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Graphical abstract

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Calcium phosphate microcrystals in the renal tubular fluid accelerate chronic kidney disease progression

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Abstract:
The western pattern diet is rich not only in fat and calorie but also in phosphate. Negative impacts of excessive fat and calorie intake on health are widely accepted, whereas potential harms of excessive phosphate intake are poorly recognized. Here we show the mechanism by which dietary phosphate damages the kidney. When phosphate intake was excessive relative to the functioning nephron number, circulating fibroblast growth factor-23 (FGF23), a hormone that increases phosphate excretion per nephron, was increased to maintain phosphate homeostasis. FGF23 suppressed phosphate reabsorption in renal tubules and thus raised the phosphate concentration in the tubular fluid. Once it exceeded a threshold, microscopic particles containing calcium phosphate crystals appeared in the tubular lumen, which damaged tubular cells through binding to Toll-like receptor-4 expressed on them. Persistent tubular damage induced interstitial fibrosis, reduced the nephron number, and further boosted FGF23 to trigger a deterioration spiral leading to progressive nephron loss. In humans, progression of chronic kidney disease (CKD) ensued when the serum FGF23 level exceeded 53 pg/mL. The present study identified the calcium phosphate particles in the renal tubular fluid as an effective therapeutic target to decelerate nephron loss during the course of aging and CKD progression.
Introduction

Chronic kidney disease (CKD) is defined as any abnormality of the kidney structure and/or function, regardless of its cause, lasting for 3 months or longer (1). CKD progression can be viewed as a process of decrease in the functioning nephron number, which occurs during the natural cause of aging and is accelerated by renal diseases or systemic disorders causing renal complications, most notably diabetes and hypertension. Accordingly, CKD is prevalent in the aging society. Elderly people with low estimated glomerular filtration rate (eGFR) and CKD patients whose eGFR is less than 60 mL/min/1.73 m² account for more than 10% of the total population (2, 3). Once CKD progresses to an advanced stage, renal replacement therapy (dialysis or renal transplantation) becomes necessary, which burdens healthcare worldwide (4). Since hyperphosphatemia was identified as a major risk for cardiovascular events and poor prognosis (5, 6), restriction of dietary phosphate intake and inhibition of intestinal phosphate absorption by administration of phosphate binders have been applied for CKD patients with hyperphosphatemia. However, these therapies aiming at lowering phosphate in the blood are not to prevent progression of early-stage CKD to end-stage renal disease (ESRD), because hyperphosphatemia is a terminal symptom observed only in ESRD patients (7). No remedy is currently available to prevent CKD progression other than identifying and controlling the disorders that have caused the kidney damage in individual patients. Hence, it is of critical
importance to identify a novel therapeutic target universally applicable to the aged and early-stage CKD patients to prevent nephron loss.

Dietary phosphate load has been known to induce renal tubular damage and interstitial fibrosis in rodents (8), which recapitulate pathologies universally observed in the aged and CKD patients (9, 10) at least in part. Notably, it is not only the amount of phosphate intake but also the nephron number that determine the severity of the kidney damage. Specifically, rats or mice whose nephron number had been reduced by partial nephrectomy developed severer kidney damage than sham-operated animals when placed on the same high phosphate diet (8, 11). In humans, elderly people and CKD patients were reported to be at high risk for developing acute kidney injury and the subsequent CKD induced by phosphate gavage for colon cleansing as a pretreatment for colonoscopy (12, 13). These observations are consistent with the notion that a low nephron number is a risk for the kidney damage induced by phosphate intake, although the mechanism remains unclear.

In this study, we elucidate the molecular mechanism by which an increase in phosphate excretion per nephron induces renal tubular damage and identify multiple points of therapeutic interventions. In addition, we provide clinical evidence suggesting that these interventions may
benefit approximately 1/4 of adults over 45 years old and the majority of CKD patients.
Results

Calcium phosphate particles, but not phosphate, damage renal tubular cells

We previously characterized mice placed on diet containing different amount of inorganic phosphate and determined the time-course and the dose-responsiveness of renal tubular damage and interstitial inflammation/fibrosis induced by dietary phosphate load (11). In response to increase in dietary phosphate intake, circulating levels of FGF23 were increased. FGF23 is a hormone secreted from the bone and acts on the kidney to increase urinary phosphate excretion (14). FGF23 is indispensable for maintaining phosphate homeostasis as evidenced by the fact that mice and humans defective in FGF23 or its obligate co-receptor Klotho develop hyperphosphatemia and ectopic calcification (15-18). Because FGF23 induces phosphaturia through suppressing phosphate reabsorption in renal proximal tubules (19), FGF23 should increase phosphate concentration in the proximal tubular fluid. Indeed, phosphate concentration in the tubular fluid collected by micropuncture was reported to increase along the course of the proximal tubule in rats infused with phosphate (20). Therefore, we hypothesized that proximal tubular cells might be damaged when exposed to high extracellular phosphate from the apical side. To test this hypothesis, we cultured renal proximal tubular cells (HK-2) and increased phosphate concentration in the medium. A dose-dependent decrease in cell viability was observed (Figure 1A). During this experiment, we noticed that the medium became slightly cloudy as the
phosphate concentration was increased. Electron microscopic observation of the high phosphate medium identified numerous electron-dense particles (Figure 1B). These particles contained calcium phosphate crystals, because they bound to bisphosphonate (21) and disappeared by treatment with acid or a calcium chelator (Figure 1C). To test the possibility that the phosphate-induced cell damage might be attributed not to phosphate but to these calcium phosphate particles, we centrifuged the high phosphate medium at 16,000 g for 2 hours to precipitate and remove these calcium phosphate particles (Figure 1C), cultured HK-2 cells with the supernatant, and confirmed that the cell viability was not reduced (Figure 1D). These results indicate that the calcium phosphate particles are responsible for cell death induced by high extracellular phosphate. Furthermore, we confirmed that addition of alendronate, which inhibits the amorphous-to-crystalline phase transition of calcium phosphate, suppressed the phosphate-induced cell death in a dose-dependent manner (Figure 1A). Conversely, addition of synthesized calcium phosphate particles to the regular medium reduced cell viability in a dose-dependent manner (Figure 1E). These observations indicate that formation of calcium phosphate crystals is necessary and sufficient for high extracellular phosphate to damage proximal tubular cells.

Calcium phosphate particles appear in the proximal tubular lumen in vivo

We hypothesized that mice suffering from tubulointerstitial damage during dietary phosphate
load should have calcium phosphate crystals in the tubular lumen. To test this hypothesis, we
performed ex vivo imaging of the kidney using a fluorescent bisphosphonate (OsteoSense) that
binds to crystalline calcium phosphate (22). We injected OsteoSense intravenously into mice fed
high phosphate diet and detected numerous OsteoSense signals in the tubular lumen at the
cortico-medullary junction. Such signals were absent in mice fed regular diet (Figure 2A, B, C).
To demonstrate that these calcium phosphate crystals were in the tubular lumen, we repeated ex
vivo imaging using FITC-Lotus Tetragonolobus lectin (LTL) instead of FITC-dextran to label the
apical membrane of proximal tubules and confirmed the OsteoSense signals within the tubular
lumen (Figure 2C). Furthermore, we performed scanning electron microscopy (SEM) with energy
dispersive X-ray spectroscopy (EDS) and confirmed that particles consisting of calcium and
phosphate were attached to the brush borders (Figure S1). The localization of calcium phosphate
particles is consistent with the fact that interstitial inflammation and fibrosis induced by dietary
phosphate load started primarily from the cortico-medullary junction (11). We next asked if the
pathology induced by the dietary phosphate load would be alleviated by inhibiting formation of
calcium phosphate crystals with bisphosphonate. Subcutaneous injection of alendronate erased
calcium phosphate particles from the cortico-medullary junction (Figure 2D). We also asked if
lowering urine pH would have the same effect, because calcium phosphate can be dissolved with
acid. As expected, urine acidification by giving ammonium chloride solution as drinking water
reduced the amount of calcium phosphate particles (Figure 2E). Accordingly, both
bisphosphonate treatment (Figure S2) and urine acidification (Figure S3) alleviated the
inflammation and fibrosis in the kidney. We unexpectedly found that the mice treated with
ammonium chloride had significantly lower FGF23 and higher phosphate levels in the blood than
the mice given tap water (Figure S3), suggesting that the ammonium treatment might have
suppressed FGF23 secretion/production and increased serum phosphate levels, although the
mechanism is not clear. Thus, ammonium treatment may have suppressed formation of calcium-
phosphate crystals in the tubular fluid and alleviated tubular damage not only through urine
acidification but also through reducing phosphate load per nephron. Conversely, urine
alkalization by giving 0.15 M sodium bicarbonate solution as drinking water to mice fed the same
high phosphate diet exacerbated the kidney damage (Figure S4), possibly through facilitating
formation of calcium phosphate crystals in the tubular fluid.

**Calcium phosphate particles bind to TLR4**

We speculated that cytotoxic signals of calcium phosphate particles might be mediated by a cell-
surface receptor. The putative receptor for calcium phosphate particles should meet at least the
following two criteria. First, it should bind to calcium phosphate crystals and thus likely belongs
to pattern-recognition receptors. Second, it must be expressed in the cortico-medullary junction in
the kidney. We identified Toll-like receptor-4 (TLR4) as a candidate that fulfilled these two
criteria. TLR4 is known as a pattern-recognition receptor and is expressed mainly in renal tubules
in the cortico-medullary junction, where the distal portion of proximal tubules (the S3 segment)
is distributed (23). To determine whether calcium phosphate particles might physically interact
with TLR4 in vitro, we performed surface plasmon resonance analysis by running the
extracellular domain of TLR4 protein over synthesized calcium phosphate particles immobilized
on a sensor chip. We observed direct interaction between calcium phosphate particles and TLR4
ectodomain with a dissociation constant 9.63 nM (Figure S5A) in the absence of myeloid
differentiation factor-2, a cofactor necessary for TLR4 to bind to lipopolysaccharide or fetuin-A
(24). It is noteworthy that high phosphate diet feeding induced accumulation of TLR4 to the
apical membrane of tubular cells (Figure S5B-D).

We confirmed that mice lacking the Tlr4 gene were resistant to the tubular damage,
inflammation, and fibrosis induced by high phosphate diet (Figure S6). We also confirmed that
deletion of the Tlr4 gene specifically in renal tubules alleviated the kidney damage. When placed
on the high phosphate diet, mice defective in renal tubular TLR4 (i.e. mice homozygous for the
Tlr4 floxed allele carrying the Ksp-Cre transgene) had lower expression levels of markers for
renal tubular damage (Kim1, Ngal, osteopontin), inflammation (MCP1, IL1β, IL6), and fibrosis
(TGFβ1, MMP2, vimentin, α-smooth muscle actin, collagen 1α1) than control mice (i.e., mice carrying the Ksp-Cre transgene alone) (Figure 3A, B), despite the fact that the urinary phosphate excretion was not different between these mice (Figure 3C). Accordingly, the collagen area fraction in the mice defective in renal tubular TLR4 was smaller than that of the control mice and similar to that of the control mice fed regular diet (Figure 3E-G). Taken together, expression of TLR4 in renal tubules is necessary for dietary phosphate load to induce kidney damage in vivo.

**Endocytosis of calcium phosphate particles disturbs endosomal trafficking**

To explore the cellular mechanism of tubular damage induced by calcium phosphate particles, we profiled gene expression in cultured proximal tubular cells (HK-2) before and after exposure to calcium phosphate particles. The KEGG pathway analysis identified enrichment of pathways relevant to inflammation (Toll-like receptor, TGF-β, and IL-17 signaling) and cell adhesion/junction (Rap1 signaling and gap junction) within 6 hours, followed by endocytosis, apoptosis, and necrosis pathways among others within 24 hours (Figure S7). At the protein level, we observed increase in phosphorylation and nuclear accumulation of phosphorylated p38 and NFκB (Figure 4A-D). We also observed increased expression and secretion of osteopontin, a cytokine that not only regulates biomineralization and inflammation (25) but also serves as a marker for renal tubular damage (26) (Figure 4E). These observations suggested that calcium
phosphate particles might induce these inflammatory responses through binding and activating TLR4. However, a cell-permeable inhibitor for the TLR4 signaling (TAK242) attenuated secretion of osteopontin but not phosphorylation of NFkB and p38 (Figure S8), indicating that the inflammatory responses were induced through both TLR4-dependent and independent signaling pathways. Prolonged exposure (6-48 hours) additionally induced endocytosis and accumulation of calcium phosphate particles in late endosomes, lysosomes, and autophagosomes around the nuclei (Figure 4A-F, Figure S9). The perinuclear clustering of lysosomes, which was reminiscent of that in cells under starvation (27), was associated with decrease in early and recycling endosomes (Figure S10), decrease in cell viability (Figure 5G), and increase in tubular cell damage (Figure 5H). In summary, calcium phosphate particles trigger inflammatory responses in proximal tubular cells, and thereafter induce endocytosis, disturbed endosomal trafficking, and cellular damage/death.

Notably, the cell damage/death induced by calcium phosphate particles in vitro was independent of TLR4 activation, because it was not attenuated by TAK242 (Figure 5G, H). Therefore, we conclude that the TLR4-dependent renal tubular damage in vivo is caused not by activation of the TLR4 signaling by calcium phosphate particles, but by tethering of the calcium phosphate particles on the surface of proximal tubular cells for 6 hours or longer against the tubular fluid.
flow, which facilitated the endocytosis to induce disturbed endosomal trafficking and tubular cell damage. Consistent with this conclusion, calcium phosphate particles were barely detectable in the tubular lumen in mice lacking the \textit{Tlr4} gene fed high phosphate diet (Figure 2G). Another finding consistent with this conclusion includes the fact that mice lacking Myd88, one of the major intracellular adaptor proteins that mediate the TLR4 signal transduction, developed renal fibrosis when placed on high phosphate diet unlike mice lacking the \textit{Tlr4} gene (Figure S11). Of note, calcium phosphate particles were present in the cortico-medullary junction. This can be explained by the fact that TLR4 is expressed predominantly in the tubules in the cortico-medullary junction (Figure S5) and by the fact that the phosphate concentration in the proximal tubular fluid in the S3 segments in the cortico-medullary junction can be higher than that in the S1 segments in the cortex upon phosphate loading (20).

\textbf{FGF23 correlates with renal tubular damage}

Given that formation of calcium phosphate particles in the renal tubular fluid is required for the tubular damage, a threshold should exist in the phosphate concentration of the proximal tubular fluid (PTFp) above which calcium phosphate precipitation and tubular damage occur. Because it is technically challenging to obtain the tubular fluid from the cortico-medullary junction in live mice, we estimated PTFp from concentration of phosphate and creatinine in the blood and urine
based on the following assumptions. First, phosphate concentration in the glomerular filtrate is equal to that in the blood. Second, proximal tubules reabsorb 70% of the filtrated water by default (28), which causes 3.33-fold increase in concentration of the solutes. Third, phosphate reabsorption takes place almost exclusively in proximal tubules (29). Lastly, fractional excretion of phosphate (FEp), which is defined as the ratio of phosphate clearance to creatinine clearance, indicates the fraction of phosphate that was not reabsorbed at proximal tubules. Thus, the product of serum phosphate concentration and FEp multiplied by 3.33 should reflect PTFp (Figure S12A), which we define as estimated PTFp (ePTFp). Namely,

\[
ePTFp \equiv Sp \times FEp \times 3.33 = Sp \times \frac{Up \times V}{Sp} \times 3.33 = \frac{Up}{Ucr} \times Scr \times 3.33 \quad \ldots (1)
\]

where \( V, Sp, Up, Scr, \) and \( Ucr \) represent the 24-hour urine volume, the concentration of serum phosphate, urine phosphate, serum creatinine, and urine creatinine, respectively. We confirmed that ePTFp served as an approximation of the actual PTFp determined by direct measurement of the phosphate concentration in the proximal tubular fluid collected by micropuncture in living rats (Figure S12B) (20). It should be noted that the serum phosphate concentration is cancelled out in the equation (1) and thus has no contribution to ePTFp.

We changed PTFp as an independent variable by placing mice with or without uninephrectomy
on diet containing either 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for 12 weeks and quantified serum FGF23 levels and relative mRNA levels of markers for renal tubular damage, inflammation, and fibrosis as dependent variables (Figure 6A). In double logarithmic plots, the relation between ePTFp and these variables fitted with two-segmented linear regression with the slope of the first segment being zero (Figure 6B-I). Specifically, serum FGF23 levels started elevating when ePTFp was increased beyond 5.18 mg/dL (Figure 6B). Concurrently with the FGF23 increase, expression of tubular damage markers (Ngal and osteopontin) started increasing (Figure 6B-D), which was followed by increase in expression of inflammatory markers (MCP1 and TNFα). Increase in fibrosis marker expression and decline of renal function (decrease in creatinine clearance) became evident when ePTFp reached approximately 10 mg/mL.

Taken together, we propose the mechanism of renal tubular damage associated with increase in FGF23 (Figure 6J). An increase in phosphate intake and/or a decrease in the nephron number must be accompanied by an increase in phosphate excretion per nephron to maintain the phosphate homeostasis. This demand is met by increasing FGF23, a hormone that increases phosphate excretion per nephron. However, the increase in FGF23 raises the PTFp and increases the risk for formation of calcium phosphate particles in the tubular fluid, which induce tubular damage through binding to TLR4 expressed on the tubular cells. If the tubular damage kills the
nephron, FGF23 must be further increased to compensate for the decrease in the nephron number unless phosphate intake is reduced, thereby triggering a deterioration spiral that leads to progressive nephron loss.

**Phosphate load reduces the nephron number**

To test whether the kidney damage induced by an increase in phosphate excretion per nephron indeed causes nephron loss, we developed a method for estimating the nephron number. The same experiment as in Figure 6A was repeated, except that the duration of the phosphate load was shortened to 2 weeks instead of 12 weeks. Because mice have approximately 10,000 nephrons per kidney (30), we calculated the phosphate excretion per nephron of individual mice by dividing the amount of 24-hour urinary phosphate excretion by 10,000 in the uninephrectomized mice or by 20,000 in the sham-operated mice. In double logarithmic plots, serum FGF23 levels started increasing when the phosphate excretion per nephron exceeded 0.80 µg/day (Figure 7A). Based on the regression line, the nephron number in mice with serum FGF23 levels higher than 290 pg/mL (= y-intercept of Figure 7A) can be calculated by the following equation:

$$
\log[\text{Nephron#}] = \log[U \times V] - \frac{\log[\text{FGF23}]}{2.536} \quad \cdots (2)
$$
Using the equation (2), we compared the nephron number in mice receiving the short-term (2 weeks) phosphate load and that in mice receiving the long-term (12 weeks) phosphate load. During the additional 10 weeks of phosphate load, significant decrease in the nephron number (Figure 7B) and reciprocal increase in the serum FGF23 level (Figure 7C) were observed in the sham-operated mice and the uninephrectomized mice placed on 1.5% or 2.0% phosphate diet. The phosphate excretion per nephron of these groups was 1.1 µg/day or over on average at the 2 weeks (Figure 7D). Hence, we conclude that nephron loss occurs when phosphate excretion per nephron exceeds 1.1 µg/day. However, a higher level of phosphate excretion per nephron was required to impair renal function within the 12 weeks, because a decrease in the average creatinine clearance was observed only in the uninephrectomized mice fed 2.0% phosphate diet (Figure 7E) whose phosphate excretion per nephron exceeded 3.4 µg/day on average (Figure 7D).

**FGF23 as a clinical parameter for progressive CKD**

Lastly, we asked if the relation between ePTFp and FGF23 observed in mice (Figure 6B) would be also observed in humans. Non-dialysis CKD patients at various stages were recruited to measure ePTFp and serum FGF23 (31). In this cross-sectional study, the relation between ePTFp and FGF23 fitted with a two-segmental linear regression as observed in mice. Serum FGF23
levels started increasing when ePTFp exceeded 2.32 mg/dL (Figure 8A). These findings suggested that patients whose serum FGF23 levels are higher than 53 pg/mL (= the y-intercept of the regression line in Figure 8A) might have developed tubulointerstitial damages and progressive CKD.

To verify this possibility, we performed a prospective study to explore the relation between serum FGF23 levels and incident kidney events using blood samples from the EMPATHY study, in which patients with diabetic retinopathy and hyperlipidemia, but without advanced CKD (eGFR < 30 ml/min/1.73m²), were enrolled (32). The median concentration of FGF23 was 54.7 pg/mL, with an interquartile range (IQR) from 44.0 pg/mL to 69.0 pg/mL. 5,039 patients were stratified into two groups based on the cut-off point at 53 pg/ml of the serum FGF23 level. During follow-up for 5 years, the renal events defined as initiation of chronic dialysis or increase in the serum creatinine level by at least twofold (and >1.5 mg/dL) occurred in 100 patients; 0.6% in the lower FGF23 group (14 of 2,336 patients) and 3.2% in the higher FGF23 group (86 of 2,703 patients). The higher FGF23 levels at the baseline were associated with the increased renal events (Figure 8B). Cox regression analysis indicated that the high FGF23 group had significantly higher risk for the renal events than the low FGF23 group [Hazard Ratio (HR) 5.18, 95% confidence interval (CI) 2.94 – 9.11, \( P < 0.001 \) by log rank test]. This relationship remained significant after
adjusting for age, sex, body mass index, and importantly, serum creatinine (HR 2.84, 95% CI 1.57 – 5.13, $P = 0.001$).
Discussion

Kawasaki et al. performed a retrospective cohort study of 191 non-dialysis CKD patients and demonstrated that the higher ratio of urinary phosphate excretion to creatinine clearance (Ccr) was associated with the higher risk for renal events (defined as progression to ESRD and/or 50% reduction of eGFR) within 3 years (33). The ratio of urinary phosphate excretion to Ccr was actually equivalent to ePTFp, because;

\[
\frac{Up \times V}{Ccr} = \frac{Up \times V}{Ucr \times V} = \frac{Up}{Ucr} \times Scr \quad (3)
\]

The equation (1) and (3) indicate that the ratio of urinary phosphate excretion to Ccr can be converted to ePTFp by proportional calculation. We calculated ePTFp of the patients in their study and found that the median of ePTFp was 3.93 mg/dL with an IQR from 2.56 mg/dL to 6.81 mg/dL. Therefore, the patients in Quartiles 2-4 had ePTFp higher than the threshold for the FGF23 increase determined in our study (2.32 mg/dL) and thus were supposed to have serum FGF23 levels higher than 53 pg/mL (Figure 8A), although they did not measure FGF23. Their conclusion was that the patients in Quartiles 2-4 exhibited a graded increase in the risk for renal events when compared with the patients in Quartile 1. Another prospective study showed that increase in Up/Ucr was independently associated with decrease in eGFR in CKD patients in stage 2-3 (34). These findings are consistent with our conclusion that increase in the phosphate
concentration of the proximal tubular fluid beyond the threshold accelerates nephron loss.

Daily phosphate intake in industrialized countries is approximately 900-1,400 mg/day, roughly 60-70% of which is absorbed from the digestive tract (35). It should be noted that this estimation does not include phosphate in food additives, which amounts to more than 30% of the phosphate in the food itself (35). Thus, average adults on standard diet consume 1,200-1,800 mg of phosphate in total and excrete approximately 1,000 mg into urine per day. The question is whether urinary phosphate excretion of this amount may contribute to kidney damage in the aged and CKD patients. Although there are considerable individual variations, normal young adults have 600,000-1,400,000 nephrons per kidney (36). Assuming that a normal adult having 2 million nephrons excretes 1,000 mg of phosphate per day, phosphate excretion per nephron is estimated as 0.5 µg/day. The nephron number is decreased during the natural course of aging in humans approximately by 50% in their seventies (36). Unless phosphate intake is reduced with age, the phosphate excretion per nephron reaches 1.0 µg/day, which is close to the level that reduces the nephron number in mice (1.1 µg/day). Thus, in humans, dietary phosphate consumed on a daily basis is a potential driving force of the progressive nephron loss.

Therefore, individuals with decreased nephron number, such as elderly people and early stage
CKD patients whose serum FGF23 levels are higher than 53 pg/mL, are expected to benefit from phosphate restriction aiming at reducing urinary phosphate excretion to lower serum FGF23 levels below 53 pg/mL. Because this notion is based on the correlation between FGF23 and renal events (Figure 8), the causality remains to be determined by randomized control trials. Specifically, restriction of dietary intake and intestinal absorption of phosphate by avoiding phosphate-rich ingredients and food additives and by administration of phosphate binders are expected to decelerate nephron loss. However, the current paradigm of phosphate restriction aims at lowering phosphate in the blood in ESRD patients with hyperphosphatemia to reduce cardiovascular events and mortality. We propose another paradigm of phosphate restriction; phosphate restriction should be applied not only to ESRD patients with hyperphosphatemia but also to normophosphatemic CKD patients with hyper-FGF23-emia (FGF23 > 53 pg/mL) to prevent tubulointerstitial damage and nephron loss. Such patients represent approximately 1/4 of adults over 45 years old and 2/3 of stage 3 CKD patients (37, 38). Besides phosphate restriction, agents that inhibit formation of calcium phosphate crystals such as bisphosphonates may be useful for the treatment of this patient population (Figure 2, Figure 6J, Figure S2).

Calcium phosphate particles in the tubular fluid likely comprise not only calcium phosphate crystals but also serum protein fetuin-A, because fetuin-A is present in the tubular fluid (39) and
has a high capacity to adsorb calcium phosphate crystals (40). Thus far, fetuin-A molecules laden with solid-phase calcium phosphate have been reported in the blood and designated as calciprotein particles (41, 42). However, calciprotein particles in the blood are too large in size to be filtrated through glomeruli and unlikely to be the origin of calcium phosphate particles in the tubular fluid. Therefore, we speculate that calcium phosphate particles in the tubular fluid are generated in situ in the tubular lumen independently of circulating calciprotein particles. Indeed, calciprotein particles in the blood are increased with serum phosphate levels (31), whereas formation of calcium phosphate particles in the tubular fluid should be dependent on the ePTFp, which is independent of serum phosphate levels as shown in the equation (1).

Regarding the mechanism by which the bone senses the necessity for increasing phosphate excretion per nephron and induces FGF23 secretion/production, we speculate that an increase in phosphate load excreted per nephron may enhance and/or prolong postprandial increase in blood levels of phosphate and calciprotein particles, which induces FGF23 expression/secretion in osteoblasts/osteocytes (43). This hypothesis remains to be determined.

We showed that bicarbonate treatment exacerbated the kidney damage induced by dietary phosphate loading in mice (Figure S4). On the other hand, bicarbonate has been widely prescribed for CKD patients
with metabolic acidosis and shown to decelerate eGFR decline and reduce urinary albumin excretion (44). Although the mechanism of kidney damage induced by metabolic acidosis remains elusive, it has been postulated that adaptive responses that increase acid excretion, including activation of ammoniagenesis and the renin-angiotensin-aldosterone system, may eventually induce inflammation and tubulointerstitial damage (45). We speculate that a net effect of bicarbonate on renal outcomes in CKD patients may be determined by a balance between the advantage obtained by correcting metabolic acidosis and the disadvantage caused by urine alkalization that facilitates formation of calcium phosphate crystals in the tubular fluid. Indeed, some recent randomized controlled studies failed to show the benefits of bicarbonate treatment (46-48), raising the possibility that the disadvantage could dominate the advantage in some CKD patients with high phosphate load excreted per nephron or high serum FGF23 levels.

The equation (2) is derived solely from the regression line between FGF23 and phosphate excretion per nephron (Figure 7A) and thus do not take into account the concentration of ions other than phosphate, such as citrate and magnesium, which potentially affect formation of calcium phosphate crystals. Nonetheless, this equation may be useful for following up changes in the nephron number by simple blood and urine tests. It should be noted that the nephron number calculated by the equation (2) does not represent the actual nephron number, but a “virtual” nephron number assuming that all nephrons had the same single nephron GFR (snGFR). Actually,
all nephrons may not have equivalent snGFR. The hyperfiltrating nephrons with high snGFR should have greater phosphate load and thus severer tubular damage than nephrons with lower snGFR. Indeed, the fibrotic area in the kidney of mice fed high phosphate diet distributed not uniformly but segmentally along the radial tracts of nephrons (Figure 3D).

Because the number of nephrons is highly variable from individual to individual and from species to species, the amount of phosphate load necessary for inducing calcium phosphate precipitation in the tubular fluid also varies from individual to individual and from species to species. As discussed above, phosphate excretion per nephron in young adults and older adults is approximately 0.5 µg/day and 1.0 µg/day, respectively. In contrast, phosphate excretion per nephron in mice fed regular diet is approximately 0.03 µg/day (Figure 7A), which is more than one order of magnitude less than that in humans. Hence, dietary phosphate overload is necessary for mice to trigger formation of calcium phosphate crystals in the tubular fluid. Considering the body size, humans may be a species gifted with less nephrons than mice (approximately 30 vs 700 per gram body weight) and thus prone to the phosphate-induced kidney damage.
Methods

Cell culture

A human renal proximal tubular cell line (HK-2) was obtained from ATCC (ATCC® CRL-2190™) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque) supplemented with 5% fetal bovine serum (FBS, Biological Industry), 50 µg/mL penicillin and 50 µg/ml streptomycin (GMBCO). Calcium and phosphate concentrations were increased to desired final concentrations by adding 1 M CaCl₂ and 1 M phosphate buffer to the medium. Viability of HK-2 cells was quantified using MTT assay (CellTiter96 Non-Radioactive Cell Proliferation Assay, Promega) or CCK-8 assay (#CK12; Dojindo Molecular Technologies) according to the manufacturer’s instructions. Damage of HK-2 cells was quantified using LDH assay (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega). For inhibition of TLR4 signaling, 2 mM TAK242 (Merck Millipore, #614316) stock solution in DMSO was added to cells at each time point (final concentration; 2 µM).

Synthesis of calcium phosphate particles

Calcium phosphate particles were synthesized in DMEM containing 0.1~10% FBS by inoculating 1 M CaCl₂ and 1 M phosphate buffer (mixture of 1 M NaH₂PO₄ and 1 M Na₂HPO₄, pH 7.4) to increase calcium and phosphate concentration to 3 mM and 3~7 mM, respectively. The
mixture was incubated at 37°C for 16–24 hours with gentle rocking, and then centrifuged at 16,000 g for 2 hours. After removal of the supernatant, the precipitated calcium phosphate particles were suspended with regular DMEM (1.8 mM calcium, 0.9 mM phosphate) containing 5% FBS. The concentration of calcium phosphate particles was indicated as phosphorus content (µg phosphorus/mL) determined by ICP-MS Nexion 2000 (PerkinElmer) as previously described (43). For fluorescent labeling of calcium phosphate particles, 5(6)-RhR-dRIS (final concentration; 25 nM) (BIOVINC) was added and incubated for 10–60 min at room temperature.

**Measurement of particle size of calcium phosphate particles**

The particle size distribution of calcium phosphate particles in the medium was determined by nanoparticle tracking analysis (NTA) using Nanosight NS300 (Nanosight, Amesbury, UK). A bisphosphonate (alendronate) conjugated with fluorescein isothiocyanate (FITC) was synthesized by amine coupling between FITC-carboxylic acid and the primary amine in alendronate. The FITC-alendronate was purified by HPLC and then added to DMEM containing 5% FBS, 3 mM calcium, and 7 mM phosphate, at the final concentration of 1 µM. After incubation at 25°C for 24 hours, the mixture was subjected to NTA using the 488 nm laser and the 500 nm long pass filter to visualize calcium phosphate particles labeled with FITC-alendronate.
Animals.

Wild-type C57BL/6 mice, mice lacking the Tlr4 gene (B6.B10ScN-Tlr4\textsuperscript{bps-del/JthJ}), Tlr4-floxed mice (B6.Cg-Tlr4\textsuperscript{tm1.Karp/J}), Myd88-deficient mice (B6.129P2(SJL)-Myd88\textsuperscript{tm1.1Defr/J}), and Ksp-Cre (B6.Cg-Tg(Cdh-cre)91Igr/J) were obtained from Jackson Laboratories. Mice were subjected to either right uninephrectomy or sham-operation (laparotomy alone) at 8 weeks of age. After 4 weeks of the recovery period, these mice were placed on diet containing 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for up to 12 weeks and then transferred individually to metabolic cages. After urine collection and measurement of food/water consumption for 3 days, the mice were sacrificed to harvest their blood and kidneys. A part of the kidney was fixed in buffered formalin for histological examination. The rest of the kidney was snap-frozen in liquid nitrogen for RNA extraction. For bisphosphonate treatment, wild-type C57BL6 male mice at 4 weeks of age were placed on diet containing 2.0% inorganic phosphate and injected subcutaneously with either alendronate (10 mg/kg), etidronate (100 mg/kg), or vehicle (saline) every other day for 4 weeks. For urine acidification, wild-type C57BL6 male mice at 4 weeks of age were placed on diet containing 2.0% inorganic phosphate and given 0.14 M NH\textsubscript{4}Cl solution or tap water as drinking water. For urine alkalization, wild-type C57BL6 male mice were placed on diet containing 2.0% inorganic phosphate and given 0.15 M NaHCO\textsubscript{3} solution.
**Ex vivo imaging of the kidney**

Mice lacking the *Tlr4* gene and wild-type C57BL/6J mice at 8 weeks of age were fed with chlorophyll-free normal phosphate diet (0.35% inorganic phosphate) for 7 days, and subsequently switched to chlorophyll-free high-phosphate diet (2.0%) for 8-9 days for *ex vivo* imaging of the kidney. Part of the mice were treated with alendronate (10 mg/kg, every other day) by subcutaneous injection or ammonium chloride (0.14M, for the last 10 days) by water supply. After anesthetization by intraperitoneal injection with urethane (1.5 g/kg), mice were injected with OsteoSense 680EX (Perkin Elmer, 50 nmol/kg), FITC-dextran (MW 150kD, 80 mg/kg), and Hoechst 33342 (20 mg/kg) from right jugular vein to visualize calcium phosphate crystals, interstitial space, and cell nuclei, respectively. To visualize the apical membrane of proximal tubules, FITC-labeled Lutus Tetragonolobus Lectin (LTL) was used instead of FITC-dextran. Thirty minutes after the injection, the kidneys were excised and cut in half coronally. The cut surface of the kidney was attached to a glass bottom dish (MatTek) without washing nor fixing, and the dish was placed on a piezo-drive stage (Tokai Hit; Nikon) of an inverted microscope (Eclipse Ti; Nikon). The tissue was excited at a wavelength of 405, 488 and 561 nm, and XY or XYZ images were captured using an A1R system (Nikon) by galvano scanning mode. 2× (N.A. 0.10, dry), 10× (N.A. 0.45, dry) and 40× (N.A. 1.15, water immersion) objective lens (Nikon) were used. The collected images were converted to 8 bit RGB tiff files using NIS-Elements.
software (Nikon), and the number of calcium phosphate particles was determined by counting the number of OsteoSense-positive particles by the “Analyze Particle” algorithm in ImageJ. The OsteoSense-positive particles in ureter were excluded.

**Electron microscopic study**

The kidney tissues of mice fed high phosphate diet for 10 days were collected after euthanasia, cut into small pieces in Krebs-Ringer HEPES buffer, and fixed with high pressure freezing using EM ICE (Leica). The tissues were freeze-substituted in acetone containing 2% OsO4 (Nissin EM) using EM AFM2 (Leica). In the freeze substitution, the samples were kept at -80 °C for 48 hrs, -35 °C for 6 hrs, 0 °C for 1 hr and room temperature for 2 hrs, and the temperature was raised at the speed of 8.2 and 10 °C/hr when the temperature was raised to -35 and 0 °C, respectively. The substitution medium was replaced by pure acetone after the incubation at room temperature, and the samples were infiltrated with and embedded in epoxy resin. After curing, the samples in the resin were sectioned at 500 nm thickness. Some of the serial sections were stained with toluidine blue and imaged with a light microscope (AX80, Olympus). Others of the serial sections were mounted on pieces of conductive carbon nanotube tape (49), and the sections on the tape were imaged with a scanning electron microscopy (Scios2, Thermo Fisher Scientific) equipped with retractable annular backscattered electron detector and energy dispersive X-ray
spectroscopy (EDS) detector (Ultim Max 170, Oxford Instruments). The secondary electron and backscatter electron images were acquired at accelerating voltage 5 kV and probe current 0.4 nA, and EDS images were acquired at accelerating voltage 10 kV and probe current 0.8 nA.

**Histological and immunohistochemical analysis**

The mouse kidneys fixed with 10% buffered-formalin were processed to make regular paraffin sections. The kidney sections were stained with Sirius-red staining to evaluate fibrosis. The fibrotic lesions were quantified as described previously (11). In addition, the kidney sections were subjected to immunohistochemistry using an antibody against TLR4 (Santa Cruz, Dallas, TX, USA) to determine its localization.

**Quantitative RT-PCR.**

Quantitative RT-PCR was performed as previously described (43). The nucleotide sequence of the primers were as follows.

α-smooth muscle actin: CTGACAGAGGCACCACACTGAA & CATCTCCAGAGTCAGAGGAC

Collagen 1α1: GAGCGGAGAGTACTGGATCG & GTTCGGGCTGATGTACCAGT

IL6: CTGCAAGAGACTTCCATCCAGTT & AAGTAGGGAAGGCCGTGGTT

MCP1: GGCTCAGCCAGATGCTGTAAC & GCCTACTCATGGGATCATCTTG
MMP2: ACCCTGGGAGAAGGACAAGT & ATCACTCGACCAGTGTCTG

Vimentin: CTGCACGATGAAGGAGATCCA & AGCCACGCTTTTCATACTGCT

TGFβ1: TTGCTTCAGCTCCACAGAGA & TGGTTGTAGAGGGCAAGGAC

Osteopontin: TCCAAAGAGAGCCAGGAGAG & GGCTTTGGAACTTGCTTGAC

Kim1: CTGGAATGGCCTGTGACATCC & GCAGATGCCAACATAGAAGCCC

Nlrp3: GAAATATGCACAGGTATCCT & GTAATTTTGAAGTATTGCTTGTTT

TNFα: CGAGACCTCTGGGAAAAAGCT & CGAGACCTCTGGGAAAAAGCT

Cyclophilin: TGGAGAGCACAAGACAGACA & TGCCGGAGTCGACAATGAT

**Microarray experiment**

The microarray analysis was performed using the SurePrint G3 Human Gene Expression 8x60K v3 (Macrogen Corp. Japan). Cy3 labeled cRNA was prepared from 1–5 µg of total RNA using the Agilent’s Quick Amp Labeling Kit. The labeled cRNA (1.65 µg) was hybridized with the Agilent expression microarray according to the protocols provided by the manufacturer. Three different time points (3 hours, 6 hours, and 24 hours) after stimulation with or without synthesized calcium-phosphate particles (10 µg phosphorus/mL) were tested in four replications for each time point. Arrays were scanned using the Agilent Technologies G4900DA SG12494263. The data processing and analysis was performed using Agilent Feature Extraction v11.0.1.1.
Enrichment test based on KEGG pathway (https://www.kegg.jp/kegg/pathway.html) was conducted with significant gene list. Raw p-value was calculated by modified Fisher’s exact test and Bonferroni test. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE135977 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135977).

**Surface plasmon resonance**

Physical interaction between calcium phosphate particles and TLR4 was determined by surface plasmon resonance (SPR) using ProteOn XPR36 (Bio-rad). Alendronate was immobilized on a sensor chip (ProteOn GLC Sensor Chip, Bio-rad) by amine coupling. Synthesized calcium-phosphate particles were applied on the chip in the running buffer (20 mM HEPES, pH 7.4, 135 mM NaCl, 1 mM Na₂HPO₄, 2 mM CaCl₂, 0.005% Tween20) and immobilized on the chip. After washing with the running buffer, the extracellular domain of human TLR4 (R&D Systems) was applied. The kinetic analysis was performed using the Langmuir model.

**Indirect Immunofluorescence**

HK-2 cells were seeded in each well of 96-well plates (Greiner bio-one #655090). Twenty-four hours after seeding, the cells were treated with or without synthesized calcium-phosphate
particles (10 µg phosphorus/mL) for indicated time periods. The cells were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) for 20 minutes at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 3 minutes for staining with primary antibodies against APPL1 (CST, #3858), Rab5 (CST, #3547), Rab7 (CST, #9367), Rab11 (CST, #5589), EEA1 (CST, #3288), Lamp2 (Hybridoma Bank, #H4B4), GM130 (BD Transduction, #610823), phospho-p38MAPK (CST, #4511), or phospho-NFκB (Abcam, #ab86299). For staining with LC3 primary antibody ((MBL, #M152-3), the cells were permeabilized with 50 µg/mL digitonin in PBS for 5 min at room temperature. For staining with CD63 primary antibody (Abcam, #ab37149), the cells were permeabilized with 0.02% saponin for 1 min at room temperature. After washed with PBS, the cells were soaked in blocking solution (PBS containing 1% bovine serum albumin) for 30 min followed by incubation with primary antibodies in blocking solution for overnight at 4°C. The cells were then washed with PBS and incubated with Alexa 488 or Alexa 647 secondary antibody (ThermoFisher) in blocking solution for 1 hour at room temperature. For staining with osteopontin primary antibody (Abcam, #ab8448), the cells were permeabilized and blocked with PBS containing 1% BSA, 10% normal goat serum, 0.3 M glycine, and 0.05% Tween 20 for 1 hour at room temperature. Nuclei, cell membrane, and cytoskeleton for the image analysis were stained with Hoechest 33342 (ThermoFisher) and CellMask Deep Red (ThermoFisher), and β-tubulin (SIGMA, #T8328),
respectively. After washing with PBS, confocal images were acquired using a confocal laser-scanning microscope (Nikon A1).

**Image processing**

Confocal images were acquired at 1024 × 1024 pixels resolution at z-step resolution 0.65 µm with CFI Plan Apo λ 40× objective (NA = 0.95). The images of endosome marker proteins and fluorescently labeled calcium-phosphate particles were acquired using a galvano scanner (average of four images). The images of tubulin, Hoechst, CellMask, and signaling proteins were acquired without averaging step. Images of 96-well plates were acquired by JOBS mode which can run fully-automated procedures macros. The images of each well were obtained at ten different areas.

**Image analysis**

All confocal images were analyzed using NIS-Elements AR 4.5 microscope imaging software (Nikon). Maximum intensity projection (MIP) was performed before the image analysis. For detecting the area of nucleus, the signals of Hoechst were used. For detecting the cell shape, the signals of tubulin antibody or CellMask were used. To determine subcellular localization of endosomes, two areas in the cell were extracted (Figure 5E); Area 1 was defined as the
perinuclear area within 10 pixels from the nucleus boundary. Area 2 was defined as the submembrane area within 12 pixels from the plasma membrane. To calculate the mean intensity of each signal, sum intensity in Area 1 or Area 2 was divided by sum area of Area 1 or Area 2, respectively.

**ELISA**

Osteopontin levels in the conditioned medium were measured using Quantikine ELISA Kit (#DOST00, R&D Systems). HK-2 cells were seeded into 6-well plates and stimulated with synthesized calcium-phosphate particles (10 µg phosphorus/mL) or vehicle (DMEM containing 5% FBS, 3 mM CaCl₂ and 1 mM phosphate buffer). After incubation for indicated time periods, the supernatant from each well was collected, centrifuged at 13,500 × g for 10 min at 25°C, and subjected to the ELISA. Serum FGF23 levels in mice and humans were measured using intact FGF23 ELISA (Kinos) according to the manufacturers’ protocols. The FGF23 assay is reported to have a lower detection limit of < 3 pg/mL, with an interassay coefficient of variation of 2.1% at 42.4 pg/mL and 3.8 % at 19.5 pg/mL. The intra-assay coefficient of variation is 2.8 % at 28.7 pg/mL and 2.0% at 33.6 pg/mL. The upper reference limit is defined as 53 pg/mL, corresponding to the cut-off value determined by the cross-sectional study.
**Blood and urine tests**

Serum creatinine, urine creatinine, and serum creatinine in mice were quantified using Fuji Dri-Chem slides and the analyzer (Dri-Chem NX500V, Fuji). Serum creatinine, urine creatinine, urine L-FABP and urine β2-microglobulin levels in humans were measured as a part of the standard patients’ care at the Jichi Medical University laboratory (Tochigi, Japan).

**Clinical studies**

The cross-sectional study was performed using urine and serum samples obtained from 198 predialysis CKD patients recruited from outpatients at the Nephrology Department in Jichi Medical University Hospital between July 2014 and August 2017 (31). The participants were all Japanese, 69 females and 129 males, consisting of 14 stage 1 (eGFR ≥ 90 mL/min/1.73m²), 37 stage 2 (90 > eGFR ≥ 60), 84 stage 3 (60 > eGFR ≥ 30), 34 stage 4 (30 > eGFR ≥ 15), and 29 stage 5 (eGFR < 15) patients. The prospective study was performed using serum samples from the Standard Versus Intensive Statin Therapy for Hypercholesterolemic Patients with Diabetic Retinopathy (EMPATHY) study, which was registered with the University Hospital Medical Information clinical trials registry (UMIN000003486). A total of 5,107 patients with hyperlipidemia and diabetic retinopathy were randomized to either intensive or standard lipid-lowering therapy. The baseline characteristics of the participants were described previously (32). Serum samples from
5,039 patients were available for measurement of FGF23 at baseline. The outcome was the renal events defined as initiation of chronic dialysis or increase in serum creatinine level by at least 2-fold (and >1.5mg/dL).

Statistics

For statistical analysis of immunofluorescence images, we used R lawstat package software. First, we performed normality test and F-test to all imaging data. As all data did not show normal distribution and equal variance, non-parametric Brunner-Munzel test was performed to get p-value and effect size according to big sample size. For statistical analysis of the prospective clinical study, cumulative end point was derived by the Kaplan-Meier method; the groups were compared using the log-rank test. Cox proportional hazards regression was performed to identify associations with the endpoint. In multivariable analysis, potential confounders were adjusted by selecting age, sex, body mass index (BMI), and serum creatinine level. The statistical analyses were performed by R software version 3.6.0. P values of <0.05 were considered statistically significant. Statistical analysis of the other data was done using GraphPad Prism version 8.4.3.

Study approval

All animal experiments were approved by the institutional animal care and use committee from
Jichi Medical University. All clinical studies were conducted in accordance with the Declaration of Helsinki. The clinical study protocols were approved by Ethical Committee of the Jichi Medical University. Written informed consent was obtained from all participants.
Author contributions:

K. Shiizaki, Y. Miura, M. Miura, Y. Iwazu, H. Hayashi, and H. Kurosu conducted animal and tissue culture experiments. A. Tsubouchi, R. Kunishige, and M. Masutani conducted tissue culture experiments and image analysis. K. Seo, T. Kuchimaru, and H. Hayashi conducted ex vivo imaging. B. Battulga, N. Ohno, and T. Hara performed electron microscopic studies. K. Shiizaki, Y. Iwazu, K. Negishi, K. Kario, K. Kotani, T. Yamada, D. Nagata, I. Komuro, and H. Itoh performed clinical studies. H. Kurosu, M. Murata, and M. Kuro-o planned the experiments. A. Tsubouchi, K. Seo, and M. Kuro-o wrote the paper. All authors reviewed, edited, and approved the manuscript. K. Shiizaki and A. Tsubouchi contributed most to the in vivo and in vitro experiments, respectively. The corresponding author judged that the in vivo and in vitro experiments were equally important to support the main conclusion of this manuscript.
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References


Figure 1. Calcium phosphate particles induce renal tubular cell damage. (A) Human renal proximal tubular cells (HK-2) were cultured in the control medium (DMEM containing 0.1% FBS, 3 mM calcium, and 1 mM phosphate) or in the high phosphate media (DMEM containing 0.1%FBS, 3 mM calcium, and 3, 5, or 7 mM phosphate). The cell viability was quantified 24 hours later by MTT assay and expressed as percentage of the cell viability in the control medium. The same experiment was repeated in the presence of alendronate at the indicated concentrations. The data represents mean ± s.d. N = 4 for each culture condition. **P < 0.0001 vs the control in each culture condition by 1-way ANOVA with Tukey’s multiple-comparison tests. (B) Transmission electron microscopic observation of the medium containing 7 mM phosphate. Bar = 500 nm. (C) The particle size distribution of calcium phosphate particles (CaPi particles). The DMEM containing 3 mM calcium and 7 mM phosphate was incubated at room temperature for 24 hours and then subjected to nanoparticle tracking analysis before (black, CaPi particles) or after centrifugation at 16,000 g for 30 min (CFG) or treatment with HCl (at 100 mM for 30 min) or EDTA (at 50 mM for 30 min). (D) Relative viability of HK-2 cells cultured in the control medium and in the supernatant of the high phosphate medium (7 mM phosphate) after centrifugation at 16,000 g for 2 hours was determined by MTT assay. The data represents mean ± s.d. N = 9 for each culture condition. No difference was observed (n.s.) by t-test. (E) Relative viability of HK-2 cells cultured in the control medium inoculated with indicated doses of synthesized calcium phosphate particles. The data represents mean ± s.d. N = 8 for each culture condition. *P < 0.05, **P < 0.0001 vs the control by 1-way ANOVA with Dunnett’s multiple-comparison tests.
Figure 2. Calcium phosphate particles in the tubular lumen detected by *ex vivo* imaging. Calcium phosphate particles, the interstitial space, and the cell nuclei were depicted in red (OsteoSense), green (FITC-dextran), and blue (Hoechst), respectively. Mice were placed on either regular diet containing 0.35% inorganic phosphate (NP, A) or high phosphate diet containing 2.0% inorganic phosphate (HP, B) for 8-9 days. (C) To show that calcium phosphate particles were present in the tubular lumen, FITC-dextran was replaced with FITC-LTL that labeled apical membrane of proximal tubules (bar = 50 µm). (D, E) Mice fed HP were treated with alendronate (10 mg/kg, subcutaneous injection every other day, Ale, D) or given 0.14 M NH₄Cl solution as drinking water (NH₄Cl, E). Calcium phosphate particles were absent in mice lacking the Tlr4 gene (*Tlr4*[^][[^]^]del) fed NP (F) or HP (G). Bar = 1000 µm except C. (H) The number of calcium phosphate particles were indicated as mean ± s.d. *N* = 6-8 for each group. *P* < 0.01, **P < 0.0001 by 1-way ANOVA with Tukey’s multiple-comparison tests.
Figure 3. TLR4 expressed in renal tubules is required for high phosphate diet to induce kidney damage. Mice lacking the Tlr4 gene in renal tubular cells (Tlr4-Cre) and their controls (Cre; mice carrying the Cre transgene alone) at 4 weeks of age were placed on high phosphate diet containing 2.0% inorganic phosphate (HP) for 4 weeks. Relative renal mRNA levels of the markers for tubular damage and inflammation (A) and fibrosis (B) were determined by quantitative RT-PCR. (C) There was no difference in the amount of urinary phosphate excretion between Tlr4-Cre mice (N = 13) and Cre mice (N = 9). The data were indicated as mean ± s.d. **P < 0.01 vs Cre by Mann-Whitney test. αSMA; α-smooth muscle actin, IL6; interleukin-6, MCP1; monocyte chemotactic protein-1, MMP2; matrix metalloprotease-2, TGFβ1; transforming growth factor-β1, IL1β; interleukin-1β, OPN; osteopontin, Kim1; kidney injury molecule-1, Ngal; neutrophil gelatinase-associated lipocalin. Sirius Red staining of the kidney sections (D, E, F) detected patchy red areas of interstitial fibrosis in the cortex and the cortico-medullary junction (CMJ) in the control mice (HP, Cre) but not in the mice lacking the Tlr4 gene in renal
tubular cells (HP, Tlr4-Cre). Bar = 100 µm. (G) The fibrotic area was quantified in the cortex (the upper panel) and the CMJ (the lower panel) in the individual mice (N = 4 for each group). The data were indicated as mean ± s.d. **P < 0.01 by 1-way ANOVA with Tukey’s multiple-comparison tests.
Figure 4. Calcium phosphate particles activate p38 NFκB pathway and induce osteopontin secretion. HK-2 cells were incubated with synthesized calcium phosphate particles (10 µg phosphorus/mL) for the indicated time periods and subjected to immunocytochemistry using antibodies against phosphorylated p38 (pp38, A) or phosphorylated NFκB (pNFκB, B). The confocal microscopic images were analyzed using the NIS-Elements software (Nikon) to determine the intensity of fluorescent signals from the nucleus (red) and the cytoplasm (blue). The data were indicated as mean ± s.d. *N* = the number of cells analyzed for each time point. *Effect size > 0.25, **effect size > 0.3, #effect size > 0.35 vs time 0 (without stimulation with calcium phosphate particles) by Brunner-Munzel test. Representative confocal images of immunocytochemistry for pp38 (C) and pNFκB (D) were shown. Plasma membrane and nuclear membrane were indicated as blue and orange lines, respectively. Bar = 50 µm. (E) Concentration of osteopontin was measured by ELISA in the conditioned medium of HK-2 cells incubated with or without calcium phosphate particles (10 µg phosphorus/mL) for the indicated time periods. *N* = 3 for each column. *P* < 0.0001 vs vehicle by 2-way ANOVA with Šidák’s multiple comparison test.
Figure 5. Calcium phosphate particles disturb endosomal trafficking. HK-2 cells were incubated with calcium phosphate particles (10 µg phosphorus/mL) labeled with 5(6)-RhR-dRIS for indicated time periods and then subjected to immunocytochemistry using antibodies against endosomal markers Rab7 (A), Lamp2 (B), CD63 (C), LC3 (D). Confocal microscopic images were analyzed to determine the intensity of fluorescent signals in Area 1 (perinuclear region, magenta) and Area 2 (submembrane region, green) (E). The data were indicated as mean ± s.d. N = the number of cells analyzed for each time point. *Effect size > 0.5 vs time 0 (before stimulation with calcium phosphate particles) by Brunner-Munzel test. (F) Overlap between signals from calcium phosphate particles and signals from endosomal markers was shown as the ratio of the overlapping area to the total area of calcium phosphate particles. The data were indicated as violin plots with median (red) and quartiles (dotted line). N = the number of cells analyzed (G) Viability of HK-2 cells was determined by CCK-8 assay at the indicated time points after addition of calcium phosphate particles (10 µg phosphorus/mL) to the medium in the
presence of a TLR4 signaling inhibitor TAK242 (the final concentration at 2 µM, blue) or vehicle (DMSO, red). (H) As in (G), except that relative cellular damage was determined by LDH assay and expressed as percentages of the maximum cell damage induced by 0.2% Triton-X100. The data were indicated as mean ± s.d. $N = 4$ for each time point. No difference was observed between the TAK242 group and vehicle group by 2-way ANOVA with Šidák’s multiple comparison test. $*P < 0.0001$ vs time 0.5h by 2-way ANOVA with Dunnett’s multiple-comparison tests.
Figure 6. Correlation of ePTFp with serum FGF23 levels and the kidney damage in mice. (A) The experimental design. Urine, blood, and kidney were obtained from uninephrectomized mice or sham-operated mice placed on diet containing 0.35%, 1.0%, 1.5%, or 2.0% inorganic phosphate for 12 weeks. Correlation between estimated phosphate concentration of proximal tubular fluid (ePTFp) and the following parameters was shown in double logarithmic plot; serum FGF23 levels (B), relative renal mRNA levels of Ngal (C), osteopontin (D), MCP1 (E), TNFa (F), vimentin (G), collagen 1a1 (H), and creatinine clearance (I). The ePTFp values of the inflection points and coefficients of determination ($R^2$) of the segmental liner regression lines were indicated. (J) A schematic representation of the mechanism by which high FGF23 causes progressive nephron loss. Points of therapeutic intervention are indicated in blue.
Figure 7. A prolonged increase in phosphate excretion per nephron reduces the nephron number in mice. (A) Urine and blood were collected from the 8 groups of mice (uninephrectomized mice or sham-operated mice placed on either 0.35%, 1.0%, 1.5%, or 2.0% phosphate diet for 2 weeks). Relation between phosphate excretion per nephron and serum FGF23 was shown in a log-log plot. The nephron number (B), serum FGF23 (C), phosphate excretion per nephron (D), and creatinine clearance (E) at the 2 weeks (open columns) and at the 12 weeks (cyan columns) were shown in each group. The nephron number of the mice with serum FGF23 levels lower than 290 pg/mL was assumed as 20,000 and 10,000 for the sham-operated mice and the uninephrectomized mice, respectively, and shaded grey. The other data were indicated as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.0001 by 2-way ANOVA with Šidák’s multiple comparison test.
Figure 8. Serum FGF23 levels predict CKD progression. (A) Relation between ePTFp and serum FGF23 in 148 outpatients with various renal function was shown in a log-log plot. The ePTFp and FGF23 values of the inflection points and the coefficient of determination ($R^2$) of the segmental linear regression line were indicated. (B) Cumulative incidence by the renal events and FGF23 levels in patients participated in the EMPATHY study. Kaplan-Meier curves of the cumulative incidence rates of the renal events by two groups stratified by 53 pg/mL of FGF23 during the follow-up period. The patients with FGF23 levels over 53 pg/mL were associated with the increased renal events independently of serum creatinine levels.