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RESEARCH ARTICLE

# Elevated luminal inorganic phosphate suppresses intestinal Zn absorption in 5/6 nephrectomized rats

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## Abstract

Zinc (Zn) is an essential trace element in various biological processes. Chronic kidney disease (CKD) often leads to hypozincemia, resulting in further progression of CKD. In CKD, intestinal Zn absorption, the main regulator of systemic Zn metabolism, is often impaired; however, the mechanism underlying Zn malabsorption remains unclear. Here, we evaluated intestinal Zn absorption capacity in a rat model of CKD induced by 5/6 nephrectomy (5/6 Nx). Rats were given Zn and the incremental area under the plasma Zn concentration-time curve (iAUC) was measured as well as the expression of ZIP4, an intestinal Zn transporter. We found that 5/6 Nx rats showed lower iAUC than sham-operated rats, but expression of ZIP4 protein was upregulated. We therefore focused on other Zn absorption regulators to explore the mechanism by which Zn absorption was substantially decreased. Because some phosphate compounds inhibit Zn absorption by coprecipitation and hyperphosphatemia is a common symptom in advanced CKD, we measured inorganic phosphate ($P_i$) levels. $P_i$ was elevated in not only serum but also the intestinal lumen of 5/6 Nx rats. Furthermore, intestinal intraluminal $P_i$ administration decreased the iAUC in a dose-dependent manner in normal rats. In vitro, increased $P_i$ concentration decreased Zn solubility under physiological conditions. Furthermore, dietary $P_i$ restriction ameliorated hypozincemia in 5/6 Nx rats. We conclude that hyperphosphatemia or excess $P_i$ intake is a factor in Zn malabsorption and hypozincemia in CKD. Appropriate management of hyperphosphatemia will be useful for prevention and treatment of hypozincemia in patients with CKD.

**NEW & NOTEWORTHY** We demonstrated that elevated intestinal luminal $P_i$ concentration can suppress intestinal Zn absorption activity without decreasing the expression of the associated Zn transporter. Increased intestinal luminal $P_i$ led to the formation of an insoluble complex with Zn while dietary $P_i$ restriction or administration of a $P_i$ binder ameliorated hypozincemia in chronic kidney disease model rats. Therefore, modulation of dietary $P_i$ by $P_i$ restriction or a $P_i$ binder might be useful for the treatment of hypozincemia and hyperphosphatemia.

*chronic kidney disease; dietary phosphate; hyperphosphatemia; zinc deficiency; zinc transporter*

## INTRODUCTION

Zinc (Zn) is an essential trace element that, as a cofactor of enzymes and transcription factors, maintains important biological processes such as cell division and differentiation (1). Hypozincemia is often a comorbidity of chronic kidney disease (CKD) (2). The prevalence of hypozincemia varies from 25% to 70% in patients on maintenance hemodialysis (3–5). Several factors affect serum Zn levels in patients with CKD. For instance, those are decreased intestinal absorption, decreased food intake, uremic toxicity, bioavailability, abnormal distribution to some tissues, increased fecal or urinary loss, hemodialysis, aging, and multiple medications (6). Low dietary Zn intake is a significant risk factor for hypozincemia (7). On the other hand, serum Zn levels showed a negative relationship with the progression of the CKD stage (8, 9). In addition, hypozincemia in patients with CKD may increase the risk of end-stage kidney disease (10) and various complications such as hypertension, dyslipidemia, type II diabetes mellitus, taste disorders, cardiovascular disease including vascular calcification, and infectious disease (11–16). Therefore, prevention of hypozincemia is essential for delaying the progression of CKD and related complications and the decline in the patient's quality of life.

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Zn supplementation is the standard treatment for hypozincemia and can generally increase serum Zn levels in patients with CKD (6). The dose of Zn supplementation is determined according to the severity of hypozincemia, ranging from 50 to 100 mg/day for adults and 1 to 3 mg/kg/day for children (6, 17). Current supplementation criteria do not consider the background and progression of the disease, which may lead to Zn under- or overdosing. In a randomized trial of patients on hemodialysis, for example, serum Zn levels were not sufficiently elevated even when standard doses of Zn were administered (18). Moreover, overdose of Zn often causes copper (Cu) and iron deficiency, leading to anemia and neurological disorders (19–22). At present, treatments that target the cause of hypozincemia are not well established in CKD.

The mechanism underlying hypozincemia in CKD has not been elucidated; however, increased urinary Zn excretion, decreased intestinal Zn absorption, and loss of Zn in dialysis have been proposed as potential causes (9, 23). Systemic Zn levels are mainly controlled by intestinal absorption. Cellular Zn flux is regulated by transporters, including members of SLC39A [Zrt-, Irt-like protein (ZIP)], and SLC30A [Zn transporter (ZnT)] families. ZIP4 (SLC39A4) is a responsible transporter of intestinal Zn absorption (24, 25). In addition to these transporter-mediated pathways, various intestinal luminal factors derived from the diet, including animal protein, sulfur-containing amino acids, calcium, and iron, have been reported to positively or negatively affect Zn absorption (26–29). Interestingly, phosphate compounds such as phytate (inositol hexaphosphate) and calcium phosphate can inhibit Zn absorption by coprecipitation with Zn (30, 31). In CKD, hyperphosphatemia, a common complication due to decreased urinary excretion of $P_i$, can increase secretion of $P_i$ into the gastrointestinal tract (32, 33). Therefore, we hypothesized that elevated intestinal luminal $P_i$ levels caused by hyperphosphatemia and/or dietary $P_i$ intake may suppress intestinal Zn absorption in patients with CKD. In this study, we examined the mechanism underlying Zn malabsorption in a rodent model of CKD induced by 5/6 nephrectomy (5/6 Nx).

## MATERIALS AND METHODS

### Animals

All animals (male Wistar rats aged 5–8 wk) used in this study were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in a climate-controlled room (22 ± 2°C) under a 12:12-h light-dark cycle and maintained on standard chow (MF; $P_i$: 0.83%, Oriental Yeast, Tokyo, Japan) with free access to food and distilled water before induction of CKD or dietary intervention. All animals were euthanized under anesthesia between 9:00 AM and 12:30 PM for sample collection. All animal studies were approved by the animal experimentation committee of Tokushima University and were conducted in accordance with the guidelines for the management and handling of experimental animals.

### 5/6 Nx-Induced CKD Model

Five-week-old rats (Japan SLC) were randomly divided into a sham-operated group (hereafter sham rats) and a 5/6 Nx-induced CKD group (hereafter 5/6 Nx rats). Two-thirds of the left kidney mass of each 5/6 Nx rat was surgically removed under pentobarbital anesthesia (50 mg/kg ip). After 1 wk, the right kidney was removed under pentobarbital anesthesia. Sham operation was performed as described previously (34). 5/6 Nx rats were fed an MF diet (Oriental Yeast) or high-$P_i$ MF diet ($P_i$: 1.2%, Oriental Yeast) for 9 wk and then fed an MF diet for 3 wk.

### Measurement of Zn Uptake in Brush Border Membrane Vesicles

Brush border membrane vesicles (BBMVs) were prepared from the duodenum by using $Ca^{2+}$ precipitation, as described previously (35, 36). Briefly, the mucosa of duodenum section was scraped, and homogenized in homogenate buffer (50 mM mannitol and 2 mM Tris·HCl buffer, pH 7.5). The homogenates were washed repeatedly by suspension and centrifugation in suspension buffer (300 mM mannitol and 10 mM Tris-HEPES, pH 7.5) to obtain purified BBMVs. All operations were performed at 4°C. A radioactivity assay was performed to analyze the uptake of $^{65}Zn$ (RIKEN, Saitama, Japan) into BBMVs by a rapid filtration technique (37). In brief, 10 μL of vesicle suspension was added to 90 μL of incubation solution (100 mM mannitol and 20 mM Tris-HEPES buffer, pH 7.0) containing $^{65}Zn$ (0.2 μCi/mL), and the preparation was incubated at 20°C for 1 min. The reacted BBMV was filtrated on a nitrocellulose membrane and washed out free $^{65}Zn$. The radioactivity [counts per minute (cpm)] of $^{65}Zn$ in the BBMV-bound membrane was measured by a gamma counter (Hitachi Aloka Medical, Tokyo, Japan). The Zn uptake activity (nmol/min) of BBMV was calculated from the radioactivity of BBMV divided by the specific activity of $^{65}Zn$ (cpm/nmol). The protein content of BBMV was determined by the Bradford method. Finally, Zn uptake activity was expressed by nmol/mg protein/min.

### Plasma and Intestinal Intraluminal Fluid Biochemical Parameters

Blood samples were collected by inferior vena cava puncture into heparin-coated tubes (Mochida Pharmaceutical, Tokyo, Japan). After centrifugation for 15 min at 5,000 *g*, the supernatant was collected as plasma. The contents of the duodenum segment were extruded into 1.5-mL tubes. After centrifugation at 3,000 rpm for 5 min, the supernatant was collected as intestinal intraluminal fluid. The concentrations of Zn, $P_i$, calcium (Ca), and creatinine (Cre) in plasma and intestinal intraluminal fluid were determined by using a Metalloassay Kit (Metallogenics, Chiba, Japan), Phospha-C test (Wako, Osaka, Japan), Calcium-E test (Wako), and Labassay Creatinine (Wako), respectively. The concentration of blood urea nitrogen (BUN) was measured by Oriental Yeast.

### Fecal Zn Excretion

Metabolic cages were used to collect fecal samples for 72 h before euthanasia. The feces were first dried at 110°C for 12 h and then micro pulverized, after which 100-mg samples were ashed at 250°C for 3 h, 350°C for 3 h, and 550°C for 24 h. These samples were heated at 100°C for 15 min with 10 mL of 1% HCl. Extracted Zn was measured using an inductively

coupled plasma optical emission spectrometer (ICP-OES; iCAP6300, Thermo Fisher Scientific, Waltham, MA). The fecal Zn excretion rate as the ratio of daily Zn excretion to daily Zn intake was then calculated because 5/6 Nx rats showed lower food intake (Supplemental Table S1).

### Western Blot Analysis

Duodenal BBMVs were mixed in a sample buffer containing 5% 2-mercaptoethanol and subjected to SDS-PAGE. The separated proteins were transferred by electrophoresis to a polyvinylidene difluoride transfer membrane (Immobilon-P, Millipore), which was then treated with affinity-purified anti-ZIP4 (1:2,500) and mouse anti-β-actin monoclonal antibody (A5441, Sigma-Aldrich, St. Louis, MO, 1:5,000) as an internal control. Goat anti-mouse IgG (H + L)-HRP conjugate (1:4,000) was used as the secondary antibody, and signals were detected using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) and luminescent image analyzer LAS-3000 (Fujifilm Life Science, Tokyo, Japan). Signal intensity was quantified with Multi Gauge V3.0 software (Fujifilm).

### Real-Time PCR

Total RNA was isolated from harvested duodenum using RNAiso plus (Takara Bio, Shiga, Japan) and then digested by recombinant DNase I (Worthington Biochemical, Lakewood, NJ). First-strand cDNA was synthesized from 1.0 μg of total RNA by using M-MLV reverse transcriptase (Nippon Gene, Tokyo, Japan), oligo (25) dT primer (Invitrogen), and dNTP mixture (Promega). After cDNA synthesis, real-time PCR was performed with the appropriate forward and reverse primers and Fast SYBR Green master mix (Applied Biosystems) using a real-time PCR system (StepOne Plus, Applied Biosystems). Real-time PCR primers (forward and reverse, respectively) were as follows: *Zip4* (*Slc39a4*), 5′-CAGCTACTGCAGAAGATTGAGG-3′ and 5′-CTTGGAAGCAGGATCCATTAAG-3′; *Znt1* (*Slc30a1*), 5′-ACACGCTAGTGGCTAA-CACC-3′ and 5′-AGACTGTCTGACTCCTGGATGA-3′; *Mt1*, 5′-TCTTGTCGCTTACACCGTTG-3′ and 5′-AGCACTGTTCGTCACTTCAG-3′; and *18 s rRNA*, 5′-GGGGAACGCGTGCATTTATC-3′ and 5′-CTCTCCGGAATCGAACCCTG-3′. Using the comparative $C_t$ method, each gene was quantified as mRNA level normalized to 18S rRNA.

### Plasma Zn Concentration-Time Curve After Zn Administration

Total Zn absorption capacity was measured in sham, 5/6 Nx, and 8-wk-old normal rats. Sham rats and 5/6 Nx rats were fed an MF diet (Oriental Yeast) for 9 wk. Next, sham rats were fed an altered AIN-93G diet (Oriental Yeast) containing 0.6% $P_i$, and 5/6 Nx rats were fed an altered AIN-93G diet containing 1.2% $P_i$ for 3 wk. A catheter (PE-50, Natsume Seisakusho, Tokyo, Japan) was inserted into the femoral vein of sham, 5/6 Nx, and normal rats under pentobarbital anesthesia. For sham and 5/6 Nx rats, 1 mL of 40 mg/mL $ZnSO_4$ solution was administered into the stomach by gavage, and plasma was collected at 0, 5, 10, 20, 30, and 40 min. For normal rats, 1 mL of 15 mM Zn solution was administered into the stomach by gavage without or with 15, 45, or 150 mM $P_i$ and 3% lanthanum carbonate (La; No. 325767, Sigma-Aldrich), a potent binder of phosphate, and plasma was collected at 0, 10, 20, 30, 40, and 60 min. $P_i$ concentrations administered to the rats were selected to be equivalent to 3 or 10 times the intestinal luminal $P_i$ levels in the sham rats. Zn levels were measured to obtain a plasma Zn concentration-time curve and the incremental area under the curve (iAUC) was determined.

### Zn Solubility in the Presence of $P_i$

Zn solution (30 mM) was mixed with an equal volume of $P_i$ solution (0–300 mM). The solution was passed through a 0.22-μm filter (Wako). Zn in the filtrate was estimated as soluble Zn (31). Zn was analyzed by ICP-OES, and the solubility of Zn was calculated as the ratio of filtered Zn to Zn before filtration.

### Effect of Dietary $P_i$ Restriction and La on Hypozincemia

The effect of $P_i$ restriction was measured in both 5/6 Nx- and adenine-induced CKD models. Sham and 5/6 Nx rats were fed an MF diet for 12 wk. Subsequently, 5/6 Nx rats were divided into a 5/6 Nx-control $P_i$ (CP) group fed an altered AIN-93G diet (Oriental Yeast) containing 1.03% $P_i$ (CP diet) and a 5/6 Nx-low $P_i$ (LP) group fed an altered AIN-93G diet containing 0.2% $P_i$ (LP diet) for 2 wk. Sham rats were fed a CP diet for 2 wk. Eight-week-old normal rats were randomly divided into a control group fed a CP diet for 40 days and an adenine group fed a CP diet containing 0.2–0.6% adenine (adenine-CP diet, A8626, Sigma-Aldrich) for 33 days. Subsequently, the adenine group was further divided into an adenine-CP group fed an adenine-CP diet and an adenine-LP group fed an LP diet containing 0.2–0.6% adenine for 7 days. In addition, 8-wk-old rats were randomly divided into a control group fed an MF diet and an adenine group fed an MF diet containing 0.4% adenine for 4 wk. The adenine group was then fed an MF diet containing 0.4% adenine with or without 6% La for 2 wk. Zn levels were measured in plasma samples as described above in *Plasma and Intestinal Intraluminal Fluid Biochemical Parameters*.

### Statistical Analysis

Data are presented as means ± SE. GraphPad PRISM software (version 5, GraphPad Software, San Diego, CA) was used for all statistical analyses. For comparison between two groups, a two-tailed unpaired *t* test was used. For multiple comparisons, one-way ANOVA followed by Tukey's tests was used. *P* values of $<0.05$ were considered statistically significant.

## RESULTS

### Imbalance in Zn Flux in the Intestine of 5/6 Nx Rats

In 5/6 Nx rats, plasma $P_i$, BUN, and Cre levels were significantly elevated, and Zn and Ca levels were significantly decreased compared with sham rats (Table 1), similar to the trend in biochemical parameters observed in patients with CKD. To assess intestinal Zn flux, we verified Zn excretion into feces. The fecal Zn content was increased, but there was no difference in daily fecal Zn excretion between sham and 5/6 Nx rats (Table 1). However, the fecal Zn excretion rate was significantly higher in 5/6 Nx rats than in sham rats (Table 1). Therefore, we hypothesized that Zn absorption is substantially decreased in CKD.

**Table 1.** *Comparison of plasma biochemical parameters and fecal Zn excretion between Sham and 5/6 Nx rats*

| Biochemical Parameter | Sham | 5/6 Nx |
|---|---|---|
| Plasma | | |
| Zn, μg/dL | 102 ± 15.5 | 58.9 ± 3.67* |
| Pi. mg/dL | 6.52 ± 0.45 | 10.2 ± 0.87* |
| Ca, mg/dL | 8.99 ± 0.26 | 6.51 ± 0.18* |
| BUN, mg/dL | 17.3 ± 0.65 | 93.1 ± 10.8* |
| Cre, mg/dL | 0.79 ± 0.09 | 1.83 ± 0.11* |
| Fecal Zn[1] | | |
| Content, mg/g | 0.24 ± 0.01 | 0.27 ± 0.01* |
| Excretion, mg/day | 0.56 ± 0.03 | 0.53 ± 0.03 |
| Excretion rate, % | 63.4 ± 1.85 | 74.6 ± 1.41* |

[1]Amount of Zn, daily Zn excretion, and daily Zn excretion rate (Zn excretion/Zn intake) in feces were measured. Data are expressed as means ± SE ($n$ = 5–7). Statistical analysis was performed using a two-tailed unpaired $t$ test. *$P < 0.05$. BUN, blood urea nitrogen; Pi, inorganic phosphate. $n$ indicates the number of samples for each experiment.

### Enhanced Zn Transport by Duodenal BBMVs in 5/6 Nx Rats

ZIP4, a ZIP family Zn transporter, mainly localizes to the intestinal brush border membrane (BBM) and plays a central role in intestinal Zn absorption (38). Zn is most actively absorbed from the jejunum in humans (39) and the duodenum in rats (40); therefore, we evaluated the uptake of Zn by the duodenum BBM using a $^{65}$Zn radioisotope. In 5/6 Nx rats, the Zn uptake activity of BBMVs was significantly increased compared with sham rats, contrary to our hypothesis (Fig. 1*A*). We also measured the expression of ZIP4, which is usually expressed as a 75-kDa protein. Under Zn deficiency, however, ZIP4 is intracellularly processed into a 37-kDa protein that accumulates on the BBM and upregulates Zn absorption from the diet (38, 41–43). Consistent with the activation of Zn uptake, ZIP4 protein levels were significantly elevated in BBMVs from 5/6 Nx rats (Fig. 1, *B* and *C*).

Zn is transported from the cytoplasm into the blood by ZnT1, which localizes to the basolateral membrane (BLM) of enterocytes (44). Intracellular Zn is stored in a form bound to metallothionein (MT), a Zn-binding protein (45). We found that mRNA levels of *Znt1* and *Mt1* in the duodenum were significantly decreased in 5/6 Nx rats compared with sham rats (Fig. 2, *A* and *B*). Both *Znt1* and *Mt1* gene expression have been reported to depend on dietary and intracellular Zn levels (46–48). These results suggest that enterocytes may not take up Zn.

### Suppression of Elevated Plasma Zn Levels After Zn Administration in 5/6 Nx Rats

To evaluate the total Zn absorption capacity in 5/6 Nx rats, including transporters and intestinal luminal factors, we calculated the iAUC after Zn administration. Plasma Zn levels were significantly lower in 5/6 Nx rats than in sham rats at all timepoints after Zn administration (Fig. 3*A*). The iAUC also decreased in 5/6 Nx rats (Fig. 3*B*), suggesting that total Zn absorption capacity is reduced in 5/6 Nx-induced CKD.

### Decreased iAUC After Zn Administration by $P_i$-Induced Reduction of Zn Solubility

To determine the cause of decreased total Zn absorption in 5/6 Nx rats, we focused on the intestinal intraluminal environment. We observed that intestinal intraluminal $P_i$ levels in sham and 5/6 Nx rats were 15.0 ± 1.89 and 22.1 ± 2.14 mM, respectively (Fig. 4*A*). To examine the effect of intestinal intraluminal $P_i$ on Zn absorption, we evaluated the iAUC after Zn administration with or without $P_i$ in normal rats, which showed that the iAUC decreased in a $P_i$ concentration-dependent manner (Fig. 4, *B* and *C*). Similar to the known properties of Zn and $P_i$ (49), the solubility of Zn was decreased by the concentrations of $P_i$ used in this study (Fig. 4*D*). Furthermore, La, a potent binder of phosphate, significantly ameliorated the $P_i$-induced reduction in the iAUC (Fig. 5, *A* and *B*). Collectively, these results suggest that elevated intestinal intraluminal $P_i$ in CKD inhibits Zn absorption by reducing Zn solubility.

### Amelioration of Hypozincemia in CKD Rats by Dietary $P_i$ Restriction and $P_i$ Binder

Lastly, we examined whether dietary interventions other than Zn supplementation might ameliorate hypozincemia. We found that 5/6 Nx rats fed an LP diet had significantly higher plasma Zn levels compared with 5/6 Nx rats fed a CP

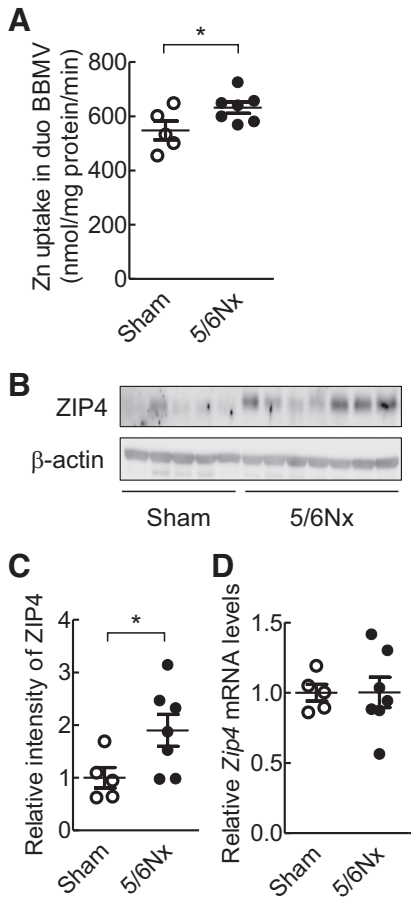


**Figure 1.** Elevated Zn transport activity in the duodenal BBMVs of 5/6 Nx rats. *A*: measurement of Zn transport activity by uptake of $^{65}$Zn in duodenal BBMVs from sham rats (open circles) and 5/6 Nx rats (closed circles). *B*: Western blot of ZIP4 in duodenal BBMVs. Each lane was loaded with 20 μg of BBMVs. *C*: Western blot band intensities. ZIP4 protein levels were normalized to β-actin levels. 5/6 Nx intensities were normalized to Sham values. *D*: measurement of *Zip4* (*Slc39a4*) mRNA levels in the duodenum by real-time RT-PCR. Data are expressed as means ± SE ($n$ = 5–7). Statistical analysis was performed using a two-tailed unpaired $t$ test. *$P < 0.05$. BBMVs, brush border membrane vesicles. $n$ indicates the number of samples for each experiment.

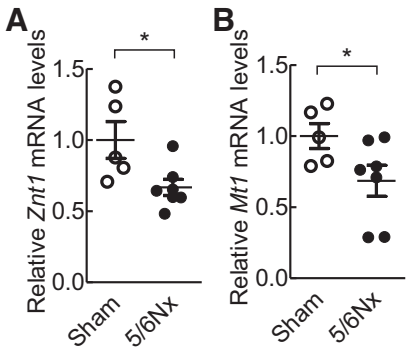


**Figure 2.** mRNA expression of *Znt1* and *Mt1* in the duodenum. Relative mRNA levels of *Znt1* (*Slc30a1*; *A*) and *Mt1* (*B*) in the duodenum of sham rats (open circles) and 5/6 Nx rats (closed circles) were measured by real-time RT-PCR. Data are expressed as means ± SE ($n = 5$–7). Statistical analysis was performed using a two-tailed unpaired *t* test. *$P < 0.05$. *n* indicates the number of samples for each experiment.

diet (Fig. 6*A*). In addition, the LP diet significantly ameliorated the decrease in plasma Zn levels observed in adenine-induced CKD rats (Fig. 6*B*). Furthermore, administration of La improved hypozincemia in adenine-induced CKD rats (Fig. 6*C*). These results suggest that appropriate Zn supplementation, dietary $P_i$ restriction, and $P_i$ binder may also be effective for improving hypozincemia in CKD.

## DISCUSSION

This study has demonstrated that elevated intestinal luminal $P_i$ concentration suppresses intestinal Zn absorption in 5/6 Nx rats, a model of CKD. One of the mechanisms of Zn malabsorption was a reduction of Zn solubility by excess intestinal luminal $P_i$. We showed that both solubility and absorption of Zn were decreased even under the condition of a 1:1 molar ratio of Zn to $P_i$. We found that the basal intestinal intraluminal $P_i$ levels were ~1.5 times higher in 5/6 Nx rats than in sham rats. These results suggest that $P_i$-mediated suppression of Zn absorption can occur in a physiological environment and may be one of the causes of hypozincemia in CKD.

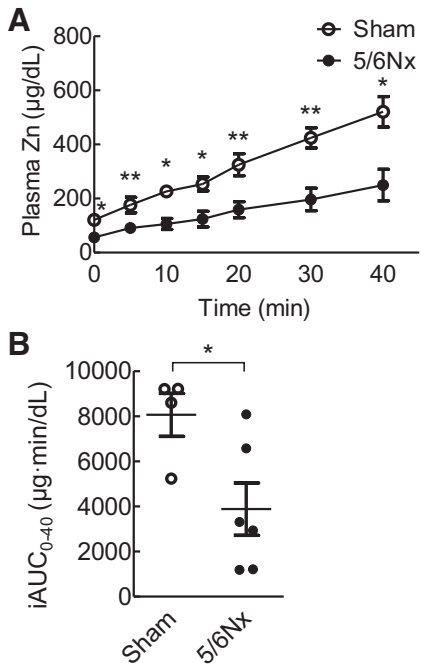


**Figure 3.** Low plasma Zn levels after Zn administration in 5/6 Nx rats. *A*: plasma Zn levels in sham rats (open circles) and 5/6 Nx rats (closed circles) at 0, 5, 10, 15, 20, 30, and 40 min after Zn administration. *B*: incremental area under the plasma Zn concentration–time curve (iAUC) in Sham and 5/6 Nx rats. Data are expressed as means ± SE ($n = 4$–6). Statistical analysis was performed using a two-tailed unpaired *t* test. *$P < 0.05$, **$P < 0.01$. *n* indicates the number of samples for each experiment.

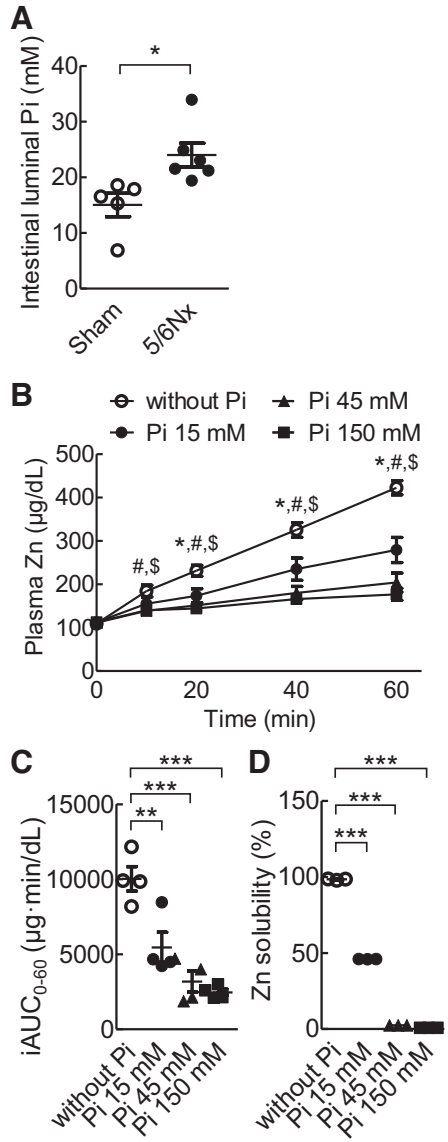


**Figure 4.** Inhibition of Zn absorption and decrease in Zn solubility by excess $P_i$. *A*: intestinal intraluminal $P_i$ levels in the duodenum of Sham (open circle) and 5/6 Nx (closed circle) rats. Data are expressed as means ± SE ($n = 5$ or 6). Statistical analysis was performed using a two-tailed unpaired *t* test. *$P < 0.05$. *B*: plasma Zn levels at 0, 10, 20, 30, 40, and 60 min after Zn administration. Eight-week-old rats were administered Zn solution without $P_i$ (open circles) or containing $P_i$ at 15 mM (closed circles), 45 mM (closed triangles), or 150 mM (closed squares). Statistical analysis was performed using one-way ANOVA followed by Tukey's tests. *$P < 0.05$ vs. $P_i$ 15 mM; #$P < 0.05$ vs. $P_i$ 45 mM; \$$P < 0.05$ vs. $P_i$ 150 mM. C: iAUC. *D*: solubility of Zn in the mixed solution (15 mM Zn and 15, 45, or 150 mM Pi) evaluated as the ratio of Zn pre- and post-filtration. Data are expressed as means ± SE ($n = 3$ or 4). Statistical analysis was performed using one-way ANOVA followed by Tukey's tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. iAUC, incremental area under the plasma Zn concentration-time curve. *n* indicates the number of samples for each experiment.

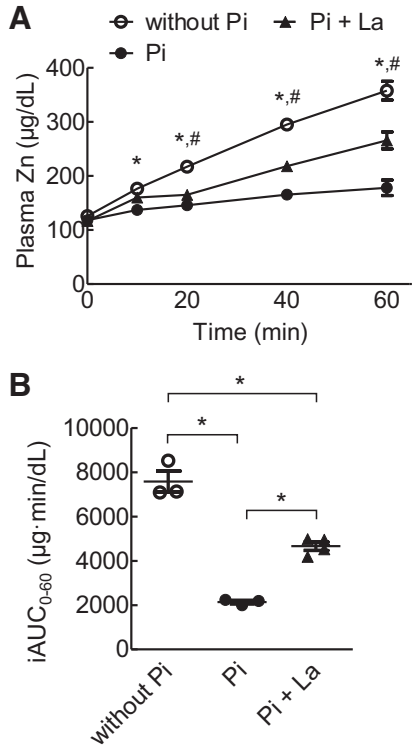


**Figure 5.** Lanthanum carbonate improved the $P_i$-induced reduction of Zn absorption. *A*: plasma Zn levels in 8-wk-old rats at 0, 10, 20, 30, 40, and 60 min after Zn administration with or without 45 mM $P_i$ and 3% La. *$P < 0.05$ vs. Pi; #$P < 0.05$ vs. $P_i$ + La. *B*: incremental area under the plasma Zn concentration-time curve (iAUC) in the rats. Data are expressed as means ± SE ($n = 3$ or 4). *$P < 0.05$. All statistical analysis was performed using one-way ANOVA followed by Tukey's tests. $P_i$, inorganic phosphate. *n* indicates the number of samples for each experiment.

In general, ZIP4 protein is upregulated to increase intestinal Zn absorption in situations of Zn deficiency or hypozincemia. We found that total intestinal Zn absorption was still suppressed in 5/6 Nx rats, although hypozincemia occurred and ZIP4 expression in the duodenum was elevated. These changes suggest that $P_i$ causes intestinal Zn malabsorption by the reduction of Zn solubility and consequently induces the compensatory response to Zn deficiency in 5/6 Nx rats. Although the Zn compounds used in Zn supplement therapy, such as gluconate, citrate, and acetate, are readily dissociated and absorbed in the gastrointestinal tract, $P_i$ can form an insoluble complex with Zn (49). We found that administration of La ameliorated the $P_i$-mediated reduction of Zn absorption. Collectively, these results suggest that suppression of Zn absorption in CKD may be, in part, induced by the formation of a Zn-$P_i$ insoluble complex in the intestinal lumen.

Thus, our study has indicated that elevated $P_i$ levels in the intestine negatively affect intestinal Zn absorption independently of the Zn transport activity of the duodenal BBM. However, there are several limitations in our study. First, we did not directly examine the amount of Zn-$P_i$ complex formed in the intestinal lumen. Because the ease of formation of the Zn-$P_i$ complex under CKD conditions is unknown, further studies will be needed to assess its contribution to the progression of hypozincemia in patients with CKD. Second, we did not evaluate the Zn transport activity of the intestinal BLM in 5/6 Nx rats. We found that the mRNA levels of *Znt1* were decreased in 5/6 Nx rats, but its protein levels remain unclear. A recent study reported that ZnT1 protein levels are downregulated by hepcidin (50), a peptide hormone secreted from the liver, and increased in CKD. Furthermore, it has been reported that albumin on the basolateral side enhances Zn absorption in a coculture of intestinal cell line Caco-2 and the mucin-producing goblet cell line HT-29-MTX (51). Thus, Zn transport by the BLM may be involved in the downregulation of total Zn absorption in CKD. In our study, total Zn absorption in 5/6 Nx rats was decreased to about half that in sham rats, and we consider that an increase in $P_i$ levels in the intestine is not the only mechanism that downregulates intestinal Zn absorption in

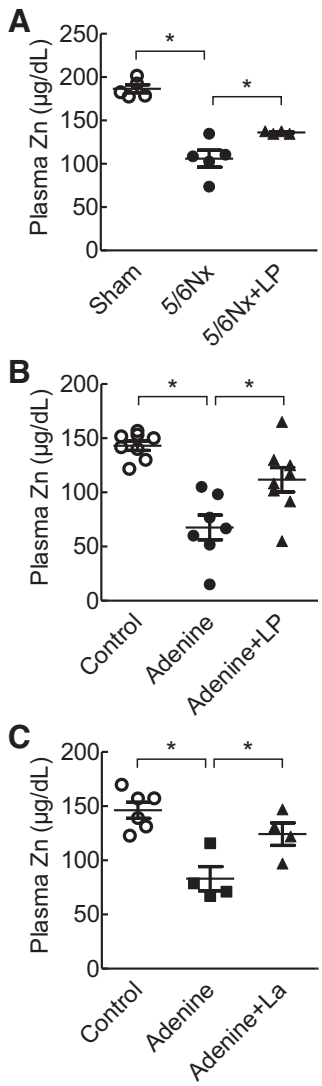


**Figure 6.** Amelioration of hypozincemia in CKD model rats by dietary $P_i$ restriction and La. *A*: plasma Zn levels in 5/6 Nx rats fed a CP diet or LP diet for 2 wk. *B*: plasma Zn levels in adenine-induced CKD rats fed a CP diet or LP diet containing adenine for 7 days. *C*: plasma Zn levels in adenine-induced CKD rats fed an MF diet containing 0.4% adenine with or without La for 2 wk. Data are expressed as means ± SE ($n = 4$–8). Statistical analysis was performed using one-way ANOVA followed by Tukey's tests. *$P < 0.05$. CKD, chronic kidney disease. *n* indicates the number of samples for each experiment.

CKD. Third, we evaluated the changes in plasma Zn levels after Zn administration as a surrogate index of total Zn absorption capacity. Plasma Zn levels reflect not only intestinal absorption but also kidney excretion and distribution to organs. In this study, the urinary excretion and distribution to organs of Zn would be less than the Zn absorption in the intestine, because plasma Zn concentration was linearly elevated at least for 40 min after Zn administration. Therefore, we consider that the changes in plasma Zn levels would reflect total Zn absorption capacity. Lastly, our study was based on rodent models of 5/6 Nx- and adenine-induced CKD, and the corresponding CKD stage of these models is not entirely clear. Hyperphosphatemia occurs more frequently in CKD stage 5 and dialysis (52). In a cohort study of patients with CKD, the fractional excretion of Zn into urine increased strongly at stage 3, suggesting that hypozincemia in CKD is not compensated by reduced renal Zn excretion (9). Thus, there may be different mechanisms underlying hypozincemia at each stage of CKD, and the actual contribution of $P_i$ management to blood Zn levels in patients with CKD remains unclear. Further clinical investigation will be needed.

Supplementation with Zn is a useful treatment for hypozincemia, but several clinical issues remain to be resolved. Because Zn and Cu compete for absorption in the intestine, continuous administration of high-dose Zn often causes Cu deficiency and its related symptoms (21). Serum Zn and Cu levels have been found to be inversely correlated in patients with CKD (22). Our study showed that dietary $P_i$ restriction and administration of La prevent a decrease in plasma Zn levels in CKD model rats, suggesting these approaches to hyperphosphatemia may be useful treatments that avoid the problem of current Zn supplementation therapy in hypozincemia. In this study, we did not evaluate the effect of $P_i$ management on the expression of Zn transporters. Additional studies will be needed to elucidate the detailed mechanisms of improved hypozincemia by $P_i$ management including Zn transporters.

In conclusion, an elevation in intestinal luminal $P_i$ concentration due to hyperphosphatemia or excess $P_i$ intake may suppress Zn absorption and cause hypozincemia in CKD. Dietary $P_i$ restriction and administration of La can ameliorate hypozincemia in CKD model rats. Appropriate management of hyperphosphatemia might be useful for the prevention and treatment of hypozincemia in patients with CKD.

### Perspectives and Significance

Zn plays a pivotal role in various biological processes. CKD often leads to hypozincemia, which is a risk factor for the progression and complications of CKD. The current treatment for hypozincemia is Zn supplementation, which often causes Cu deficiency. We demonstrated that suppression of Zn absorption in CKD may be, in part, induced by the formation of a Zn-$P_i$ insoluble complex in the intestinal lumen. In addition, dietary $P_i$ restriction can ameliorate hypozincemia in a rat model of CKD. Appropriate management of hyperphosphatemia may be useful for prevention and treatment of hypozincemia in patients with CKD, although further clinical investigation will be needed to clarify completely the relationship between dietary $P_i$ and Zn absorption capacity or hypozincemia in this disorder.

## DATA AVAILABILITY

Data will be made available upon reasonable request.

## SUPPLEMENTAL DATA

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.24234079.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Y.O. and Y.T. conceived and designed research; Y.O., K.A., S.S., Y.M., M.T., and M.M. performed experiments; Y.O., K.A., and S.S. analyzed data; Y.O., Y.K., Y.M., Y.A., M.T., A.K., H.O., K.O., M.M., T.K., and H.Y. interpreted results of experiments; Y.O. and K.A. prepared figures; Y.O. and Y.T. drafted manuscript; Y.O. and Y.T. edited and revised manuscript; Y.O. and Y.T. approved final version of manuscript.

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