Original Article

Fluctuating plasma phosphorus level by changes in dietary phosphorus intake induces endothelial dysfunction

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High serum phosphorus (P) impairs endothelial function by increasing oxidative stress and decreasing nitric oxide production. Serum P levels fluctuate due to circadian rhythms or dietary P intake in healthy people and due to dialysis in end-stage chronic kidney disease patients. Here we examined whether fluctuating plasma P caused by changes in dietary P intake may be involved in endothelial dysfunction, resulting in increased cardiovascular risk. Rats were fed a diet containing 0.6% P for 16 days (control group), or a diet alternating between 0.02% P and 1.2% P (LH group) or between 1.2% P and 0.02% P (HL group) every 2 days; the total amount of P intake among the groups during the feeding period was similar. In the LH and HL groups, endothelial-dependent vasodilation significantly decreased plasma 8-(OH)dG level significantly increased, and the expression of inflammatory factors such as MCP-1 increased in the endothelium as compared with the control group. These data indicate that repetitive fluctuations of plasma P caused by varying dietary P intake can impair endothelial function via increased oxidative stress and inflammatory response. Taken together, these results suggest that habitual fluctuation of dietary P intake might be a cause of cardiovascular disease through endothelial dysfunction, especially in chronic kidney disease patients.

Key Words: dietary phosphorus, phosphorus spike, circadian rhythm, oxidative stress, inflammation

Cardiovascular disease (CVD) is the most important complication reducing life expectancy among chronic kidney disease (CKD) patients and dialysis patients.[1–4] At any age, patients on dialysis more frequently succumb to cardiovascular death as compared with non-CKD patients, and >50% of all deaths among CKD patients are due to cardiovascular events.[5] Recent epidemiological studies suggest that a higher serum phosphorus (P) level in patients with CKD is an independent risk factor for CVD.[6–9] Recent studies reported that a higher serum level of P, even if within the normal range, was associated with the development of atherosclerosis and mortality in patients with normal kidney function.[10,11]

Endothelial dysfunction is a well-known pathological feature in the early stages of atherosclerosis progression.[12,13] Endothelial dysfunction causes a micro-environmental inflammatory response and activation of monocytes and macrophages, resulting in the development of atherosclerosis.[12,13] Some studies have reported that repetitive fluctuations of postprandial hyperglycemia can induce endothelial dysfunction and can enhance monocyte adhesion to the endothelium in diabetes mellitus, even when the fasting glucose level is within the normal range.[14–18] *In vitro* studies have

also demonstrated that, as compared with constant levels of high glucose, repetitive fluctuation between high and normal glucose impairs endothelial function due to enhanced intracellular oxidative stress.[19]

Elevation of extracellular P levels can lead to the generation of reactive oxygen species (ROS) and decreased production of nitric oxide (NO) in bovine aortic endothelial cells (BAECs),[20–21] and can also induce apoptosis in human endothelial cells.[22] Transient dietary P-loading in healthy men was found to induce postprandial elevation of serum P above the normal range and to also cause an impairment of flow-mediated vasodilation.[21] Serum P levels fluctuate due to circadian rhythms or dietary P intake in healthy people,[23] and due to dialysis in patients with end-stage CKD.[24]

Taking these observations together, we hypothesized that fluctuations of serum P level may be involved in endothelial dysfunction. As a result, the aim of this study was to investigate the effect of fluctuations of plasma P levels on endothelium-dependent vasodilation in healthy rats. Our results indicated that fluctuations of plasma P impaired endothelial function with reference to increased oxidative stress, inflammatory factors and monocyte/macrophage infiltration.

Materials and Methods

Animals. The Animal Experimentation Committee of the University of Tokushima approved the study experiments. Male Sprague-Dawley (SD) rats were obtained from Japan SLC (Shizuoka, Japan) at the age of 10 weeks and individually housed in cages. Extra-pure water was available *ad libitum* for all rats. The animal room was kept on a 12-h light/dark cycle (light, 8:00 AM to 8:00 PM; dark, 8:00 PM to 8:00 AM) and maintained at constant temperature (22 ± 1°C) throughout the experimental period.

Experimental design. Fig. 1 shows the experimental design of this study. We prepared three kinds of diet—namely, a control diet (P: 0.6%, Ca: 0.6%), low P diet (P: 0.02%, Ca: 0.6%) and high P diet (P: 1.2%, Ca: 0.6%)—using mineral mix (Oriental Yeast, Osaka, Japan) and an altered AIN93-G diet (Oriental Yeast, Osaka, Japan) derived from casein, $CaCO_2$ and KH_2PO_4.[25] All rats were given their diet from 4:00 PM to 10:00 AM. Before grouping, all rats were fed MF. At the age of 11 weeks, they were divided into five groups and fed diets containing different P for 16 days. The control group ($n = 4$, CP group) was fed the control diet,

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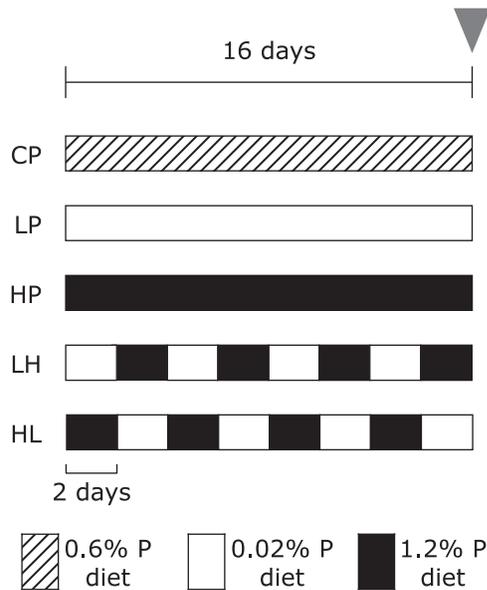
Fig. 1. Experimental design. The control (CP), low (LP) and high (HP P diet groups were continuously fed with 0.6% P, 0.02% P and 1.2% P diets, respectively, through the experimental period (16 days). Two alternating diet groups (LH and HL) were fed with either a 0.02% P diet or 1.2% P diet every 2 days during the experimental period as indicated. LH group rats were fed with the high P diet for the last two days, whereas HL group rats were fed with the low P diet for the last two days.

the low P diet group (*n* = 4, LP group) was fed the low P diet, and the high P diet group (*n* = 4, HP group) was fed the high P diet throughout the experimental period. In addition, two alternating diet groups (*n* = 5, LH group; *n* = 5, HL group) were alternately fed diets containing 0.02% or 1.2% P every 2 days. The LH group finished with the high P diet, whereas the HL group finished with the low P diet to exclude the effect of the dietary P intake on the last day of the experimental period. Each rat was given the experimental diet in accordance with a pair-feeding protocol. Therefore, daily food intake, and energy, protein, and fat intake did not differ among the groups.

Blood and urine biochemical analysis. Blood samples were taken from the tail vein between 10:00 AM and 11:00 AM everyday throughout the experimental period. Blood and urine samples were also taken at sacrifice. Before sacrifice, the rats were fasted for 12 h. Plasma and urine P, Ca and creatinine levels were measured by the Wako Phospha-C test kit, the Wako Calcium-E test kit and the Wako Creatinine test kit (Wako Pure Chem. Ind., Ltd., Osaka, Japan), respectively. Plasma intact-PTH levels were measured by the Rat Intact PTH ELISA kit (Immunotopics, San Clemente, CA). Plasma intact-FGF23 levels were measured by a sandwich ELISA for human FGF23 protein (KAINOS Laboratories, Inc., Tokyo, Japan). Plasma BUN levels were measured by the Wako BUN test (Wako Pure Chem. Ind.). Plasma 8-hydroxydeoxyguanosine (8-OHdG) levels were measured by the Highly Sensitive 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). Plasma 1,25-(OH)$_2$D was measured by SRL Co., Ltd. (Tachikawa, Japan).

Evaluation of vasorelaxation responses to acetylcholine. The thoracic aorta was carefully dissected and excised from the aortic arch to the point of the diaphragm. The thoracic aorta was dissected free from connective tissue and cut into ring segments of 2–3 mm in length. Each ring was placed in a 3-ml organ bath (Micro Easy Magnus, Kishimoto Medical, Kyoto, Japan) and

mounted on two stainless steel wires, one of which was fastened to the bath and the other of which was connected to a force transducer so that isometric tension was recorded as previously described.[21,26] The bath was filled with Krebs-Ringer bicarbonate (KRB) buffer solution pH 7.4 at 37°C and bubbled with a mixture of 95% O_2/5% CO_2. The KRB buffer contained 118 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose. The rings were equilibrated for 60 min under a resting tension of 1 g, and solution was changed at 30-min intervals.[26] After equilibration, the rings were pre-contracted with phenylephrine ($1*10^{-6}$ M) for 5 min and then concentration-dependent responses to the endothelium-dependent agonist acetylcholine ($1*10^{-9}$ to $1*10^{-6}$ M) were measured for 5 min for each concentration.

RT-PCR analysis. The thoracic aorta was carefully isolated from rats, the connective tissue was removed in ice-cold Krebs buffer, and the aorta was placed in RNA later (RNA stabilization Reagent; Life Technologies Japan, Tokyo, Japan). Endothelium-enriched fractions were detached by rubbing the intimal surface with tweezers,[27] and then washing off into Buffer RLT Plus (QIAGEN Japan, Tokyo, Japan). The Buffer RLT Plus was collected and used to examine the RNA expression profile of the endothelium-enriched fraction. Total RNA was extracted from this fraction by using the RNeasy Plus Mini Kit (QIAGEN) according to the instructions provided by the manufacturer.

Next, cDNAs were synthesized by using Superscript II RNase H Reverse Transcriptase and oligo-dT primers. The resulting cDNAs were amplified by using a SYBER Green PCR kit (Life Technologies Japan). Quantitative PCR was performed on an ABI PRISM 7700 sequence detection system (Life Technologies Japan). The relative abundance of mRNAs was calculated by the comparative cycle of threshold (CT) method with β-actin mRNA as the invariant control.

Immunoblot analysis. Total protein was extracted from rat thoracic aorta as described previously,[27] and 15 μg of protein was subjected to immunoblot analysis as described previously.[21,27]

Immunohistochemistry. Aortic arches were perfusion-fixed in 4% paraformaldehyde and embedded in paraffin. Immuno-labeling was performed on 5-μm paraffin sections, which were used for immunostaining and for staining with hematoxylin. The sections were placed in citrate buffer in a microwave at 100°C for 5 min, blocked with 1.0% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, and incubated with rabbit polyclonal to MCP-1 (Abcam, Cambridge, UK) or rabbit polyclonal IgG F4/80 (Santa Cruz Biotechnology, Dallas, TX) antibodies overnight at 4°C. The slides were then incubated with anti-rabbit Dako EnVision+ System HRP Labelled Polymer (Dako Diagnostics Japan, Tokyo, Japan) for 1 h at room temperature. Primary antibodies were diluted in the same blocking solution using anti-MCP-1 (1:100) and anti-F4/80 (1:100) in respective combinations. The MCP-1-positive area and ratio of F4/80-positive cells were measured by using Image-Pro Plus 6.0 Software (Nippon Roper, Tokyo, Japan) as previously reported.[28]

Statistical analysis. We determined statistical significance of differences between the groups by ANOVA followed by post-hoc testing using Fisher's protected least significant difference procedure for multiple comparisons. For the vasoconstriction and vasodilation studies, we performed a linear regression analysis to compare the dose-response curves. We estimated and compared slopes and intercepts, and have stated the *p* value where there was a significant difference between the slopes or intercepts. We performed all statistical analyses using Statview 5.0 (SAS Institute, Cary, NC) or PRISM 5 (Graph Pad Software, La Jolla, CA), and considered a *p* value of less than 0.05 as statistically significant.

Results

Changes in daily plasma P levels during the experimental period.
Continuous administration of the CP diet, LP diet or HP diet led to different daily dietary P intake during the experimental period (Fig. 2A). The LP diet group showed lower plasma P levels as compared with the CP diet group immediately after beginning ingestion of LP diet; on the other hand, ingestion of the HP diet did not increase plasma P levels during the experimental period (Fig. 2B). In the alternating diet groups, when the rats were fed the HP diet, both daily P intake and plasma P levels were elevated (Fig. 3). Conversely, when the rats were fed the LP diet, both daily P intake and plasma P levels were decreased (Fig. 3). Importantly, total P intake during the experimental period in the alternating diet groups was almost same as that of CP diet group. Thus, the alternating diet groups showed a clear fluctuation of dietary P intake and plasma P levels.

Mineral metabolism in the experimental groups.
To examine the effects of fluctuating dietary P intake for 16 days on plasma and urine markers of P metabolism, we measured plasma P, Ca, intact-PTH, intact-FGF-23, 1,25-$(OH)_2D$, creatinine, BUN, urine P and Ca on day 16 (sacrifice day). As shown in Table 1, there was no significant difference in body weight among the 5 groups, despite the different plasma P levels on day 16. Plasma P was significantly lower in the LP group than in the CP group, and plasma Ca, 1,25-$(OH)_2D$ and urinary Ca/Cre were significantly higher in the LP group than in the CP group. Plasma P and calcium levels did not significantly change in the HP group as compared with the CP group, but plasma intact-PTH and intact-FGF23 were significantly increased in the HP group.

Plasma P and urine P/Cre were significantly higher in the LH group than in the control group but there was no difference in plasma Ca, PTH, 1,25-$(OH)_2D$, intact-FGF23 and urine Ca/Cre between these two groups. Plasma P, intact-PTH, intact-FGF23 and urine P/Cre were significantly lower in the HL group than in the CP and LH groups. By contrast, plasma Ca, 1,25-$(OH)_2D$ and urine Ca/Cre were significantly higher in the HL group than in the CP and LH groups.

Effects of fluctuating plasma P level by changes in dietary P intake on endothelium-dependent vasodilation.
To determine whether an impairment of endothelium-dependent vasodilation is caused by fluctuating plasma P levels or sustained high P-loading, we investigated the effects of fluctuating plasma P levels on acetylcholine-induced vasodilation using thoracic aorta rings obtained from rats in each dietary group. As shown in Fig. 4A, vasorelaxation significantly deteriorated in both alternating groups as compared with the CP group. Interestingly, there was no difference between the alternating groups even though they differed in high and low plasma P levels on the last day. On the other hand, the HP group with sustained high P-loading also
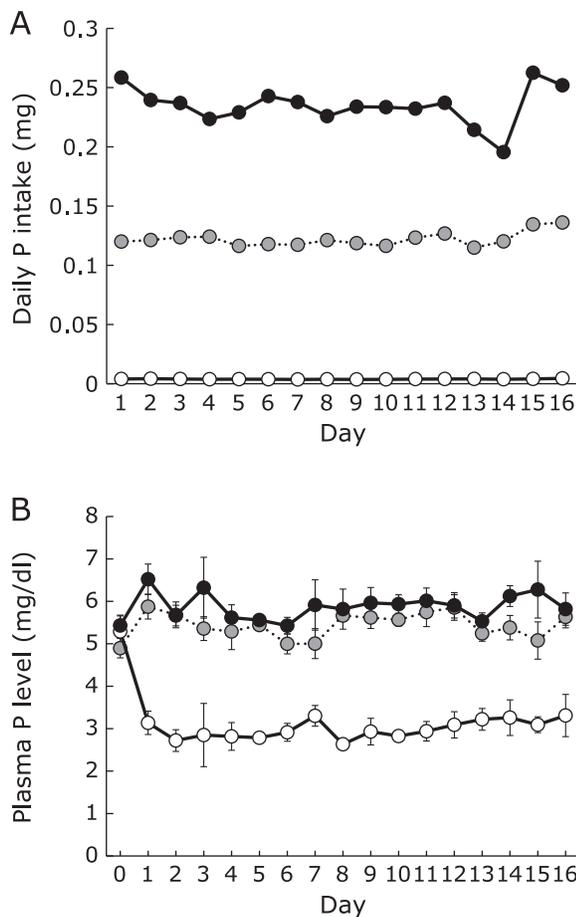
Fig. 2. Daily P intake (A) and daily plasma P level (B) in the low P (open circle), control (hatched circle), high P (closed circle) groups during the experimental period. Daily P intake was calculated from daily food intake and the P content in each experimental diet. Blood samples were taken from the tail vein. Results are expressed as mean ± SEM (*n* = 5).
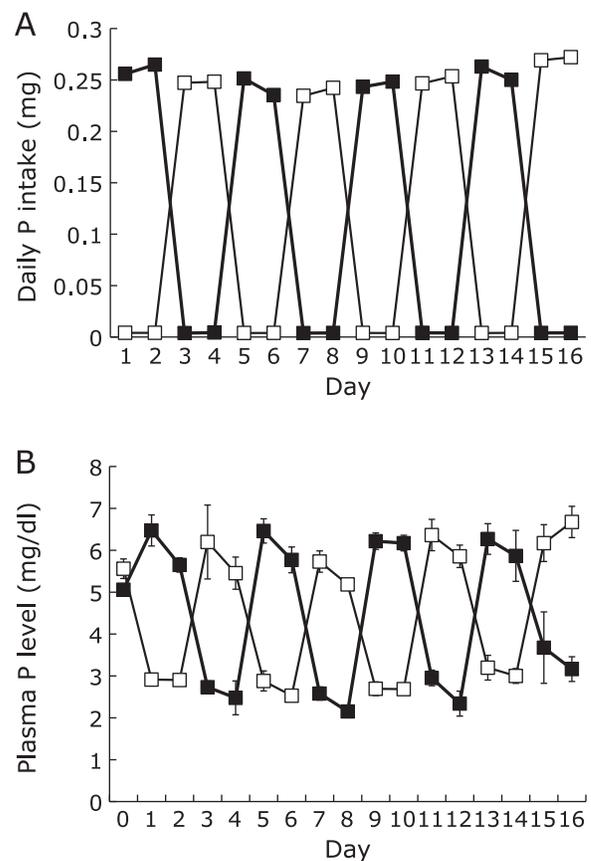
Fig. 3. Daily P intakes (A) and daily plasma P levels (B) in alternating low P and high P diet groups during the experimental period. LH (open square) indicates alternate administration of low and high P diet; HL (closed square) indicates alternate administration of high and low P diet. Daily P intake was calculated from daily food intake and the P content in each experimental diet. Blood samples were taken from the tail vein. Results are expressed as mean ± SEM (*n* = 5).

Table 1. Body weight and biochemical data of blood and urine samples collected at sacrifice day

	CP	LP	HP	LH	HL
Body weight (g)	395 ± 5.3	367 ± 8.2	392 ± 6.3	387 ± 8.3	387 ± 5.6
Plasma					
P (mg/dl)	4.23 ± 0.22	3.08 ± 0.59*	7.14 ± 0.60[†]	7.49 ± 0.16[†]	2.37 ± 0.18*[†§f]
Ca (mg/dl)	11.4 ± 0.34	14.8 ± 0.20*	12.7 ± 0.30[†]	11.5 ± 0.24[†]	12.5 ± 0.51*[†]
PTH (ng/ml)	86.0 ± 14	nd	255 ± 86*[†]	82.4 ± 23[§]	28.0 ± 40[§]
1,25D (pg/ml)	355 ± 70	886 ± 46*	313 ± 41[†]	369 ± 30[†]	532 ± 61*[†§f]
FGF23 (pg/ml)	240 ± 27	15.7 ± 1.2	449 ± 62*[†]	331 ± 24	43.9 ± 7.4*[§f]
Cre (mg/dl)	0.86 ± 0.04	0.91 ± 0.06	0.88 ± 0.07	0.98 ± 0.04	1.06 ± 0.07
BUN (mg/dl)	11.4 ± 1.8	15.5 ± 1.31	15.0 ± 0.72	12.8 ± 1.2	13.5 ± 1.3
Urine					
P/Cre	0.84 ± 0.32	nd	11.0 ± 5.3[†]	9.30 ± 4.6*[†§]	0.02 ± 0.02[§]
Ca/Cre	0.17 ± 0.12	3.03 ± 0.13*	0.44 ± 0.49[†]	0.08 ± 0.04[†]	1.87 ± 0.14*[†§f]

CP, control P diet group; LP, low P diet group; HP, high P diet group; LH, alternating low and high P diet group; HL, alternating high and low P diet group; P, phosphorus; Ca, calcium; PTH, intact parathyroid hormone; 1,25D, 1,25-dihydroxyvitamin D; FGF23, intact fibroblast growth factor 23; Cre, creatinine; BUN, blood urea nitrogen; P/Cre, urine P/Cre ratio; Ca/Cre, urine Ca/Cre ratio; nd, not determined. Data are mean ± SEM (n = 4–5). *$p<0.05$ vs CP, [†]$p<0.05$ vs LP, [§]$p<0.05$ vs HP, [f]$p<0.05$ vs LH.
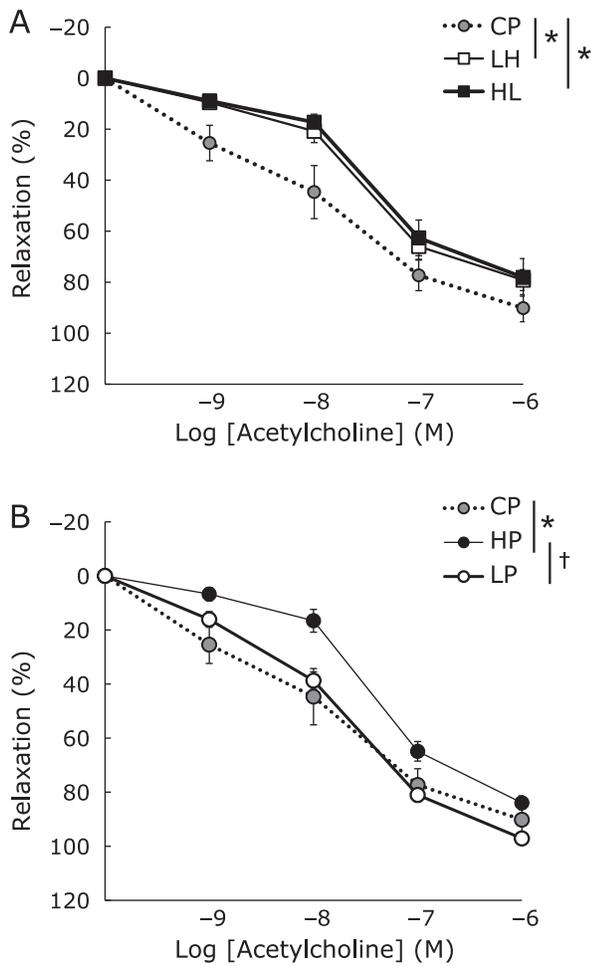
Fig. 4. Acetylcholine-dependent vasorelaxation responses. (A) Effect of alternate administration of low P and high P diet (LH; open square) and HL; closed square), or control diet (hatched circle) on acetylcholine-dependent vasorelaxation response. (B) Effect of continuous administration of low P (LP; open circle), control (CP; hatched circle), high P (HP; closed circle) on acetylcholine-dependent vasorelaxation response. Results are expressed as mean ± SEM (n = 4–5). There was significant difference in intercepts between the curves. *$p<0.005$ vs CP, [§]$p<0.001$ vs CP, [†]$p<0.0001$ vs LP.
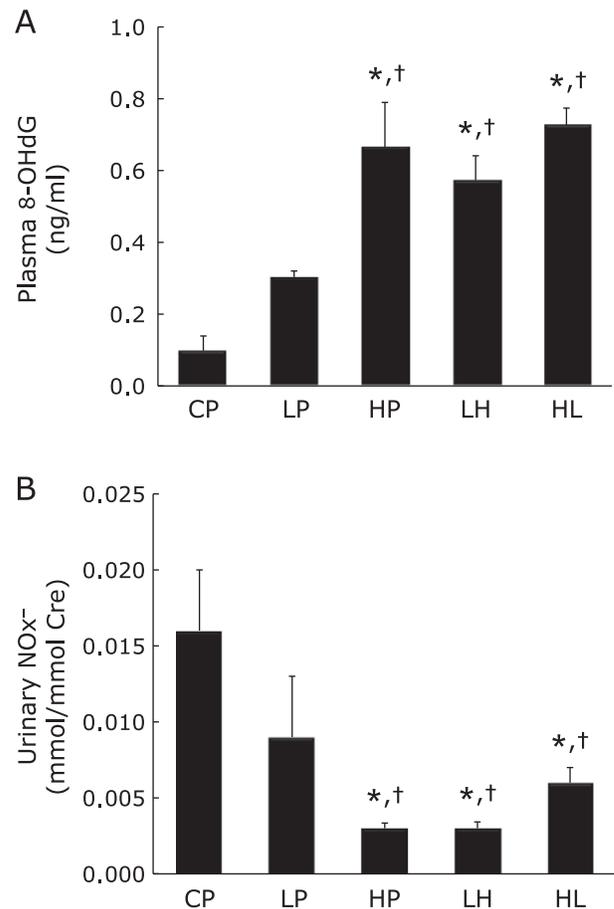
Fig. 5. Effect of continuous and alternating administration of high and low P diets on 8-OHdG (A) and urinary NOx$^-$ excretion (B). CP, control P diet group; LP, low P diet group; HP, high P diet group; LH, alternating low and high P diet group; HL, alternating high and low P diet group. Results are expressed as mean ± SEM (n = 4–5). *$p<0.05$ vs CP, [†]$p<0.05$ vs LP.
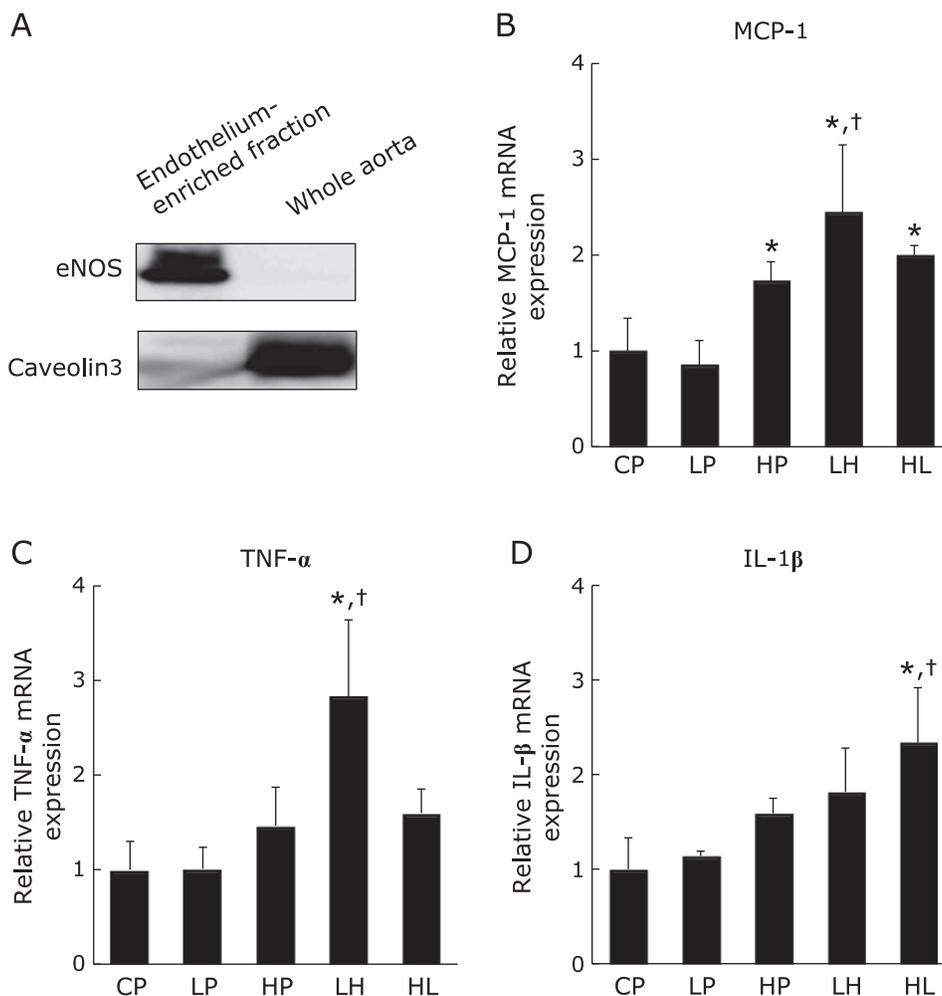
Fig. 6. Effects of continuous and alternating administration of high and low P diets on the expression of inflammatory chemokines and cytokines in the endothelium-enriched fraction of rat thoracic aorta. (A) Enriched expression of eNOS as an endothelium marker and decreased expression of caveolin3 as a marker of muscular cells in the endothelium-enriched fraction as compared with the whole aorta. (B–D) Effects of dietary intervention on mRNA expression of MCP-1 (B), TNF-α (C) and IL-1β (D) in the endothelium-enriched fraction. CP, control P diet group; LP, low P diet group; HP, high P diet group; LH, alternating low and high P diet group; HL, alternating high and low P diet group. Results are expressed as mean ± SEM (n = 4–5). *$p<0.05$ vs CP, †$p<0.05$ vs LP.

showed impaired vasorelaxation as compared with the CP group and LP group (Fig. 4B).

Effect of fluctuating plasma P level by changes in dietary P intake on oxidative stress and NO production. We previously reported that dietary P loading can increase oxidative stress and decrease NO production.[20,21] To investigate the effect of fluctuating plasma P levels on oxidative stress and the production of NO, we measured plasma 8-hydroxydeoxyguanosine (8-OHdG) as an oxidative stress marker, and urinary NOx as a marker of systemic NO production. Plasma 8-OHdG levels significantly increased in the HP, LH and HL groups as compared with the CP and LP groups (Fig. 5A). Urinary NOx excretion was significantly lower in the HP, LH and HL groups than in the CP and LP groups (Fig. 5B).

Increased expression of inflammatory factors in the endothelium-enriched fraction. The increase in oxidative stress and decrease in NO production and vasodilation suggested that endothelial cell damage must have occurred. We therefore hypothesized that an inflammatory response must be induced in the damaged endothelial cells. We prepared an endothelium-enriched fraction from the thoracic aorta as described previously.[27]

First, we checked the expression of eNOS, an endothelium-specific nitric oxide synthetic enzyme, and caveolin 3, a vascular smooth muscle cell-specific protein, by western blotting (Fig. 6A). Positive expression of eNOS was detected in the endothelium-enriched fraction, but not in the whole aorta. On the other hand, positive expression of caveolin 3 was detected in the whole aorta, but not in the endothelium-enriched fraction. These findings indicate that we had obtained the endothelium-enriched fraction from the thoracic aorta.

Next, to investigate the expression of inflammatory factors caused by fluctuation of plasma P level, we estimated the expression levels of MCP-1 (Fig. 6B), TNF-α (Fig. 6C) and IL-1 (Fig. 6D) using RNA isolated from the endothelium-enriched fraction of the thoracic aorta in each group. The mRNA expression levels of MCP-1 were significantly higher in the LH, HL and HP groups than in the CP and LP groups. In addition, although not quite reaching statistical significance, TNF-α and IL-1β mRNA expression were higher in the LH, HL and HP groups than in the CP and LP groups. Taken together, these results suggest that fluctuating plasma P levels increased the inflammatory response in the endothelium-enriched fraction of the thoracic aorta.
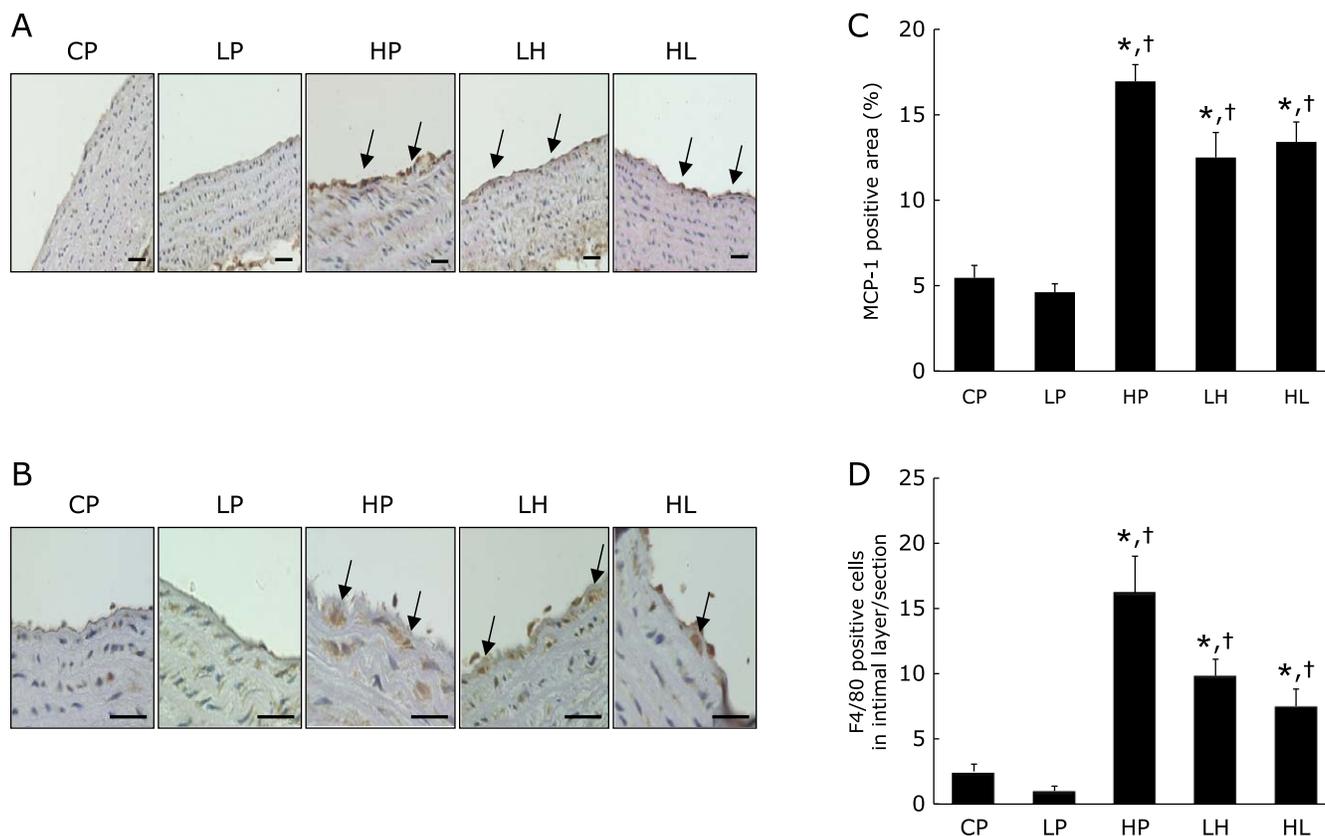
Fig. 7. Effects of continuous and alternating administration of high and low P diets on immunohistochemical staining of MCP-1 in the aortic arch and F4/80-positive cells in the intimal layer in the aortic arch. Representative immunohistochemical images of MCP-1 (A and C) and F4/80 (B and D) expression. CP, control P diet group; LP, low P diet group; HP, high P diet group; LH, alternating low and high P diet group; HL, alternating high and low P diet group. Arrows indicate MCP-1 (A) and F4/80 (B) positive areas in the intimal layer. Calibration bar indicates 50 μm (A and B), Quantification of MCP-1 positive area (C) and F4/80-positive cells (D) in the intimal layer of the aortic arch. The MCP-1-positive area and ratio of F4/80-positive cells in the intimal layer were quantified as percentages using imaging software. The area and cells were calculated in two serial sections per aorta. Results are expressed as mean ± SEM (*n* = 4–5). *$p < 0.05$ vs CP, †$p < 0.05$ vs LP.

Fluctuating plasma P levels by changes in dietary P intake enhanced monocyte adhesion in the intimal layer of the aortic arch. Endothelial dysfunction and increased MCP-1 expression in the endothelium suggested that macrophage infiltration might be increased in the HP, LH and HL groups. To investigate macrophage infiltration in the intimal layer, we performed immunohistochemical analysis in the aortic arch of each rat. The intensity of MCP-1-positive staining was higher in the HP, LH and HL groups than in the CP and LP groups (Fig. 7A); the number of F4/80-positive cells also increased (Fig. 7B). Next, we quantitated the positive area of MCP-1 expression and the number of macrophages in the intimal layer of the aortic arch (Fig. 7C and D). The results suggested that the MCP-1-positive area and the number of F4/80-positive cells significantly increased in the LH, HL and HP groups as compared with the CP and LP groups. Taken together, these results indicate that fluctuating plasma P levels might be involved in the inflammatory response in the intimal layer.

Fluctuating plasma P levels by changes in dietary P intake decreased NO production in the endothelium-enriched fraction. Finally, we attempted to elucidate the inhibitory mechanism underlying the impaired NO production and endothelial function caused by fluctuating plasma P levels by changes in dietary P intake. We previously reported that elevation of extracellular P levels can induce inhibitory phosphorylation at Thr[495] on eNOS in bovine aortic endothelial cells.[21] Thus, we hypothesized that fluctuation of plasma P might also modulate eNOS activity via phosphorylation of its activation/inactivation pathway. To address this, we investigated whether fluctuating P levels phosphorylated Thr[495] of eNOS. Rats in the LH, HL and HP groups showed significantly increased phosphorylation of eNOS at Thr[495] as compared with the CP and LP groups in the endothelium-enriched fraction (Fig. 8).

Discussion

The present study has indicated that fluctuation of plasma P due to dietary P intake alternating between low and high P can impair endothelial function in rats. In addition, although the total amount of P intake was similar among the HL, LH and CP groups, the administration of an alternating high and low P diet led to increased oxidative stress and inflammatory response, resulting in a deterioration in endothelium-dependent vasodilation, as observed in the HP group with continuous administration of the high P diet. Repetitive fluctuation of plasma P also increased plasma levels of 8-OHdG, which is well known as an oxidative stress marker of DNA damage, mRNA expression of inflammatory factors such as MCP-1 and TNF-α in the endothelium, and macrophage infiltration into the intimal layer. These results suggest that increased oxidative stress and inflammatory factors caused by fluctuation of plasma P levels due to alternating dietary P intake may suppress endothelial-dependent vasodilation.

These results have similarities to studies on diabetes mellitus that reported that repetitive fluctuation of postprandial hyperglycemia impaired endothelial function, although the fasting
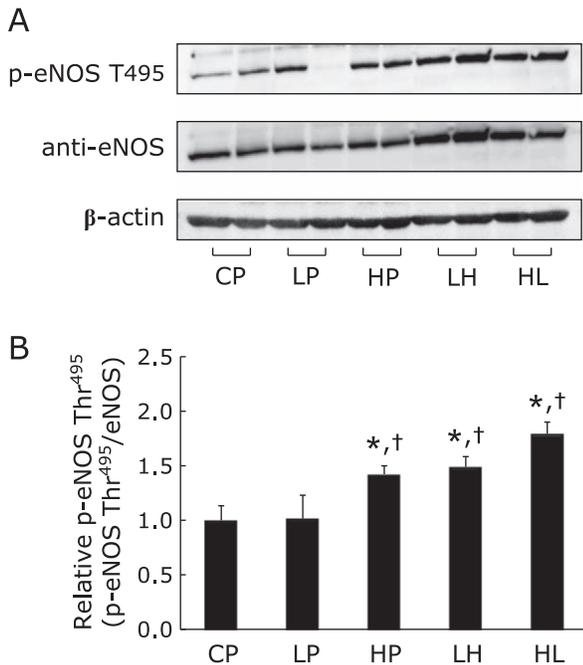
Fig. 8. Effects of continuous and alternating administration of high and low P diets on phosphorylation of eNOS at Thr^{495}. Immunoblot analysis of phosphorylated eNOS at Thr^{495} (p-eNOS T495), total eNOS (anti-eNOS) and β-actin. Representative blots from two separate experiments (A) and quantified analysis data (B) are shown. Data are expressed as mean ± SEM (n = 4–5). *$p < 0.05$ vs CP, †$p < 0.05$ vs LP.

blood glucose level remained within the normal range.[14–18] For example, Azuma et al.[18] demonstrated that a repetitive glucose spike induced by diets fed only for 1 h twice a day markedly promoted intimal thickening of the rat thoracic aorta as compared with stable hyperglycemia in a rat model of non-obese type 2 diabetes. Incidentally, in vitro studies demonstrated that intermittent high glucose loading increased intracellular oxidative stress and enhanced apoptosis more than constant high glucose medium in cultured human umbilical vein endothelial cells.[19] Thus, repetitive postprandial hyperglycemia is well known to play an important role in endothelial dysfunction and the development of atherosclerosis.

The present results suggest that plasma P spikes may be a factor in the development of cardiovascular disease by inducing endothelial dysfunction and inflammatory response, similar to glucose spikes. However, the deleterious effect of fluctuating plasma P on endothelial function has not been clarified as yet. In this study, we investigated the effect of repetitive hyperphosphatemia caused by alternative administration of high or low dietary P on endothelial function in rats. Because serum P levels are maintained within the normal range by various hormones, such as PTH, FGF23 and 1,25-(OH)$_2$D, consistent hyperphosphatemia rarely occurs in a healthy individual who has normal kidney function.[29,30] However, serum P levels do fluctuate due to circadian rhythms, even in healthy individuals.[23] In addition, recent clinical studies have shown that higher serum P levels within the normal range can be a risk factor for CVD in individuals with normal kidney function.[10,11] Our previous study reported that high P-loading increases ROS production through PKC and NAD(P)H oxidase in endothelial cells, and decreases NO production through phosphorylation of eNOS; in addition, postprandial P elevation was associated with percent flow-mediated dilation (%FMD) in young healthy men,[21] suggesting that dietary P loading or elevation of serum P impairs endothelial function. In this study,

continuous administration of high P diet also impaired endothelial function without a significant increase in plasma P levels. We measured plasma P levels in a fasting state at sacrifice. Thus, plasma P levels may be elevated during the postprandial period during continuous administration of the high P diet, and elevated plasma P levels may be involved in endothelial dysfunction.

Other possible factors would be PTH and FGF23. Both hormones were significantly increased in the HP group rats, presumably to maintain plasma P levels within the normal range. PTH is known to be associated with endothelial dysfunction. For example, Baykan et al.[31] observed a decrease in %FMD in primary hyperparathyroidism patients with low serum P levels and high serum Ca levels. Walker et al.[32] also reported that increases in carotid intra-media thickness and stiffness were observed in primary hyperparathyroidism patients. Therefore, increased plasma PTH may be involved in the endothelial dysfunction observed in our model. Recently, serum FGF23 was associated with the incidence of cardiovascular disease among CKD patients and directly induced left ventricular hypertrophy in a mouse model.[33] Because we did not investigate the direct effect of PTH and FGF23 on endothelial function in this study, we cannot elucidate the role of these hormones in the endothelial dysfunction induced by fluctuating P levels due to alternate administration of the HP and LP diet. Therefore, we cannot exclude the effect of those hormones on endothelial function at this moment.

Changes in plasma Ca and vitamin D level may be possible factors associating with the endothelial dysfunction in this study. Generally speaking, elevation of plasma P level causes decrease in plasma Ca level. In the present study, plasma Ca level was significantly changed in LP and HL groups, but not in HP and LH groups. Thus, changes in plasma Ca level may not be associated with endothelial dysfunction. Additionally, recent report demonstrated that insufficient 25-(OH)D levels may be a good marker of oxidative stress, inflammation, and endothelial dysfunction associated with the changes in serum P and Ca.[34] Generally, plasma 1, 25-(OH)$_2$D level reflects physiological response to changes in plasma Ca and P levels, but plasma 25-(OH)D level reflects nutritional status of vitamin D.[35] Therefore, we estimated plasma 1,25-(OH)$_2$D level, but not 25-(OH)D level. Vitamin D deficiency has been known as cardiovascular risk,[36,37] and is also associated with endothelial dysfunction in patients with type 2 diabetes.[38] On the other hand, Yiu et al.[39] reported that vitamin D supplementation did not ameliorated endothelial dysfunction, inflammation and oxidative stress in type 2 diabetes patients. Therefore, plasma 25-(OH)D level may be a good marker for endothelial dysfunction and oxidative stress, but it might be causal factor. We don't know whether changes in dietary P intake can affect plasma 25-(OH)D levels, in other words vitamin D status, through oxidative stress or other unidentified mechanisms directly or indirectly associating with the endothelial dysfunction. Further study will be needed to clarify the role of vitamin D status in the endothelial dysfunction.

In the clinical setting, dialysis causes fluctuation of serum P levels. In dialysis patients, not only serum P but also BUN fluctuates with dialysis. The postdialytic P level decreases by approximately 3.0 mg/dl as compared with the predialytic P level, and serum P gradually returns to the predialysis level after 48 h.[24] Thus, fluctuating serum P levels in dialysis patients or CKD patients may cause progression of CVD, which is a leading cause of death in CKD patients. Further investigation will be needed to elucidate the association of fluctuating serum P levels with endothelial function or incidence of CVD among CKD patients.

In conclusion, fluctuation of plasma P levels caused by alternating administration of a high P and low P diet can cause endothelial dysfunction through increased oxidative stress and inflammatory response. These results suggest that habitual large fluctuations of dietary P intake in healthy people and large fluctuations of serum P levels in dialysis patients might promote

the development and pathogenesis of atherosclerosis caused by endothelial dysfunction, and might be a risk factor for CVD. They indicate that not only the restriction of dietary P intake but also the suppression of fluctuating dietary P might be important in preventing CVD even in healthy people. These findings may also contribute to development of a nutritional approach for CKD patients and dialysis patients, in addition to guidance regarding appropriate intake of P.

Acknowledgments

Conflict of Interest

No potential conflicts of interest were disclosed.

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