A high-phosphorus (P) diet has several effects on calcium (Ca) and P metabolism. It increases parathyroid hormone (PTH) secretion (1, 2), which causes an increase in bone resorption and a decrease in bone mineral density (BMD) (3–5). Our previous study demonstrated that elevated PTH secretion from a high-P diet increases mRNA expression of the receptor activator of NF-κB (RANKL), a mediator of osteoclastic bone resorption (4). Moreover, it is well known that a high dietary P concentration is an important etiologic factor in the development of nephrocalcinosis and diminished kidney function. A high-P diet is reportedly associated with increased kidney Ca and P concentrations, urinary N-acetyl-β-D-glucosaminidase activity, and urinary β2-microglobulin excretion, and decreased bone mineral content and bone mineral density of the femur and tibia. Dietary Ca supplementation improved the parameters of bone metabolism and kidney function in rats fed the high-P diet, while there were no significant differences in kidney Ca or P concentrations between the HP and HPCa groups. These results suggest that dietary Ca supplementation prevented the bone loss and decline in kidney function induced by a high-P diet, whereas dietary Ca supplementation did not affect kidney mineral concentrations in rats fed the high-P diet.

**Key Words** high-phosphorus diet, dietary calcium supplementation, bone metabolism, kidney mineral concentration, kidney function

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samples. Serum and urine samples were stored at 
80˚C until further analyses. The femur and bone marrow were used for analysis of RANKL mRNA expression. Total RNA was isolated from the homogenized femur using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s specifications. The amount and purity of the RNA were assessed using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) with TaqMan gene expression assays (Applied Biosystems) for rat RANKL (Assay ID: Rn00589289_m1) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay ID: Rn01775763_g1). Real-time PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). The RANKL mRNA expression was normalized to GAPDH mRNA as a housekeeping gene. The value of the C group was considered as 1.00.

Statistical analysis. Results were expressed as the mean±SE for each group of 6 rats. Two-way analysis of variance (ANOVA) was used to determine the effects of the high-P and high-Ca diets. Then, Tukey’s post hoc test was used to analyze differences between the groups. Differences were considered to be significant when the p value was <0.05. Statistical analysis was performed using SPSS statistics ver. 22 (IBM, Chicago, IL).

Results

Body weight and food intake

Final body weight was significantly lower in the HP group than in the C group and in the HPCa group than in the HCa group, and was significantly higher in the HPCa group than in the HP group (Table 2). Food intake was significantly lower in the HP group than in the C group and tended to be lower (p=0.06) in the HPCa group than in the HCa group, and was significantly higher in the HPCa group than in the HP group. There were no significant differences in final body weight or food intake between the C and HCa groups.

Kidney dry weight and kidney Ca and P concentrations

Kidney dry weight was significantly higher in the HP group than in the C group and in the HPCa group than in the HCa group, and was significantly lower in the HPCa group than in the HP group (Table 2). There was no significant difference in kidney dry weight between the C and HCa groups. Kidney Ca and P concentrations were significantly higher in the HP and HPCa groups than in the C and HCa groups, respectively. Dietary Ca supplementation had no significant effect on kidney Ca or P concentrations.

Table 1. Composition of the experimental diets.

|          | C    | HP   | HCa  | HPCa |
|----------|------|------|------|------|
| Ca level | 0.5% | 0.5% | 1.0% | 1.0% |
| P level  | 0.3% | 1.5% | 0.3% | 1.5% |
| Casein   | 200.0| 200.0| 200.0| 200.0|
| Cornstarch| 529.486| 476.755| 517.000| 464.269|
| Sucrose  | 100.0| 100.0| 100.0| 100.0|
| Soybean oil | 70.0 | 70.0 | 70.0 | 70.0 |
| Cellulose powder | 50.0 | 50.0 | 50.0 | 50.0 |
| Mineral mixture1 | 35.0 | 35.0 | 35.0 | 35.0 |
| Vitamin mixture2 | 10.0 | 10.0 | 10.0 | 10.0 |
| l-Cystine | 3.0  | 3.0  | 3.0  | 3.0  |
| Choline bitartrate | 2.5  | 2.5  | 2.5  | 2.5  |
| tert-Butylhydroquinone | 0.014 | 0.014 | 0.014 | 0.014 |
| KH2PO4 | —    | 52.731| —    | 52.731|
| CaCO3 | —    | —    | 12.486| 12.486|

1 AIN-93G mineral mixture.
2 AIN-93 vitamin mixture.
Table 2. Body weight, food intake, kidney dry weight, and kidney Ca and P concentrations.

|                          | C       | HP      | HCa     | HPCa    | Two-way ANOVA |
|--------------------------|---------|---------|---------|---------|---------------|
| Initial body weight (g)  | 122.2±1.7 | 122.7±1.3 | 122.7±1.2 | 122.6±1.1 |               |
| Final body weight (g)    | 267.0±3.7a | 220.3±4.6b | 259.6±3.4a | 239.4±4.8b | P, P×Ca       |
| Food intake (g)          | 358.8±4.2a | 289.3±10.7b | 367.2±7.6a | 337.8±6.8a | P, Ca, P×Ca   |
| Kidney dry weight (g/100 g body weight) | 0.087±0.002a | 0.132±0.003b | 0.083±0.002a | 0.115±0.005c | P, Ca         |
| Kidney Ca (mg/g dry weight) | 0.43±0.01a | 6.73±1.06b | 0.45±0.01a | 6.96±0.63b | P             |
| Kidney P (mg/g dry weight) | 12.51±0.06a | 18.75±0.76b | 13.53±0.02a | 17.86±0.48b | P, P×Ca       |

The data indicate the mean±SE of 6 rats.

Significant effect (p<0.05): P, effect of high-P diet; Ca, effect of high-Ca diet; P×Ca, effect of interaction.

Table 3. Serum and urine parameters.

|                       | C       | HP      | HCa     | HPCa    | Two-way ANOVA |
|-----------------------|---------|---------|---------|---------|---------------|
| Serum Ca (mg/dL)      | 10.24±0.19a | 8.52±0.19b | 11.88±0.26c | 9.38±0.15d | P, Ca         |
| Serum P (mg/dL)       | 8.55±0.20a | 11.27±0.41b | 6.08±0.26c | 9.10±0.28a | P, Ca         |
| Serum PTH (pg/mL)     | 69.1±18.7a | 1074.1±78.7b | 5.4±1.9a | 91.8±17.7a | P, Ca, P×Ca   |
| Serum osteocalcin (ng/mL) | 98.3±7.2a | 145.2±4.7b | 108.2±9.4a | 108.1±10.4a | P, P×Ca       |
| Urine CTx (µg/mmol creatinine) | 14.7±2.0a | 334.6±26.1b | 21.9±3.6a | 121.9±12.5c | P, Ca, P×Ca   |
| Urine albumin (mg/g creatinine) | 36.0±5.7a | 177.4±46.4b | 18.3±2.3a | 104.0±19.5b | P              |
| Urine NAG activity (U/g creatinine) | 28.5±0.5a | 37.0±1.5b | 23.3±1.0a | 32.0±1.3a | P, Ca         |
| Urine β2-microglobulin (mg/g creatinine) | 2.20±0.48a | 5.32±0.68b | 0.85±0.07a | 2.53±0.32a | P, Ca         |
| Urine creatinine (mg/24 h) | 7.39±0.49ac | 6.56±0.20a | 9.24±0.33b | 8.00±0.37bc | P, Ca         |
| Creatinine clearance (mL/min/100 g body weight) | 0.32±0.02a | 0.33±0.01a | 0.41±0.02b | 0.37±0.02ab | Ca            |

The data indicate the mean±SE of 6 rats.

Significant effect (p<0.05): P, effect of high-P diet; Ca, effect of high-Ca diet; P×Ca, effect of interaction.

**Serum Ca, P, and PTH concentrations**

Serum Ca concentration was significantly lower in the HP group than in the C group and in the HPCa group than in the HCa group and was significantly higher in the HPCa group than in the HP group (Table 3). Compared with the C group, serum Ca concentration was significantly higher in the HCa group. Serum P concentration was significantly higher in the HP group than in the C group and in the HPCa group than in the HCa group and was significantly lower in the HPCa group than in the HP group. Compared with the C group, serum P concentration was significantly lower in the HCa group. Serum PTH concentration was significantly higher in the HP group than in the C group and was significantly lower in the HPCa group than in the HP group. There was no significant difference in serum PTH concentration between the C and HCa groups. Urinary CTx excretion was significantly higher in the HP group than in the C group and in the HPCa group than in the HCa group; it was significantly lower in the HPCa group than in the HP group. There were no significant differences in urinary CTx excretion between the C and HCa groups. **Indicators of kidney function**

Urinary albumin excretion was significantly higher in the HP group than in the C group (Table 3). Dietary Ca supplementation had no significant effect on urinary albumin excretion. Urinary NAG activity excretion was significantly higher in the HP group than in the C group and in the HPCa group than in the HCa group, and was significantly lower in the HPCa group than in the HP group. Urinary NAG activity was significantly lower in the HPCa group than in the C group. Urinary β2-microglobulin excretion was significantly higher in the HP group than in the C group and tended to be lower (p=0.08) in the HPCa group than in the HP group. There were no significant differences in urinary creatinine or creatinine clearance between the C and HP groups. Urinary creatinine excretion and creatinine clearance were significantly higher in the HCa group.
that elevated PTH secretion results in RANKL mRNA expression in the femur and urinary CTx excretion and decreased the BMC and BMD of the femur, tibia, and lumbar vertebra. It has been suggested that dietary Ca supplementation suppressed bone resorption that was enhanced by the high-P diet. This may be explained by the suppression of the serum PTH concentration by dietary Ca supplementation. As stated above, PTH stimulates RANKL, which mediates osteoclastic bone resorption (21). In the present study, dietary Ca supplementation increased the serum Ca concentration and decreased serum P and PTH concentrations in rats fed the high-P diet. Furthermore, dietary Ca supplementation decreased RANKL mRNA expression of the femur in rats fed the high-P diet. Thus, we suggest that changes in serum Ca and P concentrations by dietary Ca supplementation suppressed serum PTH concentration, which suppressed RANKL-induced bone resorption in rats fed the high-P diet.

In contrast, dietary Ca supplementation had no influence on the BMD of the lumbar vertebra. Because the lumbar vertebra contains a large amount of trabecular bone, bone loss induced by a high-P diet might be independent of the lower food intake and body weight in the present study.

Dietary Ca supplementation has beneficial effects on bone health, by increasing BMC and BMD (12, 13). Furthermore, a decrease in the Ca/P intake ratio is associated with a decrease in BMD (14, 15). In the present study, dietary Ca supplementation decreased urinary CTx excretion and inhibited bone loss of the femur and tibia in rats fed the high-P diet. These results suggest that dietary Ca supplementation suppressed bone resorption that was enhanced by the high-P diet. This may be explained by the suppression of the serum PTH concentration by dietary Ca supplementation. As stated above, PTH stimulates RANKL, which mediates osteoclastic bone resorption (21). In the present study, dietary Ca supplementation increased the serum Ca concentration and decreased serum P and PTH concentrations in rats fed the high-P diet. Furthermore, dietary Ca supplementation decreased RANKL mRNA expression of the femur in rats fed the high-P diet. Thus, we suggest that changes in serum Ca and P concentrations by dietary Ca supplementation suppressed serum PTH concentration, which suppressed RANKL-induced bone resorption in rats fed the high-P diet.

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bone, lumbar vertebrae might be susceptible to bone loss due to a high P intake. Furthermore, a high-P diet influences not only Ca but also magnesium (Mg) metabolism. Our previous study showed that a high-P diet decreases serum Mg concentration and Mg absorption in rats (23). We did not demonstrate these in the present study, but it is possible that serum Mg concentration and Mg absorption are decreased by a high-P diet. Furthermore, a previous study showed that increased dietary Ca depresses Mg absorption (24). Many studies have shown that Mg deficiency induces bone loss with an increase in bone resorption (25, 26). Therefore, it is possible that the combination of a high-P diet and dietary Ca supplementation reduces Mg absorption further. This effect on Mg absorption might explain the lack of effect on lumbar vertebra BMD by Ca supplementation in rats fed the high-P diet. However, further examinations are necessary to clarify the effects in detail.

Nephrocalcinosis also occurs in rats fed a high-P diet. In the present study, the high-P diet increased kidney Ca and P concentrations, supporting the results of our previous studies (8, 10, 11, 22). Furthermore, urinary albumin excretion, urinary NAG activity, and urinary β2-microglobulin excretion were higher in rats fed the high-P diet than in those fed the control diet. Similarly, other researchers have reported that a high-P diet increases urinary albumin excretion, which is correlated with the kidney Ca concentration (6, 9). A previous study has shown that Ca and P deposition was evident in the proximal tubules of rats fed a high-P diet (10). In addition, urinary NAG activity and urinary β2-microglobulin excretion are used as indicators of proximal tubular function. Therefore, a high-P diet might have caused the Ca and P deposition in the proximal tubules, which diminished proximal tubular function in the present study. Consequently, depression of the proximal tubular function may increase urinary albumin excretion in rats fed the high-P diet, though albumin reabsorption occurs mainly in the proximal tubules (27). Based on previous studies, we believe that a high-P diet leads to increased kidney Ca concentrations, resulting in impaired kidney function. It is likely that dietary Ca supplementation can prevent impaired kidney function by inhibiting the increase in kidney Ca concentration. However, we found that dietary Ca supplementation had no effect on kidney Ca or P concentrations in rats fed the high-P diet, while urinary NAG activity and urinary β2-microglobulin excretion were decreased by dietary Ca supplementation. These results indicate that dietary Ca supplementation prevented a decline in kidney function related with a high-P diet, even though dietary Ca supplementation did not prevent kidney Ca or P deposition. Furthermore, we suggest that kidney Ca and P deposition might not be the main factor behind the kidney dysfunction related with a high-P diet. However, to clarify this suggestion, further studies are necessary to elucidate the relationship between dietary Ca supplementation and kidney mineral concentrations for estimating kidney function. We investigated the effect of dietary Ca supplementation on creatinine clearance as an indicator of glomerular filtration rate. In the present study, urinary creatinine excretion and creatinine clearance were significantly higher in the HCa group than in the C group, whereas the high-P diet did not change these. Results in the present study also showed that the high-Ca diet affected urinary NAG activity and urinary β2-microglobulin excretion. Thus, it is possible that a high-Ca diet directly influences these parameters of kidney function, but the mechanisms by which a high-Ca diet affects kidney function are still unclear.

In conclusion, we demonstrated that dietary Ca supplementation suppressed serum PTH concentration and RANKL mRNA expression, which prevents bone loss in rats fed a high-P diet. Furthermore, dietary Ca supplementation may prevent a decline in kidney function related with a high-P diet, whereas dietary Ca supplementation had no effect on kidney Ca or P concentrations.

Acknowledgments
The authors thank Dr. Yoshiko Ishimi (National Institute of Health and Nutrition, Tokyo, Japan) for supporting the measurement of BMC and BMD. This work was supported by KAKENHI (Grant-in-Aid for Young Scientists (B), 18700613).

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