, Brueggen J, Hynes NE, Sellers WR, Hofmann F, Graus-Porta D (2011) FGF receptors control vitamin D and phosphate homeostasis by mediating renal FGF-23 signaling and regulating FGF-23 expression in bone. J Bone Miner Res 26:2486–2497
12. Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M (2008) Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr Rev 29:726–776
13. Hu MC, Kuro-o M, Moe OW (2013) Renal and extrarenal actions of Klotho. Semin Nephrol 33:118–129
14. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S, Yamashita T (2006) Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature 444:770–774
15. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390:45–51
16. Kuro-o M (2010) Klotho. Pflugers Arch 459:333–343
17. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, Sirkis R, Naveh-Many T, Silver J (2007) The parathyroid is a target organ for FGF23 in rats. J Clin Investig 117:4003–4008
18. Ohnishi M, Razzaque MS (2010) Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. FASEB J 24:3562–3571
19. Morishita K, Shirai A, Kubota M, Katakura Y, Nabeshima Y, Takeshige K, Kamiya T (2001) The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. J Nutr 131:3182–3188
20. Mitobe M, Yoshida T, Sugiura H, Shirota S, Tsuchiya K, Nihei H (2005) Oxidative stress decreases klotho expression in a mouse kidney cell line. Nephron Exp Nephrol 101:e67–e74
21. Hu MC, Shi M, Zhang J, Quiñones H, Griffith C, Kuro-o M, Moe OW (2011) Klotho deficiency causes vascular calcification in chronic kidney disease. J Am Soc Nephrol 22:124–136
22. Reeves PG, Nielsen FH, Fahey GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951
23. Liu QL, Sato S, Kishikawa T, Matsuzaki H, Yamanaka N (2001) Effectiveness of a traditional Chinese medicine, Wulingsan, in suppressing the development of nephrocalcinosis induced by a high phosphorus diet in young rats. Med Electron Microsc 34:103–114
24. Matsuzaki H, Katsumata S, Uehara M, Suzuki K, Miwa M (2007) High-phosphorus diet induces osteopontin expression of renal tubules in rats. J Clin Biochem Nutr 41:179–183
25. Donate-Correa J, Muros-de-Fuentes M, Mora-Fernández C, Navarro-González JF (2012) FGF23/Klotho axis: phosphorus, mineral metabolism and beyond. Cytokine Growth Factor Rev 23:37–46
26. Zhao Y, Banerjee S, Dey N, LeJeune WS, Sarkar PS, Brobey R, Rosenblatt KP, Tilton RG, Choudhary S (2011) Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation. Diabetes 60:1907–1916
27. Moreno JA, Izquierdo MC, Sanchez-Niño MD, Suárez-Alvarez B, Lopez-Larrea C, Jakubowski A, Blanco J, Ramirez R, Selgas R, Ruiz-Ortega M, Egido J, Ortiz A, Sanz AB (2011) The inflammatory cytokines TWEAK and TNFα reduce renal klotho expression through NFκB. J Am Soc Nephrol 22:1315–1325
28. Yamada S, Tokumoto M, Tatsumoto N, Taniguchi M, Noguchi H, Nakano T, Masutani K, Ooboshi H, Tsuruya K, Kitazono T (2014) Phosphate overload directly induces systemic inflammation and malnutrition as well as vascular calcification in uremia. Am J Physiol Ren Physiol 306:F1418–F1428
29. Nabeshima Y (2009) Discovery of alpha-Klotho unveiled new insights into calcium and phosphate homeostasis. Proc Jpn Acad Ser B 85:125–141
30. Woudenberg-Vrenken TE, van der Eerden BC, van der Kemp AW, van Leeuwen JP, Bindels RJ, Hoenderop JG (2012) Characterization of vitamin D-deficient klotho(-/-) mice: do increased levels of serum $1,25(OH)_2D_3$ cause disturbed calcium and phosphate homeostasis in klotho(-/-) mice? Nephrol Dial Transplant 27:4061–4068
31. van de Graaf SF, Hoenderop JG, Bindels RJ (2006) Regulation of TRPV5 and TRPV6 by associated proteins. Am J Physiol Ren Physiol 290:F1295–F1302
32. Alexander RT, Woudenberg-Vrenken TE, Buurman J, Dijkman H, van der Eerden BC, van Leeuwen JP, Bindels RJ, Hoenderop JG (2009) Klotho prevents renal calcium loss. J Am Soc Nephrol 20:2371–2379
33. Imura A, Tsuji Y, Murata M, Maeda R, Kubota K, Iwano A, Obuse C, Togashi K, Tominaga M, Kita N, Tomiyama K, Iijima J, Nabeshima Y, Fujioka M, Asato R, Tanaka S, Kojima K, Ito J, Nozaki K, Hashimoto N, Ito T, Nishio T, Uchiyama T, Fujimori

T (2007) Alpha-Klotho as a regulator of calcium homeostasis. Science 316:1615–1618
34. Scialla JJ, Wolf M (2014) Roles of phosphate and fibroblast growth factor 23 in cardiovascular disease. Nat Rev Nephrol 10:268–278
35. Noyola-Martínez N, Díaz L, Zaga-Clavellina V, Avila E, Halhali A, Larrea F, Barrera D (2014) Regulation of CYP27B1 and CYP24A1 gene expression by recombinant pro-inflammatory cytokines in cultured human trophoblasts. J Steroid Biochem Mol Biol 144(Pt A):106–109
36. Zehnder D, Quinkler M, Eardley KS, Bland R, Lepenies J, Hughes SV, Raymond NT, Howie AJ, Cockwell P, Stewart PM, Hewison M (2008) Reduction of the vitamin D hormonal system in kidney disease is associated with increased renal inflammation. Kidney Int 74:1343–1353
37. Pryke AM, Duggan C, White CP, Posen S, Mason RS (1990) Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. J Cell Physiol 142:652–656