Dietary Phosphorus Acutely Impairs Endothelial Function

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ABSTRACT
Excessive dietary phosphorus may increase cardiovascular risk in healthy individuals as well as in patients with chronic kidney disease, but the mechanisms underlying this risk are not completely understood. To determine whether postprandial hyperphosphatemia may promote endothelial dysfunction, we investigated the acute effect of phosphorus loading on endothelial function in vitro and in vivo. Exposing bovine aortic endothelial cells to a phosphorus load increased production of reactive oxygen species, which depended on phosphorus influx via sodium-dependent phosphate transporters, and decreased nitric oxide production via inhibitory phosphorylation of endothelial nitric oxide synthase. Phosphorus loading inhibited endothelium-dependent vasodilation of rat aortic rings. In 11 healthy men, we alternately served meals containing 400 mg or 1200 mg of phosphorus in a double-blind crossover study and measured flow-mediated dilation of the brachial artery before and 2 h after the meals. The high dietary phosphorus load increased serum phosphorus at 2 h and significantly decreased flow-mediated dilation. Flow-mediated dilation correlated inversely with serum phosphorus. Taken together, these findings suggest that endothelial dysfunction mediated by acute postprandial hyperphosphatemia may contribute to the relationship between serum phosphorus level and the risk for cardiovascular morbidity and mortality.

does not cause hyperphosphatemia, can be a risk factor for CVD. In addition, Onufrak et al. also demonstrated that serum phosphorus level was associated with carotid intima media thickness in the general population. Interestingly, continuous hyperphosphatemia in both klotho mutant mice and fibroblast growth factor 23 (FGF23)-deficient mice may also be a risk factor of a premature aging-like phenotype. This may be inferred because P restriction diet partially ameliorated the phenotype in both klotho and FGF23 deficient mice. In addition, hyperphosphatemia involved by chronic renal failure frequently causes hyperparathyroidism that affects the homeostasis of calcium and phosphate in plasma. Increased parathyroid hormone (PTH) has also been recognized as a risk factor of CVD. These factors complicate to understand the linking mechanism between serum P and CVD. Thus, a possible mechanism linking serum P and CVD risk should be clarified to gain insight into the potential impact of P on CVD and aging-related diseases. Here, we focused on the direct effect of P on endothelial function.

In this study, we reported that dietary P loading in human caused postprandial elevation of serum P and impaired endothelium-dependent vasodilation and also demonstrated that the impairment of vasodilation was mainly due to decreased NO production in endothelial cells, suggesting that postprandial elevation of serum P level deteriorates endothelial function.

RESULTS

High P Loading Increased Reactive Oxygen Species Production and Decreased Nitric Oxide Production in Bovine Aortic Endothelial Cells

We reported that elevation of extracellular P level can induce reactive oxygen species (ROS) production in endothelial cells. In this study, we also confirmed that elevation of P concentration from the control value (0.8 mM) to 2.8 mM increased ROS production by both nitro blue tetrazolium (NBT) assay and time-lapse confocal microscopy analysis with aminophenyl fluorescein (APF) in bovine aortic endothelial cells (BAECs) (Figure 1A and B). P loading significantly increased the ROS production in BAECs in a dose-dependent manner (Figure 1A). In the endothelial cells, ROS is produced mainly by the activation of NAD phosphate [NAD(P)H] oxidase, xanthine oxidase, and mitochondrial respiratory chain. NAD(P)H oxidase and xanthine oxidase pathways can mainly produce O2, and mitochondrial pathway can produce O2 and OH-. In addition, produced O2 can be converted to H2O2 by superoxide dismutase and, consequently, be converted to OH- in the presence of Fe2+. O2- is also reacted with nitric oxide (NO) to form ONOO-. Thus, we examined the effect of specific inhibitors of each pathway on the ROS production mediated by P loading. Figure 1A also demonstrates that diphenyl iodonium (DPI), a specific inhibitor of NAD(P)H oxidase decreased the ROS production. On the other hand, rotenone did not inhibit the ROS production, but oxypurinol partially inhibited it (Figure 1A). Furthermore, ebselen, a potent antioxidant, completely inhibited the ROS production induced by P loading (Figure 1B). The ROS production was also inhibited by treatment of phosphonoformic acid (PFA), which is a specific inhibitor of sodium-dependent
phosphate transporters (Figure 1B). Figure 1C demonstrates that BAECs express both PIT-1 and PIT-2, which are type III sodium-dependent phosphate transporters. Therefore, these transporters would contribute to P influx in BAECs.

**High P Loading Decreased Endothelium-Dependent Vasodilation of Rat Aorta Rings**

To confirm the impairment of endothelium-dependent vasodilation by P loading, we investigated the effects of high P loading on acetylcholine-induced vasodilation using thoracic aorta rings from rats maintained under normal condition. When aorta rings were preincubated in the medium containing 2.4 mM P for 1 h, acetylcholine-induced vasodilation decreased by 70% compared with rings preincubated in the medium containing 1.2 mM P (Figure 2A). On the other hand, when aorta rings stripped of endothelium were treated similarly, no differences were observed between the rings incubating in the medium containing different P concentrations (Figure 2B). Preincubation of intact aortic rings in the medium containing 2.4 mM P slightly increased vasoconstriction mediated by 1 μM phenylephrine; however, the difference was not statistically significant (1.374 ± 0.17 versus 1.645 ± 0.239, *P* = 0.38). We attempted to confirm the role of ROS in the impairment of endothelium-dependent vasodilation caused by high P loading. However, ebselen did not significantly ameliorate the impaired vasodilation (Figure 2C).

**High P Loading Decreased NO Production in BAECs**

To elucidate the inhibitory mechanism on vasodilation by P loading, we investigated the effect of high P loading on NO production and intracellular Ca\(^{2+}\) concentration in BAECs. High P loading completely inhibited the NO production mediated by bradykinin (Figure 3A). However, ebselen did not affect the inhibitory effect of P loading on bradykinin-mediated NO production. In addition, since intracellular Ca\(^{2+}\) increase plays an important role in the activation of endothelial NO synthase (eNOS), we investigated whether P loading can affect the receptor-mediated intracellular Ca\(^{2+}\) increase in BAECs. High P loading did not affect the intracellular Ca\(^{2+}\) increase by adenosine triphosphate (ATP) (Figure 3B).

We postulated that high P loading may modulate eNOS activity through its activation/inactivation pathway through phosphorylation. To address that, we investigated whether high P loading can phosphorylate Thr\(^{497}\) (corresponding to Thr\(^{495}\) in the human\(^{19,20}\)) of eNOS in BAECs, because the phosphorylation of this threonine causes eNOS inactivation. Loading with 3 mM P for 1 h significantly increased phosphorylation of eNOS at Thr\(^{497}\) on caveolar membranes of BAECs (Figure 3C). The phosphorylation was completely inhibited by conventional protein kinase C (PKC) inhibitor Gö6976 and partially by tempol (Figure 3C). However, protein kinase A inhibitor H-89 and PI3 kinase inhibitor wortmannin did not inhibit the phosphorylation (data not shown). Sulfate ion did not increase the phosphorylation of eNOS (Figure 3C).

**Figure 2.** Effects of high P loading on aortic vasodilation. (A) Dose-response curves of vasodilation induced by acetylcholine in rat aortic rings pretreated with Krebs-Henseleit bicarbonate (KHB) buffer containing 1.2 mM P (n = 3, closed circle) or 2.4 mM P (n = 2, open circle). Data are expressed as mean ± SEM. There was significant difference in intercepts between the curves of 1.2 mM P and 2.4 mM P (*P* = 0.0067). (B) Dose-response curves of vasodilation induced by sodium nitroprusside (SNP) in rat aortic rings stripped of the endothelium, preincubated with KHB buffer containing 1.2 mM P (n = 4, closed circle) or 2.4 mM P (n = 4, open circle). Data are expressed as mean ± SEM. There was significant difference in intercepts between the curves of 1.2 mM P and 2.4 mM P (*P* = 0.0067). (C) Effect of ebselen on the inhibition of vasodilation by high P medium in rat aortic rings. Rat aortic rings were preincubated with KHB buffer containing 1.2 mM P (n = 4), 2.4 mM P (n = 3), or 2.4 mM P with 10 μM ebselen (n = 3). Data are expressed as mean ± SEM, *P* < 0.05 versus column 1.
Intracellular Ca$^{2+}$ with 10eNOS, and caveolin. BAECs were treated with control (0.9 mM P), 3 mM P, 3 mM P eNOS at Thr497. Western blot analysis of phosphorylated eNOS at Thr497, total three independent experiments. (C) Effects of high P loading on phosphorylation of as change of fluorescence ratio (Fluo-4/Fura-Red). The curves are representative of J Am Soc Nephrol loading. In addition, the translocation of PKC into the cells should be required for PKC activation in response to P loading. P loading also increased PKC activity in BAECs, as shown in Figure 4A. The activation of PKC activity was inhibited by PFA, suggesting that influx of P into cell. We evaluated the effect of dietary P loading on endothelium-dependent vasodilation in 11 young healthy men by postprandial changes of percent flow-mediated dilatation (%FMD). %FMD decreased significantly 2 h after the ingestion of a P1200 meal (containing 1200 mg P per meal) (Table 2). The serum P level exceeded the normal range (2.5 to 4.5 mg/dl) in 8 out of 11 subjects. On the other hand, postprandial serum P level after the ingestion of a P400 meal (containing 400 mg P per meal) did not change significantly. We did not measure urinary P excretion because a previous study demonstrated that urinary P excretion increased after the ingestion of the P1200 meal compared with the P400 meal. In addition, in vitro experiments suggest that increased serum P level would more directly affect on endothelial function rather than increased urinary P excretion. Serum glucose levels at 2 h after the meal were not significantly different between those receiving a P400 meal and a P1200 meal. Serum intact-PTH (iPTH) level tended to decrease after the breakfast due to circadian regulation. However, the postprandial iPTH level of subjects receiving a P1200 meal was significantly higher than those receiving a P400 meal (Table 2). Other laboratory data were not significantly different between the subjects receiving the two types of meal.

**Effect of Dietary P Loading on Serum P, Glucose, and Intact-PTH Levels in Young Healthy Men**

To confirm the effect of P loading on endothelial function in vivo, we examined the effect of dietary P loading on endothelial function in healthy men (Table 1). Mean values of all serum chemistry measurements were within the respective normal ranges in the preprandial status as shown in Table 2. Furthermore, there was no significant difference in the preprandial data between the experimental days. Serum P level increased significantly 2 h after the ingestion of a P1200 meal (containing 1200 mg P per meal) (Table 2). The serum P level exceeded the normal range (2.5 to 4.5 mg/dl) in 8 out of 11 subjects. On the other hand, postprandial serum P level after the ingestion of a P400 meal (containing 400 mg P per meal) did not change significantly. We did not measure urinary P excretion because a previous study demonstrated that urinary P excretion increased after the ingestion of the P1200 meal compared with the P400 meal. In addition, in vitro experiments suggest that increased serum P level would more directly affect on endothelial function rather than increased urinary P excretion. Serum glucose levels at 2 h after the meal were not significantly different between those receiving a P400 meal and a P1200 meal. Serum intact-PTH (iPTH) level tended to decrease after the breakfast due to circadian regulation. However, the postprandial iPTH level of subjects receiving a P1200 meal was significantly higher than those receiving a P400 meal (Table 2). Other laboratory data were not significantly different between the subjects receiving the two types of meal.

**Activating of Conventional PKC by High P Loading**

To confirm that high P loading can activate conventional PKC in endothelial cells, we investigated if the activity of PKC can be stimulated by high P loading. P loading also increased PKC activity in BAECs as shown in Figure 4A. The activation of PKC activity was inhibited by PFA, suggesting that influx of P into the cells should be required for PKC activation in response to P loading. In addition, the translocation of PKCα from cytosol to caveolae membrane was also investigated, because previous reports have demonstrated that activated PKCα can be tar-
Figure 4. Activation of conventional PKC by high P loading in BAECs. (A) Effect of high P loading on the PKC activity of BAECs. We treated BAECs with control (0.9 mM P), 1.5 mM P, 3 mM P, or 3 mM P with 200 µM PFA for 15 min, then pretreated whole cell lysates, and subjected lysates to measurement of conventional PKC activity using the TruLight PKCα Assay kit. *P < 0.05 versus column 1, †P < 0.05 versus 3 mM P (column 3). (B) Effect of high P loading on the subcellular localization of PKCα in BAECs. We treated BAECs with the indicated concentration of P or glucose in Medium 199 medium without serum for 1 h, and then fractionated the cells into cytosol and caveolar membrane fractions. We separated 20 µg of protein of each fraction by SDS-PAGE and performed western blot analysis with anti-PKCα mAb and anti-caveolin pAb. The data are representative from two separate experiments.

Table 1. Baseline characteristics of 11 healthy male subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n = 11</th>
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<tr>
<td>Age (y)</td>
<td>24.6 ± 3.4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>170.1 ± 4.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.4 ± 6.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.9 ± 2.0</td>
</tr>
<tr>
<td>Percent of body fat (%)</td>
<td>12.5 ± 3.6</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>52.7 ± 4.7</td>
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P = 0.006) (Figure 5B). With the ingested P400 meal, on the other hand, there was no significant correlation (Figure 5A). There was no statistically significant correlation between serum glucose and %FMD (r = −0.26, P = 0.09). In addition, the decreased %FMD at 2 h after the ingestion of the P1200 meal was normalized at least 24 h later (%FMD at 24 h was 8.60 ± 0.2 [NS versus before the ingestion]).

DISCUSSION

This study demonstrates that elevation of extracellular P level causes endothelial dysfunction in vitro and in vivo. In addition, in vitro experiments demonstrated that high P loading inhibited NO production through increased ROS production and eNOS inactivation via conventional PKC, resulting in impaired endothelium-dependent vasodilation. Furthermore, dietary P loading can deteriorate flow-mediated vasodilation in healthy men, suggesting that dietary P loading or elevation of serum P level may be a risk factor for CVD in healthy persons as well as CKD patients. Our data also provide a novel explanation why higher serum P levels, even although within normal range, are associated with an increased CVD risk as previously reported.²–⁴,10

Recently, we reported that P loading increased ROS production and decreased NO production in BAECs. In this study, we demonstrated that the ROS production was mainly mediated by NAD(P)H oxidase. Contribution of other ROS generators may be partial. Elevation of glucose can increase ROS production via NAD(P)H oxidase by activating conventional PKC in endothelial cells.²⁵ We found that high P loading also can activate conventional PKC in BAECs depending on P influx. Although we cannot ascertain how P influx activates the PKC pathway, our data agreed with a recent report by Di Marco et al. that high P loading increased ROS production via P influx and induced apoptosis in endothelial cells.²⁶

ROS is a scavenger of NO and depletes the available NO resulting in dysregulation of vascular tone.²⁸,²⁷,²⁸ However, antioxidants were not enough to ameliorate the impaired vasodilation mediated by high P loading, suggesting that other factor(s) rather than ROS may be involved. The activity of eNOS can be regulated by Ca²⁺ and phosphorylation.²⁹,³⁰ Increased P influx did not influence intracellular Ca²⁺ increase in response to ATP, which can activate eNOS activity via Ca²⁺ signal. Therefore, we considered that P loading may regulate phosphorylation of eNOS. We demonstrated that high P loading enhances the phosphorylation at Thr⁴⁹⁷ of eNOS via PKC. Phosphorylation of eNOS at Thr⁴⁹⁷ can inhibit eNOS activity.²⁹,³⁰ Our data suggest that elevation of serum P by dietary P loading can primarily activate conventional PKC and then decrease NO availability via eNOS inactivation. In addition, increased ROS also reduce the available NO by converting NO to ONOO⁻. These steps eventually lead to endothelial dysfunction (Figure 6).

Until now, hyperphosphatemia was recognized as a risk factor for CVD through promotion of medial calcification.²⁵,²⁶ Jono et al. demonstrated that more than 1.4 mM P dose-dependently increased calcium deposition after a 6-d incubation in human smooth muscle cells.³ Additionaly, dietary P restriction ameliorated vascular calcification in FGF23-deficient mice that show a premature aging phenotype with hyperphosphatemia and hypervitaminosis D.¹⁵ These results can explain the correlation between hyperphosphatemia in CKD patients and vascular calcification, but are not enough to explain the recent epidemiologic findings that higher serum P level within normal range (0.9 to 1.4 mM P) can be a risk factor for CVD in persons with normal kidney function.²–⁴,¹⁰ Both endothelial dysfunction and medial calcification are closely associated with development of CVD. It is well known that long-term exposure to P, generally observed in end-stage renal failure patients, can mediate vascular calcification.²⁵,²⁶ Our data demonstrated that dietary high P loading can be involved in the postprandial elevation of serum P level, and this short-term exposure to P was enough to decrease endothelium-dependent vasodilation.

In healthy persons, serum P level is maintained within normal range by various hormones, such as iPTH, and vitamin D. None-
The serum chemistry, blood pressure, and %FMD findings before and 2 h after test meal ingestion are shown in Table 2. The %FMD findings suggest that postprandial elevation of serum P was associated with a decrease in %FMD. The relationship between serum biomarkers and lifestyle-related factors, such as blood pressure and glucose, was also investigated, and the correlation between serum P and continuous elevation of serum P to the development of CVD was found to be significant.

There were some limitations in this study. First, we investigated the effect of dietary P intake on the FMD in young healthy men. Studies with various doses of P in more elderly subjects and women should be performed in the future to better understand these effects in both sexes and different age groups. Second, because this human study was carried out with a limited number of subjects, the relationship between the amount of dietary P and endothelial function and risk of CVD should be verified in the general population epidemiologically. Third, our study strongly indicates that postprandial serum P elevation was associated with %FMD in humans. Therefore, we propose that postprandial elevation of serum P should be evaluated to assess the relationship between dietary P intake and CVD, as postprandial blood glucose elevation has been well-established as a risk factor for CVD.

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Until now, the effect of dietary P intake on vascular function has been underestimated, although association of serum P and vascular dysfunction has been well investigated, because fasting serum P level could not increase in healthy persons, even if dietary P was overloaded. Generally, fasting blood in the morning was collected and analyzed in most of the epidemiologic studies to investigate the relationship between serum biomarkers and lifestyle-related diseases. However, our data demonstrate that postprandial serum P elevation was associated with %FMD in humans. Therefore, we propose that postprandial elevation of serum P should be evaluated to assess the relationship between dietary P intake and CVD, as postprandial blood glucose elevation has been well-established as a risk factor for CVD.

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we cannot exclude a coexistent defect in smooth muscle vaso-
dilator responsiveness in this study.

In conclusion, dietary P loading can cause endothelial dys-
function within a short time. Oxidative stress and decreased
NO production in endothelial cells are possible mechanisms
for the impaired endothelial function mediated by P loading.
These findings suggest that elevation of serum P level by di-
etary P loading may be a novel risk factor for endothelial dys-
function and demonstrate a novel mechanism for explaining
the relationship between higher serum P level and CVD risk or
mortality. Our findings may also contribute to development of
new pharmacologic (e.g., endothelium-specific inhibitor of P
transporter) and nutritional approach to treatment and pre-
vention of CVD.

CONCISE METHODS

Materials
We used the following inhibitors: DPI; a NAD(P)H oxidase inhibitor,
oxyurin; a xanthine oxidase inhibitor, rotenone; a mitochondrial
respiratory chain system inhibitor, PFA; phosphonoformic acid; a
sodium-dependent phosphate transporter inhibitor, G66976; a con-
ventional PKC inhibitor, H-89; a PKA inhibitor, wortmannin;
ap13Kinesin inhibitor. Other compounds were used in this study
are listed in the Supplemental Appendix.

Examination of the Effects of High P Loading on
Endothelial Cell Functions Using BAECs
We prepared BAECs as described previously.17,32 We maintained the cells
in 100-mm plastic culture dishes in Medium 199 (Sigma-Aldrich, Tokyo,
Japan) supplemented with 20% fetal bovine serum (FBS; Invitrogen,
Carlsbad, California), penicillin 50 IU/ml, and streptomycin 50 μg/ml
under humidified atmosphere of 5% CO2 at 37°C. We routinely pas-
saged the cells before they reached confluence.

P Loading Experiment in BAECs
We incubated BAECs overnight in FBS-free Medium 199. For inhibi-
tion experiments, the cells were incubated with either 10 μM G66976
for 10 min or one of the following for 30 min: 10 μM ebselen, 1 mM
tempol, or 200 μM PFA before P loading. For P loading experiments,
we added appropriate amounts of sodium phosphate buffer (0.1 M
Na2HPO4/NaH2PO4, pH 7.4) to produce final P concentrations of 0.9
mM to 3 mM as indicated in figure legends.

NBT Assay
We incubated BAECs in serum-free Medium 199 under 5% CO2 at
37°C for 12 h and then in 0.2% NBT solution (0.2% weight per vol-
ume NBT in HBSS containing different concentrations of P [0.8 mM
to 2.8 mM]) for 120 min. We washed the cells in HBSS, first by adding
2 M KOH to solubilize the cell membranes and then by adding di-
methyl sulfoxide to dissolve blue formazan with gentle shaking for 10
min at room temperature. After solubilization, we determined the
absorbance of the samples at 620 nm.

Time-Lapse Confocal Microscopy Analysis for the
Measurement of ROS, NO Production, and
Intracellular Ca2+ Change
We measured ROS and NO levels using the specific indicators APF
and 4,5-diaminofluorescein-2 diacetate (DAF-2DA) (Daiichi Chem-
ical, Tokyo, Japan), respectively. APF can specifically recognize OH-,
ONOO-, and OCl- among many types of ROS.33 We performed the
time-lapse experiments with confocal microscopy as previously re-
ported.17 For measurement of intracellular Ca2+ change, we used the
combination of fluorescence indicators Fluo-4 and Fura-red (Molec-
ular Probes, Eugene, Oregon). More detailed information is provided
in the Supplemental Appendix.

Evaluation of Vasodilation and Vasoconstriction Using
Rat Thoracic Aorta Rings
We measured vasoconstriction and vasodilation with rat aortic rings
prepared from male 12-wk-old Sprague Dawley rats by Micro Easy
Magnus (Kishimoto Medical, Kyoto, Japan) as described previous-
ly.34 The Animal Experimentation Committee of the University of
Tokushima approved these experiments. More detailed information
is provided in the Supplemental Appendix.

Reverse Transcription PCR (RT-PCR), Western Blot
Analysis, and PKC Activity Assay
We performed RT-PCR and western blot analyses by standard
method written elsewhere. We measured PKC activity by the
TruLight PKCα Assay kit (Calbiochem, Tokyo, Japan). We provide
detailed information for RT-PCR, western blot analysis, and PKC
activity assay in the Supplemental Appendix.
Cell Fractionation
After the P loading as described above, we subjected BAECs to cell fractionation as described previously. Finally, we divided the cells into a postnuclear (PNS) soup, a cytosol (Cyt) fraction, a caveolar membrane (CM) fraction, and a noncaveolar membrane (NCM) fraction.

Dietary P Loading on Healthy Human Subjects
Eleven male volunteers (21 to 33 yrs of age) without apparent health problems were recruited for this study. The participants had no evidence of diabetes, abnormal glucose intolerance, obesity, hypertension, kidney diseases, cardiovascular disease, hyperlipidemia, and bone and mineral disorders. Demographic data for the participants are provided in Table 1. All participants were nonsmokers, had normal blood pressure (BP), consumed < 30 g/d alcohol, and took no medications or antioxidant supplements. Eligibility of participants for this study was determined as reported previously by us. The study used a double-blinded crossover design on two different days separated by more than 1 d. The subjects were alternately served either a P400 meal or a P1200 meal for breakfast at 8:30 a.m. The composition of the test meals and standard dinner is provided in the Supplemental Appendix. All of the meals were consumed over 7 to 14 min. On the day before each study day, the subjects were asked to abstain from foods and beverages other than water not containing P after 1:00 p.m. They were served a standard dinner at 8:00 p.m. We calculated the area under the curve (AUC) of serum P over 8 h after the ingestion of the P400 and P1200 meals in the previous study. The AUCs after the P400 and P1200 meals was 217 ± 56 mg·min/ml and 519 ± 38 mg·min/ml, respectively. In addition, the peak value of serum P level was 3.9 ± 0.12 mg/dl at 6 h after the ingestion of the P400 meal, while the peak value was 5.0 ± 0.11 mg/dl at 2 h after the ingestion of P1200 meal. These results suggest that enough of an amount of P can be absorbed from the intestine, and this dietary intervention is useful to control serum P level without changing other nutritional elements.

We collected blood samples immediately before (0 h) and at 2 h after the test meal ingestion. Venous blood was taken from a median cubital vein for the measurement of serum P, Ca, ionized Ca, Na, K, Cl, iPTH, uric acid (UA), glucose, triacylglyceride (TG), LDL cholesterol (LDL-Chol), and HDL cholesterol (HDL-Chol) concentrations. All biochemical measurements and analyses were performed by MBC (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). We previously reported that serum P levels increased at 30 min and remained high for at least 2 h after the ingestion of a P400 meal. We measured serum chemistry, BP, and %FMD before and 2 h after the P400 and P1200 meals in the previous study. The peak value was 5.0 ± 0.11 mg/dl at 2 h after the ingestion of P1200 meal. We estimated and compared slopes and intercepts and show a significant difference between the backgrounds of P400 and P1200 meals by ANOVA followed by post hoc testing using Fisher’s protected least significant difference procedure for multiple comparisons.

For vasoconstriction and vasodilation studies, we performed linear regression analysis to compare between the dose-response curves. We estimated and compared slopes and intercepts and show a significant difference of the different between the groups by ANOVA followed by post hoc testing using Fisher’s protected least significant difference procedure for multiple comparisons.

Statistical Analysis
We tested all data for normal distribution of variables of interests using Pearson’s χ² distribution test before further parametric or nonparametric statistical analysis. If the test judged the data as a normal distribution, we performed the following statistical analysis by parametric analysis. If not, we used nonparametric analysis.

In dietary P loading experiments, we performed comparisons between preprandial and postprandial values of serum chemistry measurements in a group and effects of meals on preprandial and postprandial values of these measurements by means of repeated measurements and Tukey-Kramer methods. We determined statistical significance of the difference between the P400 and P1200 meals by the Wilcoxon signed-rank test.

To examine associations between level of serum P and %FMD and the level of serum glucose and %FMD, we performed simple regression analysis and estimated Spearman’s nonparametric correlation coefficients. We selected the nonparametric procedure to avert requirements of normal distributions and linear associations of variables of interests.

In the case of animal study, ex vivo and in vitro study, we determined statistical significance of the differences between the groups by ANOVA followed by post hoc testing using Fisher’s protected least significant difference procedure for multiple comparisons.

For vasoconstriction and vasodilation studies, we performed linear regression analysis to compare between the dose-response curves. We estimated and compared slopes and intercepts and show a P value if there was a significant difference between the slopes or intercepts.

We performed all statistical analyses using Statsview 5.0 (SAS Institute, Cary, North Carolina) or PRISM 5 (GraphPad Software, La Jolla, California), and considered a P value < 0.05 statistically significant.

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**Measurement of FMD**
We evaluated endothelial function by measurement of FMD, according to previously published guidelines. We provide details in the Supplemental Appendix.

**Statistical Analysis**
We tested all data for normal distribution of variables of interests using Pearson’s χ² distribution test before further parametric or nonparametric statistical analysis. If the test judged the data as a normal distribution, we performed the following statistical analysis by parametric analysis. If not, we used nonparametric analysis.

In dietary P loading experiments, we performed comparisons between preprandial and postprandial values of serum chemistry measurements in a group and effects of meals on preprandial and postprandial values of these measurements by means of repeated measurements and Tukey-Kramer methods. We determined statistical significance of the difference between the P400 and P1200 meals by the Wilcoxon signed-rank test.

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For vasoconstriction and vasodilation studies, we performed linear regression analysis to compare between the dose-response curves. We estimated and compared slopes and intercepts and show a P value if there was a significant difference between the slopes or intercepts.

We performed all statistical analyses using Statsview 5.0 (SAS Institute, Cary, North Carolina) or PRISM 5 (GraphPad Software, La Jolla, California), and considered a P value < 0.05 statistically significant.
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