Correlation between hyperphosphatemia and type II Na-Pi cotransporter activity in klotho mice

Hiroko Segawa,1 Setsuko Yamanaka,1 Yasue Ohno,1 Akemi Onitsuka,1 Kazuyo Shiozawa,1 Fumito Aranami,1 Junya Furutani,1 Yuka Tomoe,1 Mikiko Ito,1 Masashi Kuwahata,1 Akihiro Imura,2 Yoichi Nabeshima,2 and Ken-ichi Miyamoto1

1Department of Molecular Nutrition, Institution of Health Biosciences, The University of Tokushima Graduate School, Tokushima; and 2Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Saky, Kyoto, Japan

Submitted 2 July 2006; accepted in final form 6 September 2006

Segawa H, Yamanaka S, Ohno Y, Onitsuka A, Shiozawa K, Aranami F, Furutani J, Tomoe Y, Ito M, Kuwahata M, Imura A, Nabeshima Y, Miyamoto K. Correlation between hyperphosphatemia and type II Na-Pi cotransporter activity in klotho mice. Am J Physiol Renal Physiol 292: F769–F779, 2007. First published September 19, 2006; doi:10.1152/ajprenal.00248.2006.—Recent studies have demonstrated that klotho protein plays a role in calcium/phosphate homeostasis. The goal of the present study was to investigate the regulation of Na-Pi cotransporters in klotho mutant (kl/kl) mice. The kl/kl mice displayed hyperphosphatemia, high plasma 1,25(OH)2D3 levels, increased activity of the renal and intestinal sodium-dependent Pi cotransporters, and increased levels of the type IIa, type Ib, and type IIC transporter proteins compared with wild-type mice. Interestingly, transcript levels of the type IIa/IIc transporter mRNA abundance, but not transcripts levels of type Ib transporter mRNA, were markedly decreased in kl/kl mice compared with wild-type mice. Furthermore, plasma fibroblast growth factor 23 (FGF23) levels were 150-fold higher in kl/kl mice than in wild-type mice. Feeding of a low-Pi diet induced the expression of klotho protein and decreased plasma FGF23 levels in kl/kl mice, whereas colchicine treatment experiments revealed evidence of abnormal membrane trafficking of the type IIa transporter in kl/kl mice. Finally, feeding of a low-Pi diet resulted in increased type IIa Na-Pi cotransporter protein in the apical membrane of the proximal tubular cells, and the type IIa transporter levels in the apical membrane are increased in response to dietary restriction of Pi and 1,25(OH)2D3 and decreased in response to FGF23, PTH, or a high-Pi diet (10, 12, 24–26). This transporter is regulated by physiological stimuli; for example, type IIa transporter levels in the apical membrane are increased in response to dietary restriction of Pi, and 1,25(OH)2D3 and decreased in response to FGF23, PTH, or a high-Pi diet (10, 12, 24–26). In addition, intestinal Pi transport activity and type Ib Na-Pi cotransporter levels are upregulated by 1,25(OH)2D3 (17, 28).

The goal of the present study was to investigate the regulation of Na-Pi cotransporter in kl/kl mice.

MATERIALS AND METHODS

Animal and diet. Heterozygous klotho mutant (kl/+ ) mice were purchased from CLEA Japan (Osaka, Japan) and mated to produce wild-type (+/+) (WT mice) and homozygous klotho mutant (kl/kl) mice (klotho mice). The genetic background of the original kl/kl mouse was a mixture of C57BL/6j and C3H/J (6). Mice genotypes were confirmed using genomic DNA extracted from tail clippings and amplified by polymerase chain reaction (PCR) using primers, as described previously (11). Mice were weaned at 3 wk of age and were given free access to water and standard mice chow (Oriental, Osaka, Japan). The mice were maintained under pathogen-free conditions and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were handled in accordance with the Guidelines for Animal Experimentation of University of Tokushima School of Medicine. All animal experiments were approved by the Animal Experimentation Committee of Tokushima University.

For experiments of dietary regulation, 6-wk-old male mice (C57BL/6N) were purchased from Charles River Japan. The mice were placed on the following diets (isocaloric) for 7 days: 1) low-Pi (LP) 0.02% Pi; 2) control-Pi (CP) 0.6% Pi; and 3) high-Pi (HP) 1.2% Pi (14, 15).

Concentrations of plasma inorganic Ca, Pi, PTH, 1,25(OH)2D3, and FGF23. Concentrations of plasma inorganic Ca and Pi were determined by the Calcium-E test (Wako, Osaka, Japan) and the Phospha-C test (Wako), respectively. Concentrations of plasma PTH were determined using the PTH ELISA kit (Immunotopics, San Clemente, CA). Concentrations of plasma 1,25(OH)2D3 were determined by the radio receptor assay (SRL, Tokyo, Japan). Concentrations of plasma FGF23 proteins were determined using the FGF-23 ELISA kit (KAINOS Laboratories, Tokyo, Japan). Metabolic cages were used for 24-h urine collection from WT and kl/kl mice.

RNA extraction and quantitative PCR analysis. Total RNA was extracted from several mouse tissues using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using the Moloney murine leukemia virus, reverse transcriptase (Superscript, Invitrogen, Carlsbad, CA), and oligo(dT)12–18 primer (4, 16). Quantitative PCR was performed using a LightCycler (Roche Diagnostics). The reaction mixture consisted of 10 ml of SYBR Premix Ex Taq (Perfect Real Time, Takara, Japan) with specific primers. The PCR reactions were initiated with denaturation at 95°C for 10 s, followed by amplification with 50 cycles at 95°C for 10 s, annealing at 60°C for 15 s, and 72°C for 15 s. Data were evaluated with LightCycler run software, version 3 (4, 16).

PCR primer sequences were as follows: FGF23 (5’-ACTTGTCG-CAGAAGCATC-3’ and 5’-GTGGGCGAACAGTGTAGAA-3’); phosphate-regulating gene with homology to endopeptidases on the X chromosome (PHEX) (5’-CCGTGGTGGTCTGTGGAATC-3’ and 5’-CCTCTTCCCTTTCACATTTAAG-3’); matrix extracellular phosphoglycoprotein (MEPE) (5’-TCAGGCTCCAGAAAGCTG-3’ and 5’-CTGGTTGCATAGGCACTG-3’) (9); klotho (5’-CACTG-
GCTTTCCTCCTTTAC-3, 5′-CTTGGCCGCTTATGAG-3′ (secreted form), and 5′-TGCACTCCAGATGAC-3′ (membrane form) (21); and Pit-1 (5′-CCCAGCTGAAGGAGA-3′, 5′-GCCACTGGAGTTGATCTGGT-3′) (accession number NM_015747) and Pit-2 (5′-CTCAGAGGTCGTCAG-3′, 5′-AAACGTGACCGTCATTCCTC-3′) (accession number NM_011394).

**Northern blot analysis.** Poly(A)⁺ RNA was isolated from mouse intestine or kidney and was separated on a 1% agarose gel (3 μg/lane) in the presence of 2.2 M formaldehyde before being blotted onto a Hybond-N membrane (Amersham Bioscience), as described previously (15, 16). The specific probes for type IIa, type IIb, and type IIc Na-Pi cotransporters were labeled with [32P]dCTP using the Megaprome DNA Labeling System (Amersham Bioscience).

**Preparation of brush border membrane vesicles (BBMVs) and transport assay.** BBMVs were prepared from mouse intestine or kidney using the Ca²⁺ precipitation method, as described previously (15, 16). BBMV 32P uptake was measured by the rapid filtration technique, as described previously (16).

**Immunoblotting.** Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 2-mercaptoethanol and were subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred by electrophoresis to Hybond-P polyvinylidene difluoride transfer membranes and then treated with diluted antibodies as follows: affinity-purified anti-type IIA (1:4,000) or type IIC (1:1,000) Na-Pi cotransporters, and antiserum type IIb Na-P, cotransporter antibodies (1:1,000). Type II Na-P, cotransporter polyclonal antibodies were generated in rabbits against a COOH-terminal peptide corresponding to amino acid residue mouse type IIA, 626–637 (LALPAHHNATRL); mouse type IIb, 682–697 (QVEVLSMKALSTTVFC); and mouse type IIC, 589–601 (CYENPQVIASSQQL) (15, 17). Mouse anti-actin monoclonal antibody (Chemicon International) was used as an internal control. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as the secondary antibody (Jackson Immunoresearch Laboratories), and signals were detected using the ECL Plus system (Amersham Pharmacia Biotechnology) (15, 16). BAT1 (b₀,₅-type amino acid transporter 1) antibody was gifted from Dr. Kanai (Kyorin University, Tokyo, Japan).

**Immunohistochemical analysis.** Immunohistochemical analysis of mouse kidney sections was performed as described previously with minor modifications (14). For immunostaining, serial sections (5 μm) were incubated with affinity-purified type IIA Na-P, cotransporter antibodies (1:1,000), type IIC (1:100) Na-P, cotransporter antibodies, or rabbit anti-klotho polyclonal antibodies (1:100) (Alpha Diagnostic International, San Antonio, TX) overnight at 4°C. The Alexa Fluor 568 anti-rabbit IgG (Molecular Probes, Eugene, OR) was used as

---

**Fig. 3.** Expression of type II Na-P, cotransporters in kl/kl mice. Western blotting of BBMVs isolated from the intestine (A) and kidney (C) of WT and kl/kl mice is shown. Each lane was loaded with 20 μg of BBMVs. Actin was used as an internal control. B and D: Northern blotting. Poly(A)⁺ RNA was extracted from the intestine (B) and kidney (D) of WT and kl/kl mice. Each lane was loaded with 3 μg of RNA. GAPDH was used as internal control. Intensity of expression of the WT mice expression was designated as 1.0. Values are means ± SD; n = 5–6. *P < 0.05.
secondary antibody (incubation for 60 min at room temperature) for
detection of type II Na-Pi cotransporter protein (15). For klotho protein
detection, sections were treated with Envision (+) rabbit peroxidase
(Dako, Carpinteria, CA) for 30 min at room temperature. Immunore-
activity was detected by treatment with diaminobenzidine (0.8 mM).

Statistical analysis. Data are expressed as means ± SD. Statistical
analysis in WT mice and kl/kl mice was performed using Student t-
test, one-way ANOVA (post hoc Scheffe F-test), and two-factor factorial
ANOVA. P < 0.05 was considered to represent statistical significance.

RESULTS

Plasma and urine Ca and P, in kl/kl mice. Plasma Ca and P, concentrations were determined in 5-wk-old kl/kl and WT mice (Fig. 1, A and B). Both Ca and P, concentration were higher in kl/kl mice than in WT mice (kl/kl vs. WT mice; Ca: 10.3 ± 2.1 vs. 8.2 ± 0.8 mg/dl; P, 14 ± 2.8 vs. 7.8 ± 0.5 mg/dl), which is consistent with previous reports (6, 11, 29).

Twenty-four-hour urinary Ca (Fig. 1C) and 1,25(OH)2D, levels (Fig. 1E) were significantly higher in kl/kl mice than in WT mice, while urinary P, excretion (Fig. 1D) in kl/kl mice was lower than in WT mice, but this difference did not reach the level of statistical significance. Plasma PTH levels (Fig. 1F) were similar when comparing the two animal groups.

Intestinal and renal Na-Pi transport activity in kl/kl mice. P, uptake in intestinal (Fig. 2A) and renal (Fig. 2B) BBMVs was significantly higher in kl/kl mice than in WT mice. The Vmax value of renal P, transport was 1.75-fold higher in kl/kl mice than in WT mice (WT: 1.6 ± 0.14 vs. kl/kl: 2.8 ± 0.17 nmol/mg protein). Moreover, the Vmax value of intestinal P, transport was 1.5-fold higher in kl/kl mice than in WT mice (WT: 0.23 ± 0.02 vs. kl/kl: 0.36 ± 0.01 nmol/mg protein). K, values for intestinal P, transport were 0.12 ± 0.08 mM in WT mice and 0.13 ± 0.06 mM in kl/kl mice. Moreover, K, values for renal P, transport were 0.08 ± 0.03 mM in WT mice and 0.07 ± 0.03 mM in kl/kl mice. These data suggested that the increased P, transport activity in kl/kl mice was due to an increase in the Vmax value rather than changes in the K, value.

Expression of the intestinal type IIb Na-Pi cotransporter. The type IIb transporter protein and mRNA levels were 4.2-fold and 3.5-fold higher, respectively, in the kl/kl mice compared with the WT mice (Fig. 3, A and B). Immunohistochemical analysis demonstrated elevation of the type IIb transporter protein levels in the apical membrane of the intestinal epithelial cells (data not shown). The type III (Pit-1) mRNA levels were slightly decreased, but not significantly, in the kl/kl mice compared with WT mice (Fig. 3B).

Expression of renal type IIa/IIc Na-Pi cotransporters in kl/kl mice. Protein levels of type IIa and type IIc Na-Pi cotransporters were 2.1-fold and 2.5-fold higher, respectively, in kl/kl mice compared with WT mice (Fig. 3C). By contrast, the mRNA levels of the type IIa and type IIc Na-Pi transporters in kl/kl mice were 26 and 15%, respectively, of the levels in the WT mice (Fig. 3D). The type III (Pit-1) mRNA levels were not decreased in kl/kl mice compared with WT mice (Fig. 3D). The levels of BAT1 mRNA and protein did not differ in a comparison of kl/kl and WT mice (data not shown).

Localization of renal type II Na-Pi cotransporter proteins. In WT mice, the type IIa and type IIc immunoreactive signals were weaker in superficial nephrones compared with deep nephrones (Fig. 4, Ac and Ab and Ba and Bb). In kl/kl mice, the intensity of the type IIa and type IIc immunoreactive signals was increased in both superficial and deep nephrones compared with the WT mice (Fig. 4, Ac and Ad and Bc and Bd). The

Fig. 4. Immunofluorescent detection of renal type II Na-Pi cotransporter proteins in WT mice (a and b) and kl/kl mice (c and d). a and c: Type IIa Na-Pi cotransporter; b and d: type IIc Na-Pi cotransporter. A: magnification ×40, bar = 10 μm. B: higher magnification, bar = 10 μm.
localization patterns of these transporters in kl/kl mice were very similar to those observed in animals fed an LP diet or in thyroparathyroidectomized animals (14, 22).

**Expression of novel Pi-regulating factors.** Plasma FGF23 protein levels were ~150-fold higher in kl/kl mice than in WT mice (Fig. 5A). Real-time PCR analysis of a variety of tissues demonstrated that FGF23 expression was highest in the calvaria followed by the femur in both kl/kl and WT mice. However, FGF23 expression in the calvaria was 200-fold higher in the kl/kl mice than in the WT mice (Fig. 5B). The study of rare disorders associated with renal P_i wasting has resulted in the discovery of a number of proteins (FGF23, a PHEX, MEPE) that decrease renal Na^+–dependent P_i transport in vivo and in vitro (24, 27). PHEX and MEPE are mainly expressed in the bone. In the present study, RNA levels of PHEX (Fig. 5, C and D) and MEPE (Fig. 5, E and F) in bone tissues were similar when comparing both animal groups. The type III (Pit-1 and Pit-2) mRNA levels did not differ in a comparison of kl/kl and WT mice (Fig. 5, G–J).

**Effect of dietary P_i on klotho expression in WT mice.** Next, the effect of LP (0.02%), CP (0.6%), and HP (1.2%) diets on klotho mRNA and protein levels in the kidneys of WT mice...
was investigated. Levels of the membrane form of klotho mRNA in the kidney were unaffected by the type of diet (Fig. 6A, open bar), while levels of the secreted form of klotho mRNA were significantly higher in mice fed an LP diet than in those fed a CP diet (Fig. 6A, solid bar). Western blotting analysis demonstrated that renal klotho protein levels were similar in the HP and CP groups, but were significantly elevated in the LP diet group relative to the other diet groups (Fig. 6B). In mice fed an LP diet, immunohistochemical experiments demonstrated the presence of klotho protein in the distal tubules but not in proximal tubules (Fig. 6C).

$P_i$ restriction induces an increase in klotho mRNA and protein levels in kl/kl mice. Five weeks after birth, kl/kl mice that were fed an LP diet continued to gain body weight (Fig. 7A), which is consistent with a previous report (11). Renal levels of the secreted form of klotho mRNA were significantly higher in WT mice fed an LP diet compared with WT mice fed a CP diet (Fig. 7Ba). In kl/kl mice, membrane and secreted forms of klotho mRNA were not detected in the kidney of mice fed a CP diet (Fig. 7Bb), but both forms of klotho were present in mice fed an LP diet. In addition, Western blotting demonstrated that klotho protein was increased in the kidney in kl/kl mice fed an LP diet (Fig. 7C).

Effect of LP diet on various plasma parameters of kl/kl mice. In both WT and kl/kl mice, plasma $P_i$ levels were significantly lower in mice fed an LP diet compared with those fed a CP diet (Fig. 8Aa). In WT mice, plasma Ca levels were significantly higher in response to an LP diet compared with those fed a CP diet (Fig. 8Ab). By contrast, plasma Ca levels were similar when comparing kl/kl mice fed an LP diet compared with those fed a CP diet. Plasma Ca and $P_i$ levels in kl/kl mice fed a CP diet were abnormal, but those fed an LP diet were normal compared with WT mice fed an LP diet. In addition, plasma FGF23 levels were markedly lower in kl/kl mice fed an LP diet compared with kl/kl mice fed a CP diet (Fig. 8Ac). Bone FGF23 mRNA levels showed similar patterns of change (Fig. 8B). These data suggest that $P_i$ restriction results in increased klotho expression and decreased FGF23 expression in bone and that plasma FGF23 were positively correlated with plasma $P_i$ and inversely correlated with klotho protein levels.

Type IIa and type IIc transporter transcripts in kl/kl mice fed an LP diet. In WT mice, type IIa and type IIc transporter mRNA and protein levels were increased in mice fed an LP diet compared with those fed a normal $P_i$ diet (Fig. 9). The LP diet produced the same effect in type IIc transcripts in kl/kl mice. By contrast, diet had no significant effect on type IIa

Fig. 6. Dietary $P_i$ regulation of klotho mRNA and protein expression. A: 7 days after administration of test diets to mice, klotho mRNA levels were assessed using real-time PCR. Klotho protein levels were assessed using Western blotting (B), and immunohistochemical analysis was performed (C). LP, low-phosphate diet; CP, control $P_i$ diet; HP, high-$P_i$ diet. A: quantitative PCR of the mRNA of klotho membrane (open bars) and secreted forms (solid bars) of klotho mRNA in the kidney of mice fed an LP, CP, or HP diet. GAPDH was used as an internal control. Values are means ± SD; $n$ = 5–8. $*P < 0.05$ vs. CP diet. B: Western blot analysis of klotho protein in the kidney of WT mice (C57BL/6N) fed a LP, CP, or HP diets. Values are means ± SD; $n$ = 5. $*P < 0.05$ vs. CP diet. C: immunohistochemical analysis of klotho protein in the kidney of WT mice fed an LP (a and d), CP (b), or HP diet (c and e). Magnification ×40 (a–c), ×100 (d and e). The sections were counterstained with hematoxylin/eosin.
Fig. 7. Effect of LP diet on plasma Pi and Ca and levels of klotho mRNA and protein. Two weeks after administration of LP diet to kl/kl mice, klotho mRNA and protein levels were assessed using real-time PCR and Western blotting. 

A: growth curve (percentage body weight gain) of WT and kl/kl mice fed an LP diet or a CP diet. Values are average of body weight gain (n = 4). 

B: quantitative PCR of mRNA of klotho membrane form (open bars) and secreted form (solid bars) of klotho mRNA in the kidney of WT (a) and kl/kl mice (b) fed an LP or CP diet (n = 5–8). GAPDH was used as an internal control. Values are means ± SD. *P < 0.05 vs. CP diet. ND, not detected. 

C: Western blot analysis of klotho protein in the kidney of kl/kl mice fed an LP or CP diet (n = 5). Values are means ± SD.

Fig. 8. Effect of a LP diet on the expression of FGF23 mRNA levels. 

A: determination of plasma Pi (a), Ca (b), and FGF23 (c); n = 4–6. 

B: the levels of FGF23 mRNA were determined in bone tissue of kl/kl mice fed LP or CP diet. a: calvaria; b: femur; n = 6–10. GAPDH was used as internal control. Values are means ± SD. *P < 0.05 compared with WT mice fed a CP diet. bP < 0.05 compared with kl/kl mice fed a CP diet.
Fig. 9. Effect of LP diet on expression of renal type II Na-Pi cotransporters. A: Northern blot analysis. B: Western blot analysis. Two weeks after initiation of test diets, mRNA and protein of renal type II Na-Pi cotransporters were measured. Values are means ± SD; n = 6–10. *P < 0.05 compared with WT mice fed a CP diet. †P < 0.05 compared with kl/kl mice fed a CP diet.

Fig. 10. Analyses of endocytosis/exocytosis of the type IIa Na-Pi cotransporter and scaffolding protein in renal proximal tubule in kl/kl mice. A: protein levels of type IIa and type IIc Na-Pi cotransporters at 2 h after infusion of an HP solution (Pi Sol.; 2 M sodium phosphate buffer, pH 7.4, 20 ml/g body wt). BBMVs from mouse kidneys were immunoblotted. B: effect of microtubule disruption reagent, colchicine, on insertion of type IIa Na-Pi cotransporter. Renal BBMVs were isolated 6 or 12 h after administration of colchicine (1.0 mg/kg body wt, dissolved in saline and administered intraperitoneally). Type IIa and type IIc Na-Pi cotransporter protein levels were determined by immunoblotting. Actin was used as an internal control. Values are means ± SD; n = 4–5. *P < 0.05.
Na-Pi cotransporter protein levels in kl/kl mice. Thus type IIa/IIc transcript levels correlated with type IIa/IIc transporter protein levels in kl/kl mice fed an LP diet.

Compared with WT mice, kl/kl mice fed a CP diet showed a paradoxical decrease in mRNA levels with an increase in protein levels of the renal type IIa/IIc transporters. However, the kl/kl mice on a nLP diet showed a positive correlation between mRNA and protein levels.

Regulation of type IIa Na-Pi cotransporter in kl/kl mice. To determine whether abnormal regulation of type II Na-Pi transporters in kl/kl mice is due to a defect of klotho signaling, as opposed to endogenous trafficking systems in the renal proximal tubule cells, endocytosis of the type IIa transporter in the proximal tubular segments of kl/kl mice was investigated (Fig. 10A). Infusion of a Pi solution resulted in a significant decrease in type IIa transporter protein levels in the renal BBMVs of kl/kl mice and WT mice (Fig. 10A).

The amounts of the type IIa transporter are mediated by microtubule-dependent translocation of presynthesized type IIa protein to the apical membrane (3, 12). The microtubule-disrupting reagent, colchicine, significantly inhibited the insertion of the type IIa Na-Pi cotransporter from intracellular compartments to the apical membrane in WT mice, but not in kl/kl mice (Fig. 10B).

**DISCUSSION**

The present study demonstrated that hyperphosphatemia in kl/kl mice results from increased renal sodium-dependent Pi transporter activity and increased intestinal Pi absorption. Mutation of klotho causes an elevation in the type IIa and type IIc Na-Pi cotransporter proteins in the apical membrane, but this elevation cannot be explained by changes in PTH and FGF23. The elevation of plasma 1,25(OH)2D3 in kl/kl mice, however, is one of the most important factors in their hyperphosphatemia, because the activities of intestinal Pi absorption (type IIb transporter) and renal Pi reabsorption (type IIa and type IIc transporter) were significantly increased in kl/kl mice. We previously demonstrated that 1,25(OH)2D3 stimulates expression of the type IIa Na-Pi cotransporter gene via its vitamin D receptor element, but that 1,25(OH)2D3 does not affect cellular localization of this transporter (23). In kl/kl mice, type II transporter protein levels were significantly increased, but mRNA levels in kl/kl mice were markedly suppressed, despite the presence of high levels of plasma 1,25(OH)2D3. These data indicate that high levels of the type IIa and type IIc transporter protein in the apical membrane are not simply due to high plasma 1,25(OH)2D3 levels.

It is likely to explain that the downregulation of the type II transporter mRNA may be due to high levels of plasma FGF23. We and other investigators demonstrated that elevation of plasma FGF23 could induce a prominent decrease in the levels of the type IIa and type IIc transporter in the apical membrane of the proximal tubule cells (4, 16, 20). More recently, Yu et al. (30) demonstrated that FGF23 induces tyrosine phosphorylation and directly downregulated type II Na-Pi transporter (Npt2a) mRNA using opossum kidney cells. Furthermore, a feeding of an LP diet downregulated plasma FGF23 levels in kl/kl mice and increased the levels of the type IIa transporter mRNA. In these contexts, we suggest that the low levels of type IIa and type IIc transporter transcripts may be due to elevation of plasma FGF23 levels in kl/kl mice.

In contrast, we cannot exactly comment the reason why type II transporter proteins do not respond to FGF23 in kl/kl mice. Disruption of the microtubular network by colchicine prevented the apical localization of the type IIa transporter in WT mice, but the effect of colchicine was much less in kl/kl mice.

![Fig. 11. Putative role of klotho on renal Pi reabsorption. Klotho might be required for the recognition of FGF23 by a target cell. If this is the case, the FGF23 signal could be transduced into distal convoluted tubule cells where klotho is expressed. However, because the type IIa Na-Pi cotransporter, the major target of the FGF23 signal, is preferentially expressed in proximal convoluted tubule cells, it has been speculated that either 1) one or more signal mediators from the distal to the proximal tubule would be required; or 2) some paracrine action of secreted klotho would be necessary for this signal transduction to occur (13). FGF23/klotho/FGF receptor (FGFR) complex stimulates ERK, signal mediators may be secreted in the distal tubules, and these mediators act on proximal tubule and may affect membrane localization of type II Na-Pi cotransporters. On the other hand, FGF23 may downregulate the levels of type II transporter mRNAs in the proximal tubules directly.](http://ajprenal.physiology.org/)

AJP-Renal Physiol • VOL 292 • FEBRUARY 2007 • www.ajprenal.org
One possibility is impaired constitutive endocytosis and degradation of the type IIa transporter in kl/kl mice instead of enhanced insertion. However, a feeding of an HP solution induced the endocytosis of the type IIa transporter in WT and kl/kl mice. Although we investigated the amounts of several scaffolding proteins that regulate the membrane trafficking of the type IIa transporters, those protein levels were not changed in WT and kl/kl mice (data not shown). The present study suggests that the ability of membrane insertion of the type IIa transporter is maximally enhanced in kl/kl mice, and feeding of an LP diet had no effect on the membrane insertion in kl/kl mice. We are now investigating the mechanisms of abnormal trafficking (endocytosis and exocytosis) in kl/kl mice.

In P\textsubscript{i} homeostasis in kl/kl mice, we have to consider the next two points. 1) Why were plasma FGF23 levels markedly increased in kl/kl mice? 2) Why were plasma PTH levels not changed in kl/kl mice, despite high plasma 1,25(OH)\textsubscript{2}D\textsubscript{3} and hyperphosphatemia? A more recent study demonstrated that klotho and FGF23 function via a common signaling pathway (7). FGF23 binds to the klotho/FGFR receptor (FGFR) complex with higher affinity than it binds to FGFR or klotho alone. In addition, klotho significantly enhances the ability of FGF23 to induce phosphorylation of an FGFR substrate and ERK in various types of cells, suggesting that klotho functions as a cofactor essential for activation of FGF signaling by FGF23 (7). In these contexts, we suggest that the overexpression of FGF23 may be due to loss of a cofactor (klotho) of FGF23 signaling in kl/kl mice. In kl/kl mice, we did not observe FGF23 induce phosphorylation of an FGFR substrate and ERK (data not shown). We suggest that the presence of klotho may be necessary to play a function of FGF23 in vivo.

For the second question, plasma PTH levels were not responsive to high plasma 1,25(OH)\textsubscript{2}D\textsubscript{3} and hyperphosphatemia in kl/kl mice. A recent report indicates that klotho are present in the parathyroid glands and in kl/kl mice; PTH secretion does not respond to hypocalcemia (13). Thus klotho may be needed for the secretion of PTH in the parathyroid glands.

In this study, at least two hypotheses are considered for the physiological roles of klotho on the regulation of renal Na-P\textsubscript{i} cotransporters (Fig. 11). The first hypothesis is that klotho acts on the type IIa transporter directly. Chang et al. (2) demonstrated that klotho activates a cell surface channel (transient receptor potential vanilloid 5, entrapping the channel in the plasma membrane (2). Therefore, klotho protein may also modify sugar residues of the type II Na-P\textsubscript{i} cotransporter and thereby directly affect membrane insertion of the transporter. Since klotho is expressed in the distal convoluted tubules and the extracellular domain of klotho is shed and secreted, it may act on adjacent proximal tubules and inhibit P\textsubscript{i} transport. However, secreted klotho protein did not affect type IIa transport activity in opossum kidney cells (data not shown). Therefore, it is unlikely that klotho has a direct effect on the renal type II Na-P\textsubscript{i} cotransporter.

The second hypothesis is that klotho is a processed signal mediator(s), which is activated by the FGF23/FGFR complex in the distal tubules. Klotho has significant similarity to members of the β-glycosidase family. Putative substrates are processed to an active form by the enzymatic action of klotho, suggesting that klotho action is mediated via the action of the undefined bioactive substrate (signal mediators) (13). The signal mediator(s) is activated in distal tubules by klotho, and it is secreted and has an influence on membrane localization of type IIa transporter (abnormal trafficking) in proximal tubules afterwards. In these contexts, defect of klotho may stimulate endocytosis of the type IIa Na-P\textsubscript{i} cotransporter. Further studies are needed to clarify the role of klotho on renal P\textsubscript{i} reabsorption.

In conclusion, we investigated a correlation between hyperphosphatemia in the kl/kl mouse and the activity of the type II Na-P\textsubscript{i} cotransporters. Deficiency in klotho protein may lead to abnormal trafficking and mRNA expression of the type IIa/IiC transporter in the proximal tubule cells. The kl/kl mice may provide an important tool to study the roles of FGF23 in body P\textsubscript{i} homeostasis.

ACKNOWLEDGMENTS

We thank Noriko Yata and Makio Kinosita for technical support and Koji Morishita (Kyowa Hakko Kogyo Company) and Yuji Katakura (Kyowa Hakko Kogyo Company) for technical advice.

GRANTS

This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (Grant 15790430 to H. Segawa and Grant 11557202 to K. Miyamoto), Takeda Science Foundation (H. Segawa), and the 21st Century COE Program, Human Nutritional Science on Stress Control, Tokushima, Japan.

REFERENCES