



Calcium Overload Accelerates Phosphate-Induced Vascular Calcification Via Pit-1, but not the Calcium-Sensing Receptor

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Aim: Vascular calcification (VC) is a risk factor of cardiovascular and all-cause mortality in patients with chronic kidney disease (CKD). CKD–mineral and bone metabolism disorder is an important problem in patients with renal failure. Abnormal levels of serum phosphate and calcium affect CKD–mineral and bone metabolism disorder and contribute to bone disease, VC, and cardiovascular disease. Hypercalcemia is a contributing factor in progression of VC in patients with CKD. However, the mechanisms of how calcium promotes intracellular calcification are still unclear. This study aimed to examine the mechanisms underlying calcium-induced calcification in a rat aortic tissue culture model.

Methods: Aortic segments from 7-week-old male Sprague–Dawley rats were cultured in serum-supplemented medium for 10 days. We added high calcium (HiCa; calcium 3.0 mM) to high phosphate (HPi; phosphate 3.8 mM) medium to accelerate phosphate and calcium-induced VC. We used phosphonoformic acid and the calcimimetic R-568 to determine whether the mechanism of calcification involves Pit-1 or the calcium-sensing receptor.

Results: Medial VC was significantly augmented by HPi + HiCa medium compared with HPi alone (300%, $p < 0.05$), and was associated with upregulation of Pit-1 protein. Pit-1 protein concentrations in HPi + HiCa medium were greater than those in HPi medium. Phosphonoformic acid completely negated the augmentation of medial VC induced by HPi + HiCa. R-568 had no additive direct effect on medial VC.

Conclusion: These results indicated that exposure to HPi + HiCa accelerates medial VC, and this is mediated through Pit-1, not the calcium-sensing receptor.

Key words: Calcium, Phosphate, Mönckeberg's arteriosclerosis, Pit-1, Calcium-sensing receptor

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Introduction

Vascular calcification (VC) is common in patients with end-stage renal disease and is thought to contribute to increased cardiovascular and all-cause mortality in chronic kidney disease (CKD)^{1,2}. One of the characteristic types of calcification of uremic artery disease is Mönckeberg's arteriosclerosis. In Mönckeberg's arteriosclerosis, also called arterial medial calcification, calcium deposits are found in the muscular middle layer of arteries. Arterial medial calcification has been

observed in young and middle-aged patients without conventional atherosclerotic risk factors³. However, little is known about the adjustment processes of arterial medial calcification.

Hyperphosphatemia is prevalent in patients with CKD and it is linked to an increased risk of cardiovascular mortality in these patients⁴. Phosphate is arguably the most important biochemical parameter of CKD–mineral and bone metabolism disorder and an important inducer of VC. Phosphate levels above physiological serum levels have been shown to promote VC in experimental models *in vivo*⁵ and *in vitro*^{6,7}. Phosphate that is transported into cells is primarily mediated by sodium-dependent phosphate cotransporters. Phosphate uptake through Pit-1, a type III sodium-dependent phosphate cotransporter, is essential for calcification of vascular smooth muscle cells (VSMCs)^{8,9}.

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We previously reported that phosphate uptake through Pit-1 leads to induction of apoptosis and subsequent calcification of VSMCs in a rat aortic tissue culture model^{6, 7}. Serum phosphate control is an important component in patients with CKD.

In the clinical setting, serum calcium and phosphate are carefully controlled in patients on hemodialysis. Epidemiological studies have shown a direct correlation between serum phosphate and the calcium-phosphate product ($\text{Ca} \times \text{Pi}$) and VC in patients with end-stage renal disease^{10, 11}. Furthermore, a previous study showed that serum calcium is a risk factor associated with all-cause mortality⁵. Serum calcium levels are regulated by the action of parathyroid hormone (PTH). Major drivers of PTH hypersecretion and parathyroid cell proliferation are hypocalcemia and hyperphosphatemia. Hypocalcemia and hyperphosphatemia develop in patients with CKD and secondary hyperparathyroidism as a result of low calcitriol levels and decreased kidney function. Management of patients receiving dialysis requires control of secondary hyperparathyroidism and associated extrasosseous calcification.

Arterial medial calcification is associated with increased pulse pressure and arterial stiffness¹². In the clinical setting, the coronary artery calcium score, the cardio-ankle vascular index, and pulse wave velocity are screening tools to detect coronary artery calcification and arterial sclerosis^{13, 14}. Clinical factors associated with arterial stenosis (AS) are similar to risk factors for VC¹⁵. The $\text{Ca} \times \text{Pi}$ product is correlated with the severity of AS¹⁶. On the basis of these factors, treatment of hypercalcemia has a beneficial effect on VC and AS. However, the mechanisms underlying calcium-induced calcification are unclear.

Recently, the use of calcimimetics has become a major treatment of secondary hyperthyroidism in patients on dialysis. Calcimimetics increase the sensitivity of the calcium-sensing receptor (CaSR), thereby controlling PTH oversecretion without inducing hypercalcemia. This observation led to the hypothesis that the beneficial effect of calcimimetics on serum biochemistry contributes to slowing the progression of VC. However, whether calcimimetics also modify the progression of atherosclerosis, and whether they act only indirectly via a decrease in serum PTH, calcium, and phosphate levels, or also by a direct effect on vascular CaSRs, remain unclear.

Aim

This study aimed to examine the mechanisms underlying calcium-induced calcification in a rat aortic tissue culture model. We used phosphonoformic

acid (PFA) and the calcimimetic R-568 to determine whether the mechanism of calcification involves Pit-1 or CaSR.

Methods

Experimental Animals

We purchased 7-week-old male Sprague–Dawley rats from Kiwa Laboratory Animals Co. (Wakayama, Japan), and maintained them under specific pathogen-free conditions with a 12-h light/dark cycle. After several days (2–7 days) of acclimatization, all of the rats were sacrificed to obtain aortic tissue to culture as described below. All experimental procedures were approved by the Animal Care and Use Committee of Wakayama Medical University and conformed to NIH guidelines for the care and use of laboratory animals.

Aortic Tissue Culture

Cell cultures have many problems, such as cell transformation and lack of an extracellular matrix. Therefore, we used rat aortic tissue culture, which has the structure and matrix of a vessel, and is closer to the *in vivo* situation than cell cultures. Thoracic aortas below the arch to the diaphragm were removed from 7–8-week-old male Sprague–Dawley rats. After carefully removing the connective tissue, the aortas were cut into several 3–5-mm rings and placed in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, Waltham, MA), supplemented with 10% fetal bovine serum, 1% streptomycin, and penicillin. Aortic segments were maintained at 37°C in a 5% CO₂ incubator, and the medium was changed every other day. DMEM contained 1.8 mM Ca²⁺, 0.9 mM PO₄³⁻, with a pH of 7.2. NaH₂PO₄, Na₂HPO₄, and CaCl₂ were added to the serum-supplemented DMEM to create various phosphate and calcium concentrations. Aortic rings were cultured in DMEM with normal phosphate concentrations (CON; phosphate 0.9 mM) or high phosphate concentrations (HPi; phosphate 3.8 mM). Aortic rings in CON and HPi conditions were also cultured with normal calcium concentrations (NCa; calcium 1.8 mM) or high calcium concentrations (HiCa; calcium 3.0 mM) for 10 days, and were visualized by von Kossa stain. We added 1.0 mM PFA (Sigma-Aldrich, Japan, Tokyo, Japan) to the medium to determine whether calcium induced medial calcification with mediation through Pit-1. PFA is a specific inhibitor of sodium-dependent phosphate cotransporters. R-568 was provided by Kyowa-Hakko-Kirin Inc., Tokyo, Japan. R-568 (calcimimetic) concentrations in the medium were adjusted to 0, 10, 100, and 1,000 ng/ml. R-568 (N-(3-[2-chlorophenyl]propyl)-(R)- α -methyl-3-methoxybenzylamine) potentiates the

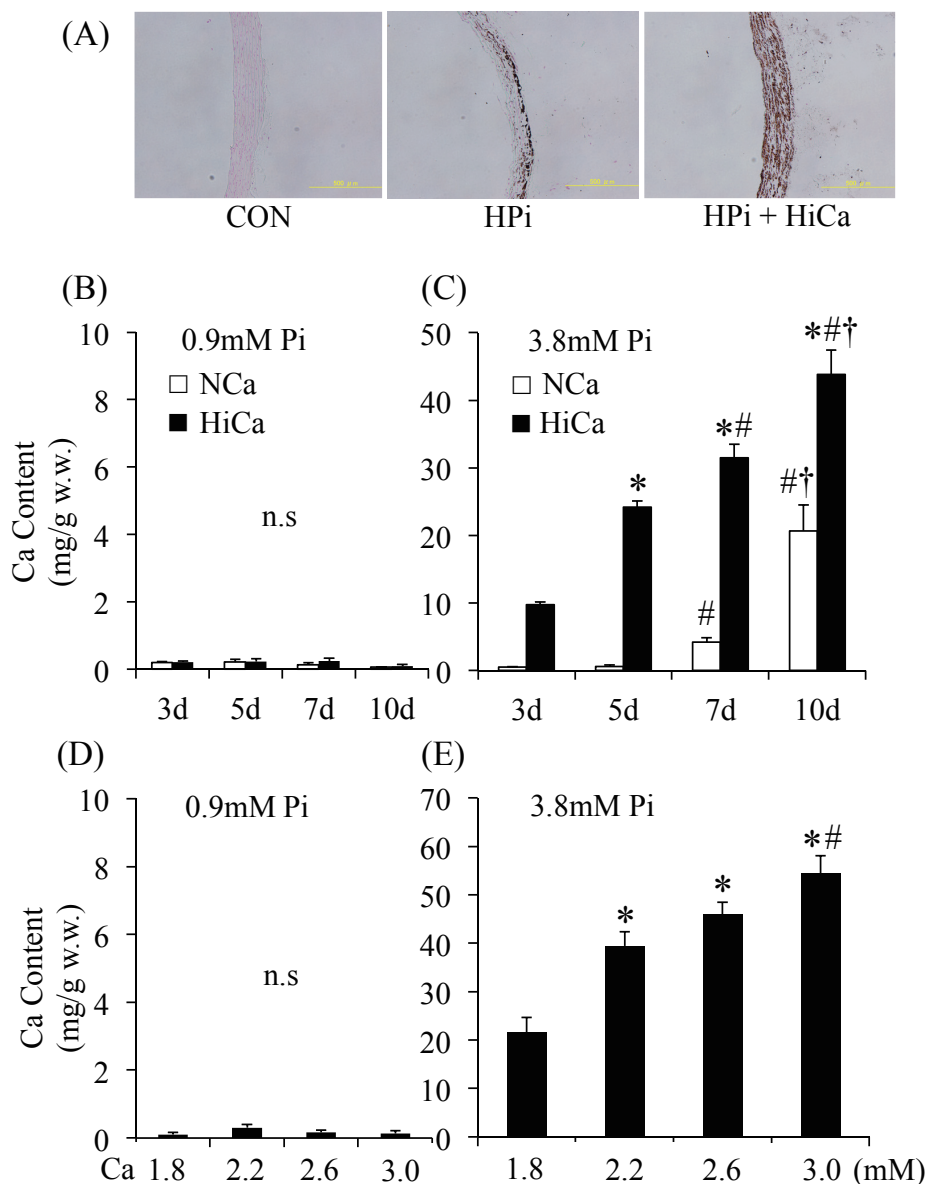


Fig. 1. von Kossa staining of aortic rings. Sections show no staining when cultured in a medium with normal concentrations of Pi (0.9 mM, left panel). Sections show moderate calcification in HPi (3.8 mM, center panel) and diffuse severe calcification in HPi + HiCa (Pi 3.8 mM + Ca 3.0 mM; right panel) (A). Calcium content of aortic rings cultured with (B) normal concentrations of inorganic phosphate (CON; 0.9 mM) or (C) high Pi (HPi; 3.8 mM). Time course of calcium accumulation during 10 days of *ex vivo* incubation with normal calcium medium (NCa; 1.8 mM) and with high Ca medium (HiCa; 3.0 mM). Results are the average of at least 10 rings and represent an independent experiment ($n=15-20$ rings obtained from 10 rats). * $p < 0.0001$ compared with day 3; # $p < 0.001$ compared with day 5; † $p < 0.05$ compared with day 7. Dose-response relationship between aortic calcium content and calcium concentration of the medium after 10 days of *ex vivo* culture with (D) normal concentrations of inorganic phosphate (CON; 0.9 mM) or (E) high Pi (HPi; 3.8 mM). Results are the average of at least 10 rings and represent an independent experiment ($n=15-20$ rings obtained from 10 rats). * $p < 0.01$ compared with 1.8 mM; # $p < 0.05$ compared with 2.2 mM.

effects of extracellular calcium on parathyroid calcium receptors and inhibits PTH secretion. Aortic segments were cultured at different time intervals according to our study requirements. After completing the culture procedure, the aortic segments were washed three

times in Hank's Balanced Salt Solution (-) and were divided for quantification and tissue analysis.

Quantification of Calcification

Aortic segments were weighed and decalcified

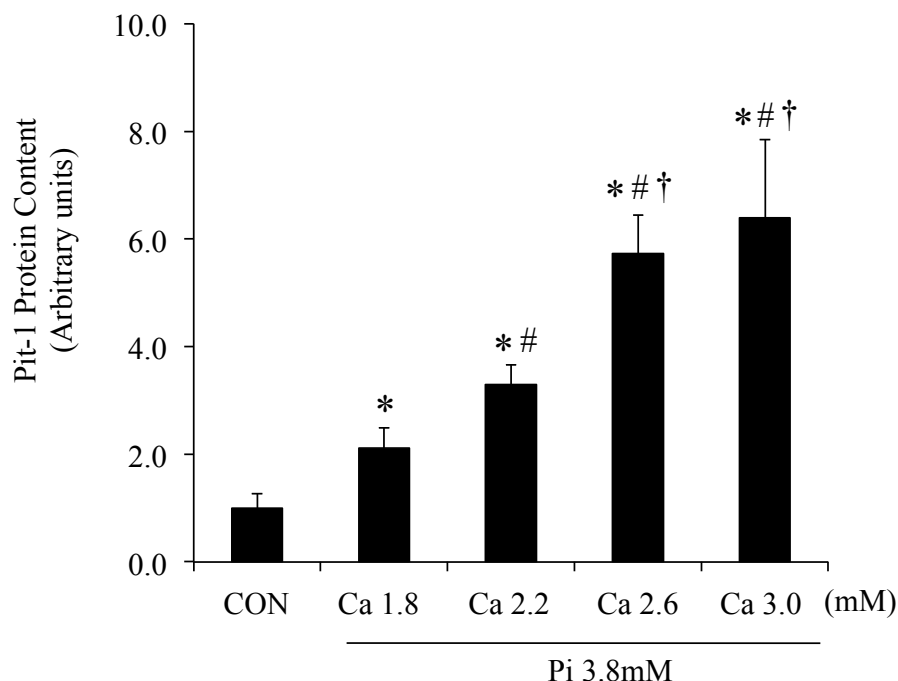


Fig. 2. Protein expression of Pit-1 in aortic rings cultured in CON and HPI with some Ca dose (Ca 1.8 mM, 2.2 mM, 2.6 mM, 3.0 mM) medium for 10 days. Pit-1 protein levels were analyzed using western blotting. Pit-1 protein levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Results are the average of at least 12 rings and represent an independent experiment ($n=12$ rings obtained from six rats). * $p<0.05$ compared with CON; # $p<0.05$ compared with Ca 1.8 mM; † $p<0.05$ compared with Ca 2.2 mM. CON, normal concentrations of inorganic phosphate (0.9 mM).

with 10% formic acid for 24 h at room temperature. The calcium content of the supernatant was determined by the methylxylene blue method using the Wako Calcium E-Test (Wako Pure Chemical Industries, Osaka, Japan). Results were corrected by wet tissue weight and expressed as mg/g wet weight of tissue.

Western Blot Analysis

After protein extraction, protein concentrations were quantified using a commercial reagent (BCA; Pierce, Biotechnology, Inc., Rockford, IL), which was normalized to 0.5 or 1 $\mu\text{g}/\mu\text{L}$ for all samples. A total of 10 μg protein was applied to each lane of a 15% polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride membrane using the Ezblot (AE-1460; Atto, Tokyo, Japan) reagent and a semi-dry blotting unit (WSE-4110 Powered BLOT One; Atto) according to the instruction manual. After transfer, the blots were blocked with a commercial blocking reagent (PVDF Blocking Reagent for Can Get Signal®; Toyobo, Osaka, Japan) for 1 h at room temperature. After being washed in Tris-buffered saline (50-mM Tris, 150-mM NaCl, pH 7.6) with 0.1% Tween

20 (TBST), the blots were cultured with primary antibodies diluted in immunoreaction enhancer solution (Can Get Signal Solution 1, Toyobo) overnight at 4°C. Primary antibodies used in this study were rabbit polyclonal anti-GAPDH (diluted 1:1500, sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-Pit-1 (diluted 1:1000, sc-98814; Santa Cruz Biotechnology). The membrane was washed in TBST and then reacted with horseradish peroxidase-conjugated donkey anti-rabbit IgG (A50-201p; Bethyl Laboratories, Inc., Montgomery, TX), which was diluted in immunoreaction enhancer solution (1:10000, Can Get Signal Solution 2; Toyobo) for 1 h at room temperature. The membrane was then washed again in TBST and processed with an enhanced chemiluminescence procedure (ECL Prime Western Blotting Detection Reagent; GE Healthcare, Japan, Tokyo, Japan). The enhanced chemiluminescence signals on the immunoblots were detected using the WSE-6100 LuminoGraph and quantified using the CS Analyzer version 1.0.2 (Atto).

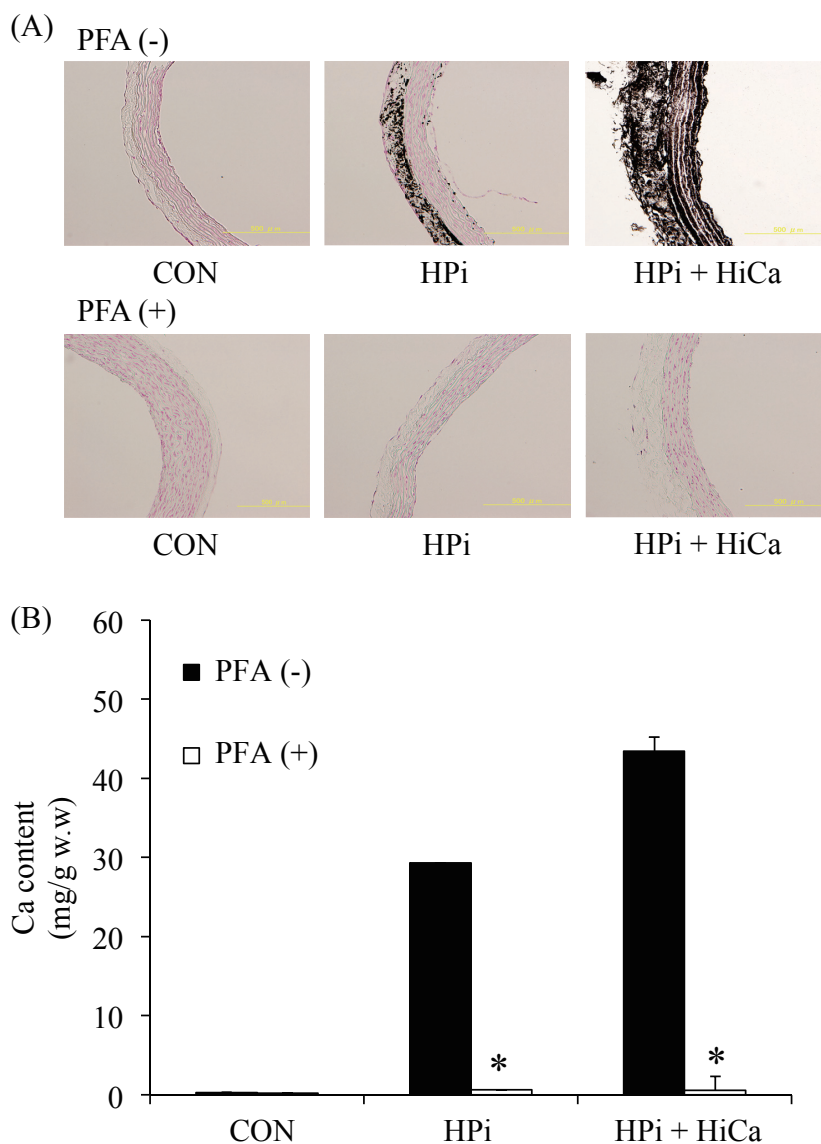


Fig. 3. von Kossa staining of aortic rings in various types of medium with/without PFA for 10 days. Culture without PFA is shown in the upper panels and culture with PFA is shown in the lower panels. Sections without PFA show no staining when cultured in a medium with normal concentrations of Pi (0.9 mM, left panel). However, moderate calcification was observed in HPi (3.8 mM, center panel) and diffuse severe calcification was observed in HPi + HiCa (Pi 3.8-mM + Ca 3.0 mM; right panel). Sections with PFA show no staining when cultured in all types of medium (A). Calcium content of cultured aortic rings in various types of medium with/without PFA for 10 days. * $p < 0.01$, calcium content cultured with PFA was significantly lower than that without PFA in the same condition. Results are the averages of at least 15 rings and represent an independent experiment ($n = 15$ rings obtained from eight rats) (B). PFA, phosphonoformic acid.

Statistical Analysis

Data are shown as mean \pm standard error (SE). The data were primarily analyzed with Bartlett's test (JMP version 9.0; SAS Institute Inc., Cary, NC) for equality of variance. When homoscedasticity was not confirmed by Levene's test, the Kruskal–Wallis test was applied to the data. On the basis of results of the Kruskal–Wallis test, Steel–Dwass multiple comparisons were further performed to compare the differ-

ences among the four conditions. A p value < 0.05 was considered to be statistically significant.

Results

Time Course

Histological images showed severe medial VC in aortic rings that were exposed to HPi + HiCa compared with those cultured in CON and HPi. Histol-

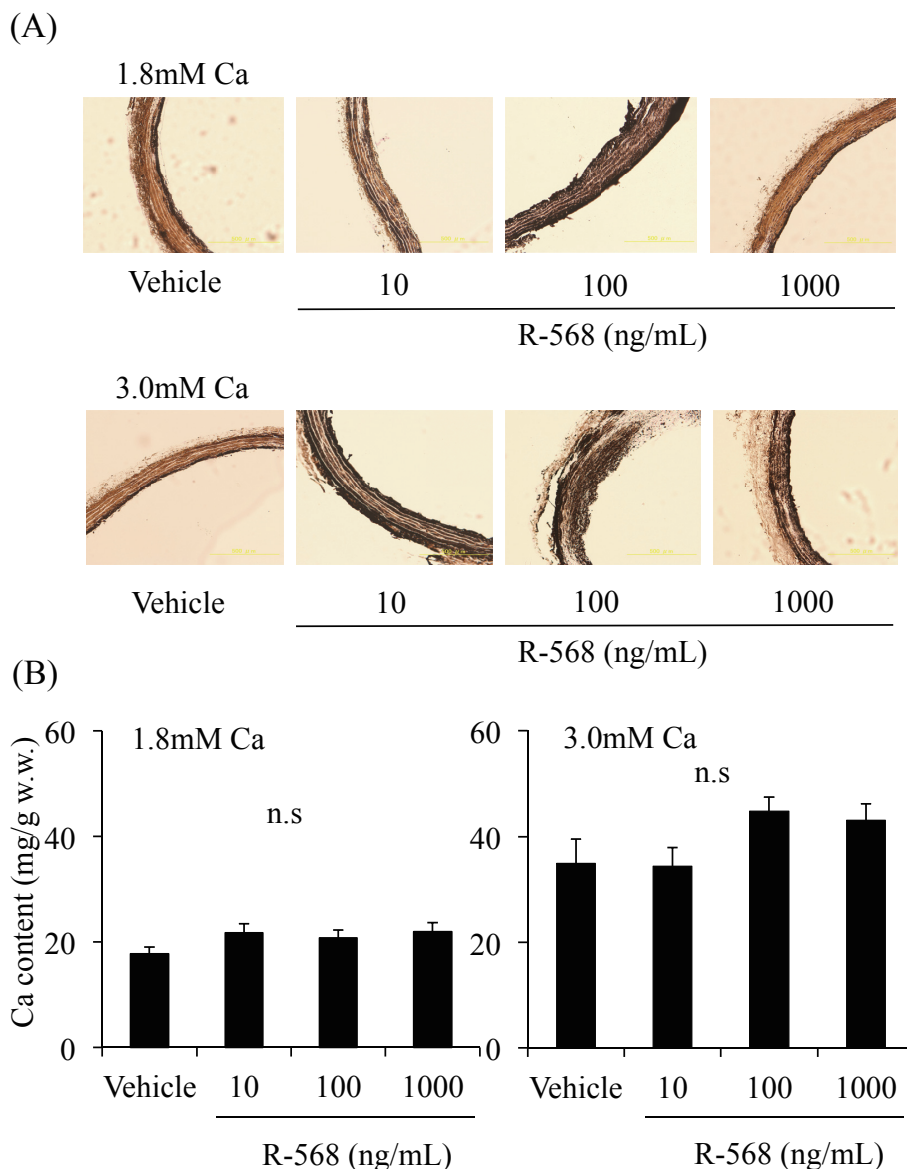


Fig. 4. von Kossa staining of aortic rings cultured in normal Ca (1.8 mM) or high Ca (3.0 mM) under HPi (Pi 3.8 mM) with or without R-568. Culture in normal Ca (1.8 mM) under HPi (Pi 3.8 mM) is shown in the upper panels. Culture in high Ca (3.0 mM) under HPi (Pi 3.8 mM) is shown in the lower panels. Sections show diffuse severe calcification when cultured in all types of medium (A). Calcium content of aortic rings cultured in normal Ca (1.8 mM) or high Ca (3.0 mM) under HPi (Pi 3.8 mM) with or without R-568. Results are the average of at least 14 rings and represent an independent experiment ($n = 14$ rings obtained from eight rats) (B).

ogy showed moderate calcification in culture with HPi and diffuse severe calcification in culture with HPi + HiCa (**Fig. 1A**). The time course of calcium accumulation during 10 days of *ex vivo* culture was quantified as aortic calcium content (**Fig. 1B, C**). There was a time course effect on calcium accumulation in the HiCa and NCa conditions. Calcium content was not increased in aortic rings that were cultured in medium with CON, regardless of HiCa during 10 days (**Fig. 1B**). In medium with HPi, calcium content was

gradually increased in a time-dependent manner (**Fig. 1C**). HiCa caused higher calcification than NCa at each time point ($p < 0.05$).

Dose-Dependent Effect of Calcium Concentrations

After 10 days of *ex vivo* culture, calcification was quantified as aortic calcium content (**Fig. 1D, E**). Calcium content was not increased in aortic rings that were cultured in medium with CON (**Fig. 1D**). In medium with HPi, calcium content gradually increased

in a dose-dependent manner (**Fig. 1E**).

Changes in Pit-1 Protein Concentrations

The amount of Pit-1 protein in aortic rings that were cultured in Pi 3.8 mM+Ca 1.8 mM medium was approximately two-fold greater than in those that were cultured in CON ($p < 0.05$). In addition, Pit-1 protein concentrations in Pi 3.8 mM+Ca 3.0 mM medium were approximately three-fold greater than those in Pi 3.8 mM+Ca 1.8 mM ($p < 0.05$, **Fig. 2**). The amount of Pit-1 protein had increased to calcium concentration of medium dependency in Pi 3.8 mM medium conditions.

Role of Pit-1 in Medial VC

We used PFA to examine if Pit-1 is involved in the mechanism of calcification induced by HiCa and/or HPi. Tissue was visualized by von Kossa stain. Histology showed medial VC with exposure to HPi and HPi + HiCa. PFA treatment markedly reduced medial VC in the HPi and HPi + HiCa groups (**Fig. 3A**). PFA treatment markedly reduced aortic calcium content in the HPi and HPi + HiCa groups ($p < 0.01$) compared with culture without PFA in the same conditions after 10 days of culture (**Fig. 3B**).

Effect of Calcimimetics in Medial VC

To examine the direct effect of calcimimetics on calcification of aortic rings, we added calcimimetics to the medium, and visualized tissue by von Kossa stain. Histology showed medial VC with exposure to Ca 1.8 mM and Ca 3.0 mM a under HPi conditions. R-568 had no additive effect on medial calcification (**Fig. 4A, B**).

Discussion

Once believed to be a passive process, the mechanism of VC is a complex of $\text{Ca} \times \text{Pi}$ that is deposited in the vascular wall. However, currently, VC is recognized as not merely being generated by a passive process of deposition^{17, 18}. Numerous studies have shown that VC is an active, highly regulated, cellular process, mainly mediated by VSMCs^{19, 20}. Among the major events involved in promotion of VC, an increase in extracellular calcium and phosphate levels leads to the transition of VSMCs from a contractile to an osteochondrogenic phenotype, with formation of a procalcifying matrix and vesicles able to nucleate hydroxyapatite²¹. VC is currently recognized as a cell-regulated process caused by loss of calcification inhibitors and involving osteoblast-like changes in vascular-cell gene expression patterns and matrix development²². In a clinical study, we previously reported that serum cal-

cium concentrations were significantly higher in the aortic calcification area index high group than in the aortic calcification area index low group in patients on hemodialysis². In the present study, we showed promotion of arterial medial calcification with addition of calcium under HPi by tissue culture. Calcification of aortic rings was enhanced not only by HiCa but also with HPi + HiCa. In a cell culture model of VC, calcium-induced calcification was inhibited by PFA, and thus depended on the activity of Pit-1 or induced expression of Pit-1, as shown by measuring Pit-1 mRNA levels²³. In our study, extracellular calcium-induced aortic tissue calcification in the presence of HPi. We also showed that Pit-1 expression increased in association with calcium concentrations under HPi by measuring expression of Pit-1 protein. This study is the first report to show increased expression of Pit-1 protein in tissue culture, indicating that calcium is able to promote calcification with HPi. In HPi + HiCa, calcification was markedly reduced using PFA. This result suggests that Pit-1 is a major factor of VC with calcium-induced calcification.

Hypercalcemia is significantly related to an increased risk of mortality²⁴. Phosphate is thought to be greatly involved in ectopic calcification and mortality. Moreover, calcium should also be considered in this process. Arterial medial calcification is closely associated with the duration of hemodialysis and calcium-containing phosphate binders, including oral doses of elemental calcium prescribed as a phosphate binder⁵. Calcium-based phosphate binders are inexpensive, effective, and the most widely used for control of secondary hyperparathyroidism; however, there is increasing concern about their association with hypercalcemia. Disturbances in mineral metabolism, such as hyperphosphatemia and hypercalcemia, contribute to progression of calcification. Compared to sevelamer, a non-calcium-containing phosphate binder, calcium-containing phosphate binders are more likely to cause hypercalcemia and progressive coronary and aortic calcification in patients on hemodialysis²⁵. Treatment with calcium-containing phosphate binders significantly decreases the survival rate compared with the use of sevelamer²⁶. Treatment with phosphate binders is independently associated with improved survival in incident hemodialysis patients, but there is not good efficacy in patients with serum calcium concentrations greater than 2.25 mmol/L²⁷. These reports suggest the importance of calcium levels and calcium component medicines in lowering phosphate levels. Serum calcium concentrations need to be kept as low as possible, even if maintained within the target value, to improve the risk of mortality in patients with CKD.

Calcimimetics are allosteric activators of CaSRs

and improve upregulation of CaSRs to extracellular calcium in parathyroid cells. In our study, we added R568 to the medium and examined calcification in a rat aortic tissue culture model. VC was not reduced with exposure to R-568. Calcium-induced calcification might not be caused through CaSR signaling. Henaut *et al.* reported that calcimimetics reduced calcification in human VSMCs²⁸⁾, which is in contrast to our finding of no reduction in calcification with R-568. Smajilovic *et al.* reported that CaSR is present in vascular intima, but not in vascular media, using a rat aortic tissue culture model²⁹⁾. Moreover, Klein *et al.* reported that CaSR is present in vascular intima and adventitia, but not in the media of the sheep aorta³⁰⁾. Therefore, this difference in finding between Henaut *et al.*'s study²⁸⁾ and our study might be because of the differences between cell and tissue culture.

There are some limitations in this study. First, we used a rat aortic tissue culture model, which is more similar to the structure and matrix *in vivo*. We found that cellular uptake of phosphate and calcium through Pit-1 involved medial VC. Calcification in this model is histologically similar to that observed in uremic human and rat vessels, but is not exactly the same condition as in *in vivo*.

Second, we found that expression of Pit-1 protein was increased in aortic tissue calcification, but we did not measure phosphate uptake in this model. Our results might not be sufficient to determine whether HiCa under HPi conditions is caused by an increase in intracellular phosphate in response to higher Pit-1 protein levels. We might be able to detect phosphate uptake in this model or other culture conditions. Therefore, further studies on this issue are required.

Conclusion

Our study shows that the amount of Pit-1 protein in aortic rings that are cultured with HPi + HiCa is greater than that in those cultured in only HPi. Our results indicate that exposure to HPi + HiCa accelerates aortic medial calcification through Pit-1, but not CaSR.

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