



Protein kinase C regulates vascular calcification via cytoskeleton reorganization and osteogenic signaling



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ABSTRACT

Vascular calcification is an active cell-mediated process that reduces elasticity of blood vessels and increases blood pressure. Until now, the molecular basis of vascular calcification has not been fully understood. We previously reported that microtubule disturbances mediate vascular calcification. Here, we found that protein kinase C (PKC) signaling acted as a novel coordinator between cytoskeletal changes and hyperphosphatemia-induced vascular calcification. Phosphorylation and expression of both PKC α and PKC δ decreased during inorganic phosphate (Pi)-induced vascular smooth muscle cell (VSMC) calcification. Knockdown of PKC isoforms by short interfering RNA as well as PKC inactivation by Go6976 or rottlerin treatment revealed that specific inhibition of PKC α and PKC δ accelerated Pi-induced calcification both in VSMCs and ex vivo aorta culture through upregulation of osteogenic signaling. Additionally, inhibition of PKC α and PKC δ induced disassembly of microtubule and actin, respectively. In summary, our results indicate that cytoskeleton perturbation via PKC α and PKC δ inactivation potentiates vascular calcification through osteogenic signal induction.

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1. Introduction

Phosphorus, mainly in the form of inorganic phosphate (Pi), makes an important contribution to cellular structure, metabolism, and homeostasis [1], and it is a key regulator of vascular calcification [2]. Hyperphosphatemia leads to vascular calcification, cardiac dysrhythmia, renal failure, and, eventually death [3]. VSMCs generally reside in the tunica media of blood vessel walls in a differentiated state. Their primary function is contractile activity, which is essential for the regulation and maintenance of blood pressure and blood flow [4]. Thus, vascular smooth muscle cells (VSMCs) are central to the process of vascular calcification, which reduces elasticity of blood vessels and increases blood pressure.

Diabetes, hypertension, hyperparathyroidism, and osteoporosis are all known risk factors for vascular calcification [5,6]. Many other factors have been reported to be involved in the process of vascular calcification, including matrix Gla protein, fetuin A, osteoprotegerin, and Klotho as inhibitory factors of vascular calcification as well as BMP2, RANKL, Runx2, reactive oxygen species, and vitamin D as stimulators of calcification [7–9]. It has been also

reported that activation of nuclear factor-kappa B pathway promoted Pi-induced vascular calcification [10] and sirtuin 1, a histone deacetylase, prevented arterial calcification [11].

Filamentous actin (F-actin) and microtubules form highly dynamic cytoskeletal systems that underlie fundamental cellular processes such as cellular morphogenesis, cell motility, cell division, and intracellular transport of organelles [12]. Actin and microtubule cytoskeletons undergo dynamic reorganization in response to various stimuli such as epidermal growth factor, thrombin, and phorbol esters, and this reorganization plays key roles in regulation of these cellular processes [13–15]. We previously reported that the cytoskeleton is involved in the process of vascular calcification by showing that microtubule stabilization reduces calcification [16]. In the present study, we explored protein kinase C (PKC) as a new target molecule capable of regulating Pi-mediated vascular calcification and showed that the PKC/cytoskeleton/osteogenic signaling axis plays a central role in vascular calcification.

2. Materials and methods

2.1. Cell and aorta organ culture

Primary VSMCs were prepared from aortas of 6-week-old male C57BL/6J mice (Central Lab Animals, Seoul, Korea) by following the

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explant method as described previously [16,17]. Briefly, aortas were removed from euthanized mice, cut into small pieces rapidly, and stripped of their adventitia. Thereafter, aortas were placed on 100-mm tissue culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 20% fetal bovine serum (FBS, Hyclone) and antibiotics under a humidified atmosphere of 5% CO₂ at 37 °C. VSMCs grew out from explants starting 10 days after aortas were placed on dishes. Once cells reached confluency, they were isolated by exposure to trypsin, maintained in DMEM supplemented with 10% FBS and antibiotics, and used for experiments at passages 4–6. For ex vivo aorta culture, entire aortas isolated from mice were cultured in DMEM supplemented with 10% FBS and antibiotics. Go6976 and rottlerin were obtained from Sigma. All other materials used were of analytical reagent grade. Animal experiments were approved by the Institutional Review Board of Yeungnam University College of Medicine and were in accordance with the Care and Use of Laboratory Animals.

2.2. Induction, detection, and quantification of calcification in vitro and ex vivo

For induction of calcification in vitro, VSMCs were incubated in calcification medium (DMEM supplemented with 10% FBS plus an additional 3 mM Pi in the form of NaH₂PO₄, pH 7.4) for 8 days or the indicated times. The medium was changed every other day. To visualize calcified cells, cells fixed with 70% ethanol for 1 h were stained with 2% alizarin red S (pH 4.2). After washing three times with water, cells were photographed. For quantification of VSMC calcification, deposited calcium was incubated with 0.6 M HCl overnight and determined colorimetrically using a QuantiChrome Calcium Assay Kit (BioAssay Systems). DC protein assay (Bio-Rad) was used to determine protein concentration, and calcium content was normalized to the total cellular protein content. To induce calcification ex vivo, entire aortas were cultured with calcification medium for 2 weeks. For detection of calcium, calcified aortas were visualized using Von Kossa staining. After fixing with 3.7% formaldehyde, aortas were rinsed with phosphate-buffered saline (PBS) and incubated in 5% silver nitrate. Aortas were dried, weighed, and cut into small pieces, from which calcium was extracted by incubation with 0.6 M HCl for 24 h. Aortic calcium content was quantified as described above and was normalized on the basis of tissue dry weight.

2.3. RNA interference

For RNA interference, control or PKC isoform-specific (PKC α , PKC β , PKC δ , or PKC θ) small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology. VSMCs cultured overnight to 60% confluency in DMEM supplemented with 10% FBS were transfected for 2 days with each siRNA at a final concentration of 60 nM using Lipofectamine 2000 reagent (Invitrogen). The cells were then subjected to quantitative RT-PCR analysis or treated with Pi as indicated.

2.4. Quantitative RT-PCR

For real-time quantitative RT-PCR analysis, total RNA was isolated from cells using Trizol reagent (Invitrogen), after which 2 μ g of RNA was subjected to reverse transcription (RT) followed by real-time polymerase chain reaction (PCR) analysis using SYBR Premix Ex Taq (Takara Bio) and an Applied Biosystems 7500 Sequence Detection System. PCR primers were synthesized by Bionics (Seoul, Korea), and their sequences are listed in [Supplementary Table 1](#). To determine relative changes in gene expression, the comparative threshold cycle method was used with glyceralde-

hyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control.

2.5. Immunoblot analyses

For immunoblot analysis, cells were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, and protease inhibitors (Complete tablets, Roche Molecular Biochemicals). The suspension was centrifuged at 10,000 $\times$ g for 10 min at 4 °C, and the protein concentration of the resulting supernatant was determined with the use of a DC protein assay (Bio-Rad). The separated proteins by SDS-polyacrylamide gel electrophoresis were transferred to a nitrocellulose membrane and exposed to primary antibodies for phosphorylated or total forms of PKC α or PKC δ (Cell Signaling Technology), or β -actin as a loading control (Santa Cruz Biotechnology). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Abfrontier).

2.6. In vivo assays of actin and tubulin

To determine relative amounts of F-actin and globular actin (G-actin), we used an F-actin/G-actin in vivo assay kit (Cytoskeleton) as described previously [16]. Briefly, cells were passed through a 26.5-gauge syringe in lysis/F-actin stabilization buffer, after which F-actin and G-actin were separated by ultracentrifugation at 100,000 $\times$ g for 1 h at 37 °C. Equal amounts of F-actin and G-actin fractions were subjected to immunoblotting analysis using antibody against actin. To analyze microtubule dynamics, the ratio of polymerized to free tubulin was determined by a microtubule/tubulin in vivo assay kit (Cytoskeleton). In brief, VSMCs homogenized in lysis/microtubule stabilization buffer were centrifuged at 100,000 $\times$ g for 30 min at 37 °C. The pellet containing polymerized tubulin was suspended in 200 μ M CaCl₂ to the same volume as the supernatant containing free tubulin for 1 h on ice with frequent vortexing. Equal proportions of the polymerized and free tubulin fractions were then subjected to immunoblot analysis with antibody against tubulin. The F-actin/G-actin ratio and polymerized/free tubulin ratio were determined using densitometric analysis software (Image-Pro Plus version 6.0, Media Cybernetics).

2.7. Statistical analysis

Quantitative results are expressed as means $\pm$ SD from at least three independent experiments and were compared among three or more groups by analysis of variance (ANOVA) as performed using SPSS 18.0 software package. Pairwise comparisons were performed with two-tailed Student's *t* test for analysis of differences among groups. Differences were considered statistically significant if *P*-value was less than 0.05.

3. Results and discussion

3.1. Inactivation of PKC signaling accelerates Pi-induced VSMC calcification

PKC plays an important role in the regulation of numerous cellular functions [18]. We first analyzed alteration of PKC signaling in VSMCs treated with Pi. The amounts of both total and phosphorylated forms of PKC α and PKC δ , which are the predominant PKC isoforms in VSMCs [19], were markedly reduced in Pi-treated VSMCs (Fig. 1A). We further screened PKC isoforms to evaluate their potential role in VSMC calcification, using RNA interference with small interfering RNAs (siRNAs) targeted to PKC α , PKC β , PKC δ ,

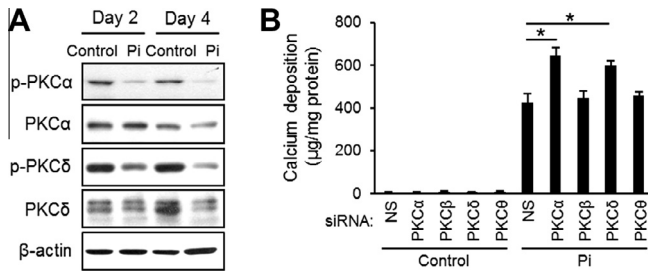


Fig. 1. Downregulation of PKC signal stimulates VSMC calcification. (A) Immunoblot analysis of phosphorylated (p-) and total forms of PKCα and PKCδ in VSMCs cultured in control or calcification medium for 2 or 4 days. Data are representative of three independent experiments. (B) VSMCs transfected with siRNAs specific for PKC isoforms (PKCα, PKCβ, PKCδ, or PKCθ) or with a nonspecific (NS) control siRNA were cultured in control or calcification medium for 8 days and then assayed for calcium deposition. Data are means ± SD from three independent experiments. **P* < 0.05.

and PKCθ mRNAs. Quantitative RT-PCR analysis confirmed the specific depletion of each PKC mRNA in cells transfected with the corresponding siRNA (Supplementary Fig. 1). Knockdown of PKCα or PKCδ resulted in enhancement of Pi-induced VSMC calcification compared with that in cells transfected with nonspecific siRNA controls (Fig. 1B). In contrast, knockdown of PKCβ or PKCθ had no effect on Pi-induced calcification. These results suggest that PKCα and PKCδ contribute to Pi-mediated VSMC calcification. To provide further support for this notion, we examined the effects of PKC inhibitors on VSMC calcification (Fig. 2A and B), as visualized by alizarin red staining and proven by calcium quantification. Although neither Go6976 (PKCα inhibitor) nor rottlerin (PKCδ inhibitor) alone induced VSMC calcification, each inhibitor signifi-

cantly enhanced the stimulatory effect of Pi on this process. These data indicate that PKCα and PKCδ signaling do not contribute to calcification under normal conditions, but participate in the regulation of calcification only under hyperphosphatemic conditions. Consistently, it has been reported that stimulatory factors of vascular calcification including reactive oxygen species, Runx2, and BMP2 could not induce calcification in the absence of Pi [7,20]. We also examined the roles of PKCα and PKCδ in vascular calcification in mouse aortas cultured *ex vivo*. When calcium deposition in aortas was visualized by von Kossa staining and evaluated by quantitative calcium assay, the effects of Go6976 and rottlerin on Pi-induced aortic calcification were similar to those observed with cultured VSMCs (Fig. 2C and D).

3.2. PKC inactivation upregulates osteogenic signaling during Pi-induced VSMC calcification

The pathogenesis of vascular calcification includes the phenotypic transition of VSMCs into osteoblast-like cells [2]. Accordingly, we investigated whether or not PKC inactivation is related to changes in osteogenic gene expression during Pi-induced VSMC calcification. As shown in Fig. 3, Go6976 treatment to VSMCs resulted in increased expression of osteoblast-specific genes such as SPARC, osteocalcin, osterix, and IBSP. Levels of mRNAs for osteopontin and IBSP were also upregulated by rottlerin treatment. Runx2 expression was not significantly altered by Go6976 or rottlerin treatment. These results suggest that PKCα and PKCδ inactivation possibly lead to a synergistic increase in Pi-induced VSMC calcification through induction of osteogenic signaling. PKC activation has been reported to suppress extracellular matrix mineralization and osteoblastic differentiation in preosteoblastic MC3T3-E1 and myoblast C2C12 cell lines [21,22], and knockdown of PKC was found to stimulate expression of osteoblast-specific marker genes [21]. Together, these results support the negative effects of PKC activity on both VSMC calcification and osteoblast mineralization.

3.3. PKC signaling regulates actin/microtubule cytoskeleton function during VSMC calcification

As PKC has been implicated as an important regulator of cytoskeletal function [23] and cytoskeletal changes play a crucial role in vascular calcification [16], we characterized the relation between PKC signaling and cytoskeleton reorganization during VSMC calcification. Whereas the ratio of F-actin to G-actin in VSMCs treated with both Go6976 and Pi was similar to that in cells treated with Pi alone, it was significantly reduced in cells exposed to both rottlerin and Pi compared to that in cells treated with Pi alone (Fig. 4A and B). Furthermore, whereas rottlerin did not affect reduction of the polymerized/free tubulin ratio induced by Pi, Go6976 markedly enhanced this effect of Pi in VSMCs (Fig. 4C and D). Given that inhibition of PKCα and PKCδ activities induced the disturbance of microtubule and actin cytoskeletons, respectively, the additive effect of the two inhibitors on Pi-induced VSMC calcification (Supplementary Fig. 2) might be due to enhanced disruption of both the actin and microtubule cytoskeletons. We further validated the effect of cytoskeletal disruption on calcification, showing that F-actin or microtubule disruption by cytochalasin D or nocodazole treatment promoted Pi-induced VSMC or aortic calcification (Supplementary Fig. 3). Collectively, these results indicate that PKCα-microtubule and PKCδ-actin signaling axes play a key role in vascular calcification.

PKC has been reported to participate in the regulation of actin and microtubule cytoskeletons and is associated with cytoskeletal proteins [23,24]. Various proteins related to actin and microtubule cytoskeletons, including talin, vinculin, troponin,

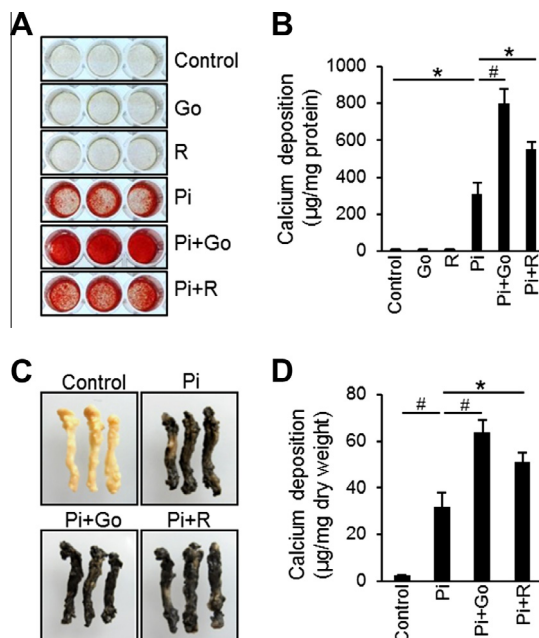


Fig. 2. Stimulatory effects of PKC inactivation on Pi-induced VSMC and aortic calcification. (A and B) Calcium deposition in VSMCs cultured for 8 days in control or calcification medium containing 1 μM Go6976 (Go) or 1 μM rottlerin (R), as indicated. Cells were stained with alizarin red S (A), and calcium content was quantified and normalized by protein content (B). Data are means ± SD from three independent experiments. **P* < 0.05, #*P* < 0.005. Mouse aortas cultured in control or calcification medium in the absence or presence of Go6976 or rottlerin for 14 days were subjected to von Kossa staining (C) and calcium quantification (D). Quantitative data were normalized by tissue dry weight and are means ± SD from five independent experiments. **P* < 0.05, #*P* < 0.005.

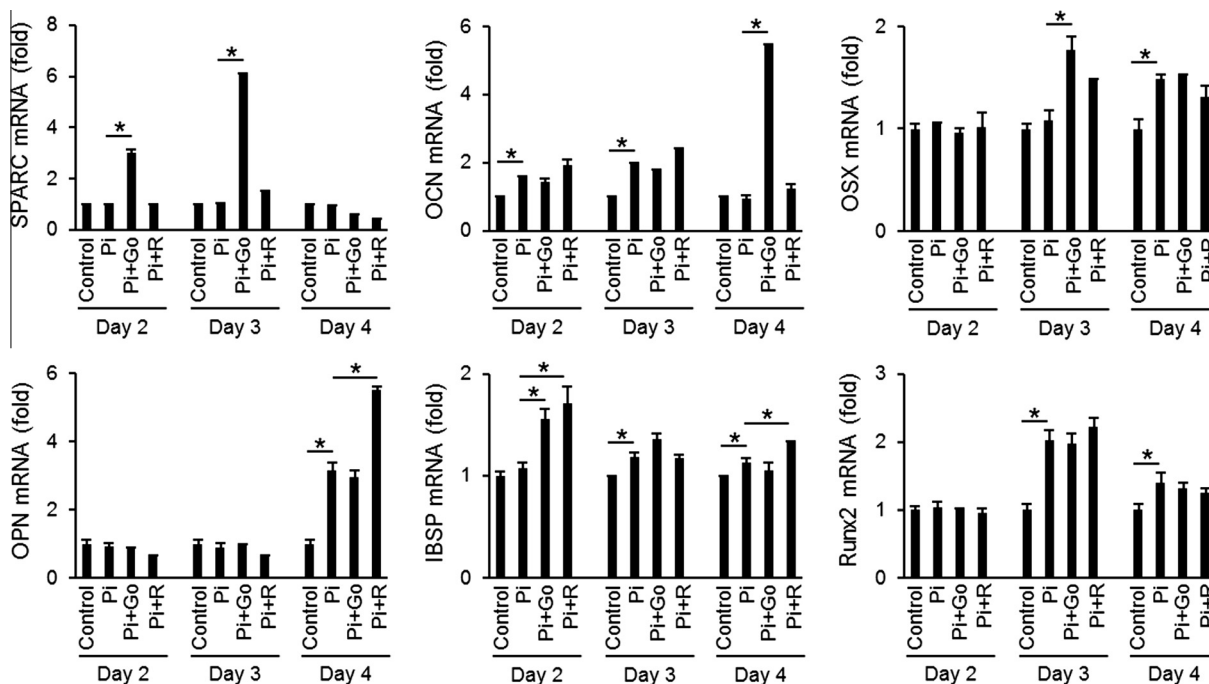


Fig. 3. Enhanced effects of PKC inactivation on osteogenic gene expression in Pi-induced VSMCs. VSMCs were cultured in control or calcification medium containing 1 μ M Go6976 (Go) or 1 μ M rottlerin (R) for the indicated times. Relative gene expression of osteogenic marker genes was analyzed by quantitative real-time PCR using GAPDH mRNA as a control. Quantification of mRNA expression is represented as a fold difference relative to control. Values are expressed as means $\pm$ SD from three independent experiments. * $P < 0.05$. OCN: osteocalcin; OSX: osterix; OPN: osteopontin.

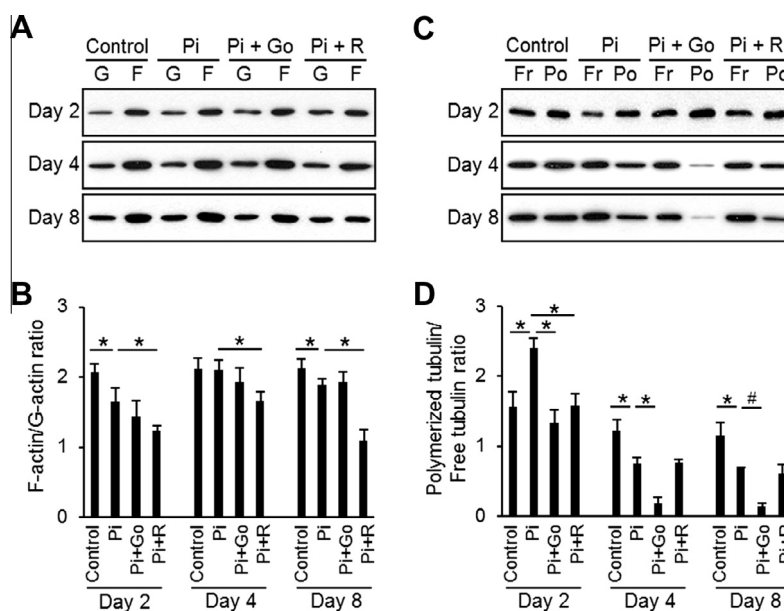


Fig. 4. PKC inactivation promotes disruption of actin and microtubule cytoskeletons in Pi-induced VSMCs. VSMCs cultured in control or calcification medium in the absence or presence of Go6976 (Go) or rottlerin (R) for the indicated times were analyzed for actin (A and B) and tubulin (C and D) polymerization. F-actin (F) and G-actin (G) fractions prepared by ultracentrifugation were subjected to immunoblotting with antibodies to actin (A) followed by densitometric analysis (B). The polymerized (Po) and free (Fr) fractions of tubulin were subjected to immunoblotting with antibodies to tubulin (C) followed by densitometric analysis (D). All densitometric data are means $\pm$ SD from three independent experiments. * $P < 0.05$, # $P < 0.005$ versus corresponding control.

tau, microtubule-associated protein 2, and α -tubulin, have been reported as substrates of PKC [23,25]. Here, we observed that inhibition of PKC α and PKC δ activities enhanced Pi-induced disruption of microtubule and actin, respectively. Consistent with our data, PKC α was shown to promote microtubule elongation and cell

motility through phosphorylation of α -tubulin in human breast cells [25], and activation of PKC promotes microtubule assembly in neuronal growth cones [26]. In conclusion, our results indicate that hyperphosphatemia-dependent VSMC calcification is mediated by inactivation of PKC α and PKC δ through disruption of

actin/microtubule cytoskeletons and stimulation of osteogenic signaling. This study thus extends our knowledge about the complicated mechanisms of vascular calcification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.10.026>.

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