

ORIGINAL

Excessive dietary phosphorus intake impairs endothelial function in young healthy men : a time- and dose-dependent study

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Abstract : Excessive dietary phosphorus (P) has been speculated to be a risk factor for cardiovascular disease (CVD). Here, we performed a double-blinded crossover study to investigate the time- and dose-dependent effects of dietary P intake on endothelial function in healthy subjects. Sixteen healthy male volunteers were given meals containing 400, 800, and 1,200 mg P (P400, P800, and P1200 meals, respectively) with at least 7 days between doses. There were no differences in nutritional composition among the experimental diets except for P content. Blood biochemistry data and flow-mediated dilation (%FMD) of the brachial artery were measured while fasted, at 0 h, 1 h, 2 h, and 4 h after meal ingestion, and the next morning while fasted. The P800 and P1200 meals significantly increased serum P levels at 1-4 h after ingestion. A significant decrease in %FMD was observed between 1-4 h, while the P400 meal did not affect %FMD. We observed no differences among meals in serum P levels or %FMD the next morning. A significant negative correlation was observed between %FMD and serum P. These results indicate that excessive dietary P intake can acutely impair endothelial function in healthy people. *J. Med. Invest.* 62 : 167-172, August, 2015

Keywords : phosphorus, endothelial dysfunction, flow-mediated vasodilation, hyperphosphatemia, chronic kidney disease

INTRODUCTION

Cardiovascular disease (CVD) is the most important complication contributing to reduced life expectancy in patients with chronic kidney disease (CKD) (1-3). Traditional and non-traditional risk factors relating to the pathogenesis of CVD in CKD patients have been identified (4, 5). Hyperphosphatemia has recently been recognized as a mediator between CKD and CVD (6, 7). Hyperphosphatemia is also an emerging problem, not only in CKD patients, but also in the healthy population. Recent studies have demonstrated that higher serum P levels, even those within the normal range, were associated with the development of atherosclerosis and mortality in the population with normal kidney function (8) and in the Framingham Offspring Study participants (9). Onufrak *et al.* also demonstrated that a high serum P level was associated with thickening of the carotid artery intima media in the general population (10). Hyperphosphatemia can induce the differentiation of vascular smooth muscle cells to osteoblast-like cells that are involved in the medial calcification of the artery, so-called Mönkeberg's arteriosclerosis (11-13). In addition, we and others have demonstrated that hyperphosphatemia can also mediate endothelial dysfunction (14-16), which is a principal cause of atherosclerosis resulting in CVD.

Our previous study demonstrated that the ingestion of a high P diet (1,200 mg P per meal) impaired flow-mediated dilation at 2 h after meal ingestion in young healthy men, compared with those given a control diet (400 mg P per meal) (14). In addition, increasing the

extracellular P level induced increased oxidative stress and decreased nitric oxide production in bovine thoracic aorta endothelial cells (14). Peng *et al.* reported that hyperphosphatemia decreased endothelial nitric oxide synthase (eNOS) expression in human umbilical vein endothelial cells (15). DiMarco *et al.* also demonstrated that an elevation of extracellular P can induce apoptosis via increased oxidative stress in human endothelial cell lines (16). These results suggest that over a high dietary intake of P may contribute to the pathogenesis of CVD. In this study, we performed a double-blinded crossover study to investigate the dose- and time-dependent effects of high dietary P intake on endothelial function in healthy human subjects.

METHODS

Subjects

Sixteen male volunteers aged 23.4±2.8 years and without apparent health problems were recruited for this study. The participants showed no evidence of diabetes, abnormal glucose intolerance, obesity, hypertension, kidney diseases, CVD, dyslipidemia, or other bone and mineral disorders. Demographic data for the participants are provided in Table 1. All participants were nonsmokers, had normal blood pressure, consumed < 30 g/d alcohol, and took no medications or antioxidant supplements. The eligibility of participants for this study was determined similarly to our previous reports (14, 17).

Study design

The study used a double-blinded crossover design, with the administration of meals containing specific amounts of P to each volunteer on 3 different days, each separated from the other test days by more than 1 week. Figure 1 illustrates the design of the study.

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Table 1. Baseline characteristics of subjects.

Age	23.4±2.8
Height (cm)	171.5±2.8
Weight (kg)	60.4±5.1
Percentage body fat (%)	14.2±7.0
Body fat (kg)	8.8±3.5
Fat free mass (kg)	51.6±4.5
Muscle mass (kg)	48.9±4.3
Total body water (kg)	35.0±3.4
BMI (kg/m ²)	20.5±2.1

Values are mean±S.E.M., n=16.

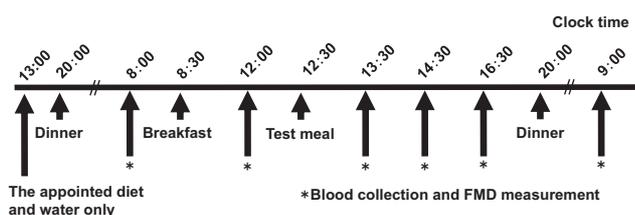


Figure 1. Study schema. The three test meals containing different amounts of P were served at 12:30 on the test day. The subjects were allowed only the standardized water and meals that we supplied after 14:00 on the day before the test day. Asterisks indicate the times at which blood collection and FMD measurements were performed.

On the day before each test day, the subjects were asked to abstain from foods and beverages other than water not containing P after 13:00. They were served a standard dinner at 20:00 on the evening before each test day, and a standard breakfast at 8:30 on each test day. On the test days, subjects were served either a P400 meal (standard lunch, which contained 400 mg of P+placebo supplement (NaCl)), a P800 meal (standard lunch+400 mg neutralized phosphate supplement), or a P1200 meal (standard lunch+800 mg neutralized phosphate supplement) for lunch at 12:30. The composition of the test meals and standard dinner and breakfast is provided in our previous study. In brief, the standard dinner consisted of 700 kcal in energy with a protein : fat : carbohydrate ratio in %energy of 15 : 19 : 66, and contained 200 mg of calcium (Ca) and 400 mg of P. The standard breakfast consisted of 700 kcal with a protein : fat : carbohydrate ratio in %energy of 14 : 21 : 65, and contained 200 mg of Ca and 400 mg of P. Standard lunch consisted of 700 kcal with a protein : fat : carbohydrate ratio in %energy of 14 : 21 : 65, and contained 200 mg of Ca and 400 mg of P.

We collected blood samples immediately before (0 h), and at 1 h, 2 h, 4 h, and 20 h after the test meal ingestion. Venous blood was taken from a median cubital vein for the measurement of serum glucose, insulin, P, Ca, Na, K, Cl, intact-PTH (iPTH) and high sensitivity-C reactive protein (hs-CRP) concentrations. All biochemical measurements and analyses were performed by LSI Medience (Tokyo, Japan). Serum monocyte-chemoattractant protein (MCP-1) and fibroblast growth factor 23 (FGF23) were measured by CCL2/MCP-1 immunoassay kit (R&D Systems Inc., Minneapolis, MN) and FGF23 ELISA kit (Kinos, Tokyo, Japan), respectively. We also measured blood pressure and flow-mediated dilation (FMD) by using UNEXEF 18G (UNEX Corporation, Aichi, Japan) according to previously published guidelines (18) immediately before (0 h), and at 1 h, 2 h, 4 h, and 20 h after the test meal ingestion.

The study protocols were approved by the Ethics Committee of

the Tokushima University Hospital. This study has been registered and opened on the UMIN-CTR database in Japan according to the ICMJE guidelines (UMIN00000803, Dietary phosphorus loading trial in human).

Statistical analysis

We tested all data for normal distribution of variables of interests by Kolmogorov-Smirnov test before further parametric or non-parametric statistical analysis. If the test judged the data to be normally distributed, we performed subsequent statistical analysis by parametric analysis. If not, we used nonparametric analysis.

Serum biochemical measurements and %FMD within groups and the effects of meals on pre-prandial and post-prandial values of these measurements were analyzed by repeated measurements analysis of variance (ANOVA) and post hoc analysis by Bonferroni's method.

For the association analysis, we performed a simple regression analysis and estimated Spearman's non-parametric correlation coefficients. We selected the nonparametric procedure, which does not require normally distributed data or linear associations of the variables of interest.

We performed all statistical analyses using SPSS Statistics 17.0.

RESULTS

1. Dose and time-dependent effects of high dietary P intake on the serum P level and other P metabolism-regulating factors

In this study, the subjects alternately received P400, P800, or P1200 meals as lunch and the serum levels of P and P metabolism-regulating factors were measured in the morning (fasting), and before and after intake of the test meal (Table 2 and 3). In spite of the differences in P content among the test meals, the serum P level was significantly increased at 1 h, 2 h, and 4 h after the ingestion of the test meals, compared with the pre-prandial serum P level. However, the serum P levels at 1 h, 2 h, and 4 h after ingestion of the P1200 meal were significantly higher than that measured following ingestion of the P400 meal (Figure 2). Area under the curve (AUC) analysis revealed post-prandial changes in the serum P level during the 4 h after test meal ingestion; the serum P level was increased accordingly with the increases in phosphorus intake (Figure 3A). In addition, the serum P levels were above the normal range at 1 h, 2 h, and 4 h after ingestion of the P800 or P1200 meals, but not after ingestion of the P400 meal. Serum P levels had reverted to a normal level when measured the next morning after ingestion of the test meals.

Serum intact-PTH levels did not show significant differences among the groups; however, they showed a biphasic peak at 1 h and 4 h after ingestion of the test meals (Table 2), as reported previously (17). The intact-PTH level at 4 h after ingestion of the P400 and P1200 meals was significantly increased compared with the pre-prandial serum intact-PTH level. The AUC for post-prandial serum intact-PTH changes over 4 h increased accordingly with the increases in the intake of P (Figure 3B). The AUC after the ingestion of the P1200 meal was significantly greater than that after the P400 meal ($P < 0.05$). FGF23 is also an important P metabolism-regulating hormone. The serum FGF23 level was not increased following ingestion of the test meals (Table 3). Serum Na, K, Cl, Ca, hs-CRP, and MCP-1 levels also were not affected by the experimental increases in P intake (Tables 2 and 3).

2. Dose- and time-dependent effects of high dietary P intake on FMD

We demonstrated that intake of the P1200 meal led to a significant decrease in %FMD compared with that measured following intake of the P400 meal at 2 h after meal ingestion (14). Here, we investigated the dose- and time-dependent effects of high dietary

Table 2. Measurements of blood and urine biochemical markers.

		%FMD	SBP (mmHg)	DBP (mmHg)	Glucose (mg/dL)	Insulin (μU/mL)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)	Ca (mEq/L)	P (mEq/L)	Intact-PTH (pg/dL)
	Normal Range		<130	<85	70-109	1.7-10.4	137-147	3.5-5.0	98-108	8.4-10.4	2.5-4.5	10-65
P400 meal	Morning	10.6±0.4	117±2.1	70.3±2.1	91.9±2.2	3.93±0.5	141±0.3	4.10±0.1	103±0.5	9.89±0.1	4.02±0.1	41.0±2.9
	Pre-prandial	11.1±0.3	114±2.5	65.9±2.6	83.4±2.4	4.90±0.7	140±0.4	4.31±0.1	103±0.4	9.90±0.1	3.82±0.1	31.9±2.6
	1 H	10.2±0.2	116±2.6	63.1±2.1	96.9±3.9	16.5±1.7*	142±0.5	4.04±0.1*	104±0.5	9.65±0.1	4.13±0.1*	33.7±2.1
	2 H	9.25±0.3	115±2.4	64.1±1.8	102±2.3	15.4±1.1*	141±0.5	4.08±0.1*	104±0.4	9.72±0.1	4.26±0.1*	32.4±2.2
	4 H	10.2±0.4	113±2.2	66.2±1.9	95.2±2.0	5.80±1.0	141±0.4	4.22±0.1	103±0.4	9.78±0.1	4.48±0.1*	40.7±2.5*
	Next morning	10.6±0.3	113±2.1	68.4±1.8	92.3±1.4	3.66±0.3	140±0.3	4.16±0.1	103±0.4	9.86±0.1	3.91±0.1	33.5±1.6
P800 meal	Morning	10.1±0.4	115±2.5	69.9±1.6	89.9±2.0	3.65±0.4	140±0.3	4.08±0.1	102±0.5	9.94±0.1	4.10±0.1	39.0±3.6
	Pre-prandial	10.8±0.2	113±2.8	65.0±2.0	82.6±2.5	6.94±2.0	140±0.4	4.38±0.1	103±0.3	9.84±0.1	3.81±0.1	33.1±2.5
	1 H	6.65±0.4*	115±2.5	63.8±1.5	95.9±4.1	16.7±1.6*	141±0.5*	4.08±0.1*	103±0.3	9.59±0.1	4.81±0.1*	39.6±2.1
	2 H	5.89±0.5*	113±2.4	62.5±1.8	101±3.9	15.6±1.7*	141±0.4*	4.08±0.1*	102±0.4	9.63±0.1	4.89±0.1*	38.1±2.0
	4 H	7.21±0.4*	115±2.3	67.4±1.5	92.1±2.2	4.46±0.4	140±0.3	4.14±0.1	102±0.4	9.71±0.1	4.86±0.1*	39.6±3.3
	Next morning	10.5±0.4	113±2.6	67.3±2.0	91.5±1.5	3.58±0.3	140±0.3	4.22±0.1	102±0.4	9.93±0.1	3.89±0.1	34.7±3.4
P1200 meal	Morning	9.99±0.3	117±2.2	70.3±1.8	90.9±2.0	3.79±0.4	140±0.5	4.14±0.1	102±0.5	9.85±0.1	4.00±0.1	40.8±3.3
	Pre-prandial	10.6±0.3	116±2.6	66.9±2.1	81.1±2.9	5.10±0.9	140±0.5	4.31±0.1	103±0.3	9.89±0.1	3.75±0.1	33.6±2.7
	1 H	5.28±0.4*	115±2.4	63.1±1.8	102±4.6	21.2±2.6*	141±0.5*	3.99±0.1*	103±0.4	9.59±0.1*	5.02±0.2*	41.7±2.5
	2 H	5.62±0.4*	116±2.4	64.9±1.7	97.1±3.3	14.1±1.0*	141±0.5*	4.01±0.1*	103±0.4	9.54±0.1*	5.26±0.2*	41.2±2.3
	4 H	7.06±0.4*	115±2.2	67.4±1.9	93.3±2.4	4.59±0.5	141±0.4	3.98±0.1*	102±0.3	9.66±0.1	5.23±0.1*	45.9±2.7*
	Next morning	10.6±0.3	116±2.4	67.3±1.4	91.6±1.7	3.83±0.4	141±0.4	4.11±0.1	103±0.3	9.81±0.1	3.93±0.1	32.5±2.1

P400, P400 meal ; P800, P800 meal ; P1200, P1200 meal ; %FMD, %flow-mediated dilation ; SBP, systolic blood pressure ; DBP, diastolic blood pressure ; Cre, creatinine. Values are mean±S.E.M. for 16 subjects. *P*<0.05 vs pre-prandial in the same meal.

Table 3. Effects of high dietary phosphorus intake on serum hs-CRP, MCP-1, and FGF23 levels.

		Pre-prandial	4 h	Next morning
P400 meal	hs-CRP (mg/dL)	0.035±0.0	0.034±0.0	0.029±0.0
	MCP-1 (pg/dL)	165.8±7.2	164.3±6.5	166.2±6.1
	FGF23 (pg/mL)	41.6±16.5	35.7±16.7	45.0±15.5
P800 meal	hs-CRP (mg/dL)	0.048±0.0	0.043±0.0	0.035±0.0
	MCP-1 (pg/dL)	165.7±6.7	157.2±6.8	160.9±5.9
	FGF23 (pg/mL)	50.8±13.5	39.3±15.6	40.7±14.7
P1200 meal	hs-CRP (mg/dL)	0.062±0.0	0.061±0.0	0.052±0.0
	MCP-1 (pg/dL)	165.8±8.3	154.7±7.8	160.9±7.7
	FGF23 (pg/mL)	60.6±16.7	40.1±16.2	49.2±18.4

Abbreviations are hs-CRP, high sensitive-C reactive protein ; MCP-1, monocyte/macrophage chemoattractant protein-1 ; FGF23, fibroblast growth factor 23.

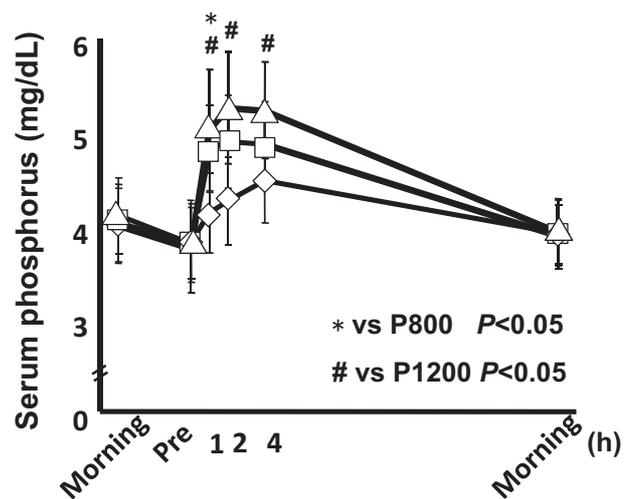


Figure 2. Effects of high dietary P intake (open diamond, P400 meal ; open square, P800 meal ; open triangle, P1200 meal) on the serum P level before and after ingestion of test meals. Data are mean±S.E.M. for 16 subjects.

P intake on FMD in young healthy men. As shown in the Figure 4, %FMD at 1 h, 2 h, and 4 h after the ingestion of P800 and P1200 meals was significantly decreased compared with that measured following ingestion of the P400 meal. The peak inhibition of FMD by P800 was observed at 2 h after meal ingestion, while that by P 1200 was at 1 h after meal ingestion. In addition, the decrease in %FMD observed after high P intake was recovered by the next morning. The rate of increase in the post-prandial serum P level between 0-4 h after meal intake was significantly correlated with the rate of decrease in %FMD (Figure 5).

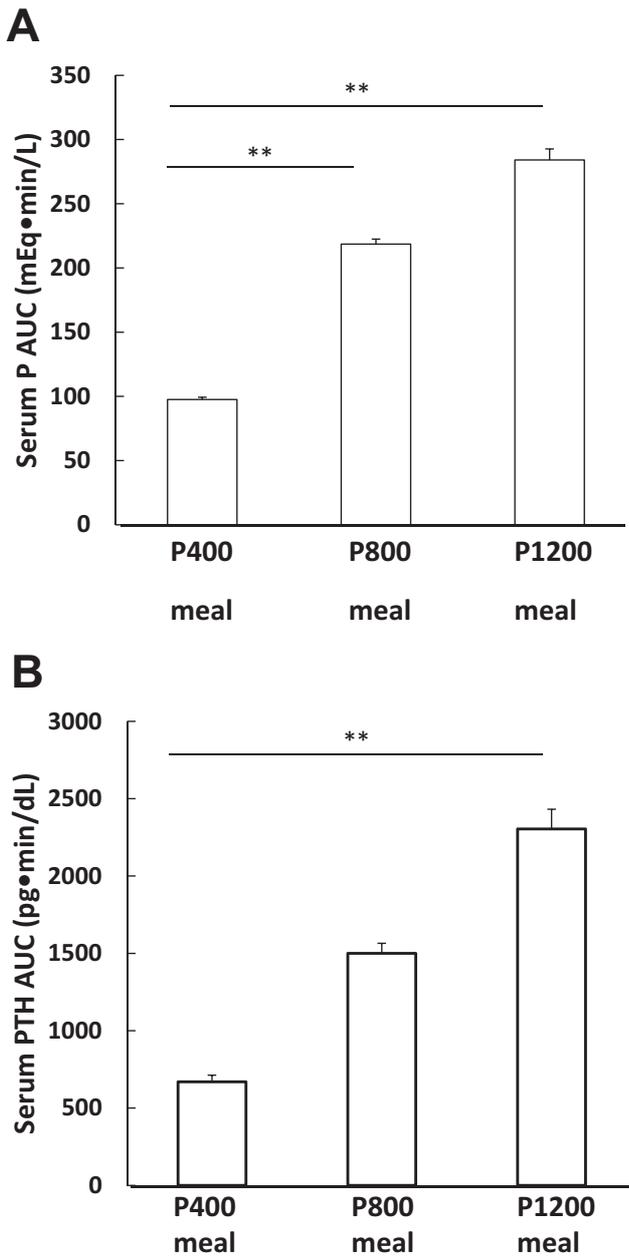


Figure 3. Effects of high dietary P intake on areas under the curve for post-prandial changes in serum P (A) and intact PTH levels (B) over 4 h after ingestion of the test meal. Data are mean ± S.E.M. for 16 subjects. ***P* < 0.01 for differences among the meals.

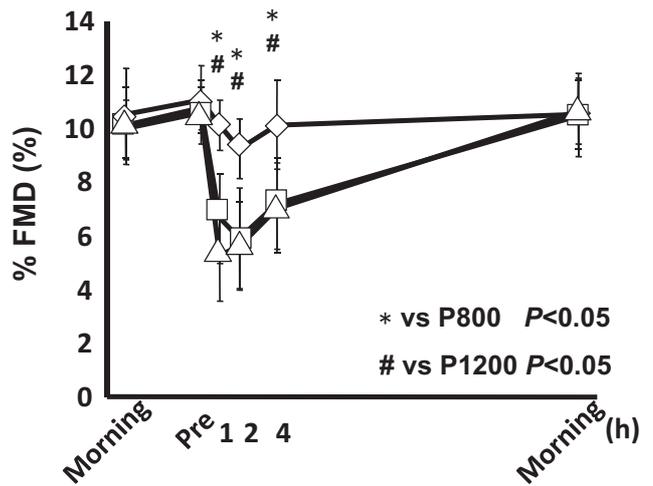


Figure 4. Effects of high dietary P intake (open diamond, P400 meal ; open square, P800 meal ; open triangle, P1200 meal) on %FMD (B) before and after ingestion of test meals. Data are mean ± S.E.M. for 16 subjects.

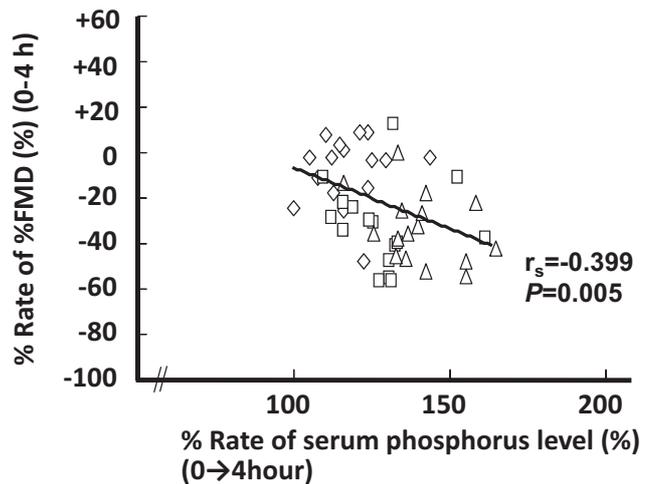


Figure 5. Univariate association analysis of the ratios of changes (%) in serum P and %FMD from pre-prandial measurements to those made 4 h after ingestion of the test meals. All variables were centralized according to the median value for each individual. Each symbol is used as in Figure 2. Spearman's correlation coefficient (r_s) and its *P*-value for $r_s=0$ are presented in the association.

DISCUSSION

In this study, we investigated the time- and dose-dependent effects of high dietary P intake on endothelial function by evaluating %FMD. We found that FMD was rapidly inhibited by high P intake, but began to be recovered at 4 h and was normalized by the next morning. We did not find any clear differences between the P800 and P1200 meals in the high dietary P intake-induced inhibition of FMD. However, the P1200 meal inhibited FMD slightly faster than did the P800 diet. In addition, the inhibitory effect of high dietary P intake could be observed at the minimum level of intake of 800 mg of phosphorus in a single meal.

The post-prandial increase in the serum P level was significantly

correlated with the degree of impairment of FMD. Our previous work demonstrated that the experimental elevation of the extracellular P level can inhibit nitric oxide production in endothelial cells via increasing oxidative stress and the inhibitory phosphorylation of eNOS (14). Therefore, a transient increase in the serum P level may be enough to lead to a deterioration of endothelial function. Another possible mechanism for the impairment of endothelial function by a high serum P level is via PTH and FGF23. The post-prandial serum PTH level was increased by high dietary P intake in a dose-dependent manner. Primary hyperparathyroidism patients have an impaired FMD (19-22), but the impairment of FMD was ameliorated after parathyroidectomy (21, 20). Parathyroidectomy or Ca channel blockade was reported to restore inhibited eNOS activity in a rat model of CKD (23). On the other hand, FGF23 also can directly impair endothelium-dependent vasodilation by increasing oxidative stress and reducing NO availability (24). However, the serum FGF23 level was not increased after a single ingestion of a high P meal in our study. Thus, the serum FGF23 level did not appear to be related to the decreases in %FMD observed in this study.

A transient increase in the serum P level may be an important atherogenic factor. Watari *et al.* demonstrated that inducing fluctuations in the serum P level by the alternating administration of high or low P diets led to a deterioration of endothelium-dependent vasodilation and an increased expression of VCAM-1 and MCP-1 in the tunica intima (25). The impairment of endothelial function by the alternating administration of high or low P diets was almost same as that produced by the chronic administration of a high P diet (25). Therefore, repeated transient increases in the serum P level may have some of the same adverse effects on endothelial cells as continuous high dietary P intake.

A chronic increase in the serum P level is a well-known risk factor for CVD, not only in CKD patients, but also in the general population (6, 10). In addition, Yamamoto *et al.* reported that a high dietary P intake was associated with left ventricular hypertrophy (26). They concluded that the highest quintile of dietary phosphorus intake (male 1,554-5,032 mg/day, female 1,346-4,069 mg/day) was associated with a greater left ventricular hypertrophy compared with the lowest quintile (male 270-687 mg/day, female 251-585 mg/day). A recent study demonstrated that high dietary P intake was associated with all-cause mortality in the NHANES III cohort (27). All-cause mortality was significantly increased in the people with high phosphorus intake (more than 1,400 mg/day) compared with low phosphorus intake (less than 1,400 mg/day). In our study, standard P400 meal corresponded to 1,200 mg of daily phosphorus consumption if the subject consumed the same meal three times per day. On the other hand, the ingestion of P800 or P1200 meal three times per day would be estimated over 1,400 mg/day. In this study, the single-time ingestion of P800 or P1200 meal significantly deteriorated endothelial function. Therefore, habitual consumption of high phosphorus diet likes P800 and P1200 meals may increase the risk of cardiovascular disease.

High phosphorus diet also causes large fluctuation of serum phosphorus levels. Portale *et al.* demonstrated that there is a circadian rhythm of the serum P level (28), with the serum P level being at its lowest during the morning fasting state and highest during the night. A high dietary intake of P increased the serum P level during both day and night, except during the morning fasting state. Thus, a chronic high phosphorus diet can widen the amount of difference between the lowest and highest serum P levels present during each circadian cycle. Such large daily fluctuations arising from continuous high dietary P intake may cause endothelial dysfunction in humans, as was previously observed in rodents (25).

This study has some limitations. Firstly, this study was carried out with a limited number, gender, and age range of subjects, although the impact of these limitations was reduced by the use of

a double-blinded crossover protocol. A further intervention study with a large number of subjects of different ages and genders should be performed to confirm our results in the future. Secondly, we could not fully clarify the effects of FGF23, PTH, or other factors on endothelial dysfunction caused by high dietary P intake. An elevation or fluctuations in the serum P level must directly inhibit endothelial function. However, PTH and FGF23 may be important as mediators of the deterioration of endothelial function produced by chronic high dietary P intake. Thus, a study investigating the effects of the chronic administration of a high P diet is needed to clarify the effects of PTH or FGF23 on the impairment of endothelial function.

In conclusion, excessive dietary P intake can acutely impair endothelial function in healthy people. Habitual excessive P intake and the resulting endothelial dysfunction may contribute to the progression of CVD or increased mortality, as is suggested by epidemiological data.

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CONFLICT OF INTEREST

We have no conflicts of interest to declare for this study.

DISCLOSURE

No conflicts of interest are declared.

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