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Vericiguat attenuates high phosphate-induced vascular smooth muscle cell calcification through the PKG/VASP/NF-kB/NLRP3 pathway

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Keywords

Vericiguat; vascular calcification; high phosphate; vascular smooth muscle cells; NF-kB; NLRP3 inflammasome

Abstract

Background

Vascular calcification is a common pathological feature of chronic kidney disease, diabetes, and atherosclerotic cardiovascular disease and is closely associated with arterial stiffness and adverse cardiovascular outcomes. High extracellular phosphate is a major driver of vascular smooth muscle cell (VSMC) calcification, promoting osteogenic phenotypic switching, inflammatory activation, and mineral deposition. Vericiguat, an oral soluble guanylate cyclase (sGC) stimulator approved for heart failure with reduced ejection fraction, enhances cyclic guanosine monophosphate signaling and exerts anti-inflammatory and cytoprotective effects. However, its effect on high phosphate-induced vascular calcification remains unclear.

Methods

A7r5 cells were exposed to 10 mmol/L β-glycerophosphate (β-GP) to establish an in vitro calcification model. Vericiguat was administered at 100 μmol/L. The PKG inhibitor Rp-8-Br-PET-cGMPS was used at 10 μM to evaluate the involvement of PKG signaling. Calcification, phenotypic switching, and inflammatory signaling were assessed by Alizarin Red S staining, calcium content assay, Western blotting, and ELISA.

Results

High phosphate significantly increased mineral deposition and calcium content in A7r5 cells. It also decreased the contractile markers α-smooth muscle actin (α-SMA) and SM22α while increasing the osteogenic markers bone morphogenetic protein 2 (BMP2) and runt-related transcription factor 2 (RUNX2). Vericiguat markedly attenuated these changes, indicating inhibition of calcification and phenotypic switching. Mechanistically, vericiguat increased the p-VASP (Ser239)/VASP ratio and reduced the p-IkBα/IkBα ratio, suggesting activation of PKG/VASP signaling and suppression of

NF-kB activity. Pharmacological inhibition of PKG partially abolished the protective effects of vericiguat on calcification and osteogenic transdifferentiation. Vericiguat also suppressed high phosphate-induced activation of the NLRP3 inflammasome, as shown by reduced expression of NLRP3, ASC, p-Caspase-1, and IL-1β, together with decreased IL-1β secretion. These anti-inflammatory effects were partially reversed by PKG inhibition.

Conclusions

Vericiguat attenuates high phosphate-induced VSMC calcification and inflammatory activation, at least in part through the PKG/VASP/NF-kB/NLRP3 signaling pathway. Vericiguat may represent a potential therapeutic strategy for phosphate-driven vascular calcification.

1.Introduction

Vascular calcification is now recognized as an actively regulated pathological process rather than passive calcium deposition. It is highly prevalent in chronic kidney disease (CKD), diabetes mellitus, atherosclerosis, and aging, and contributes directly to arterial stiffness, impaired vascular compliance, left ventricular overload, plaque instability, and cardiovascular mortality [1-4]. A central event in this process is the phenotypic switching of vascular smooth muscle cells (VSMCs) from a contractile state to an osteogenic or synthetic phenotype [8,19]. This transition is characterized by downregulation of smooth muscle lineage markers such as α-smooth muscle actin (α-SMA) and SM22α and induction of osteogenic regulators including bone morphogenetic protein 2 (BMP2) and runt-related transcription factor 2 (RUNX2) [20].

Among the known drivers of vascular calcification, phosphate overload is particularly important, especially in CKD-associated mineral and bone disorder [5,6]. Elevated extracellular phosphate promotes sodium-dependent phosphate transporter signaling, extracellular vesicle-mediated mineral nucleation, osteogenic transdifferentiation, and calcium-phosphate deposition in VSMCs [7]. Experimental studies have shown that phosphate directly induces calcification in cultured VSMCs and accelerates calcific remodeling under uremic conditions [7]. In addition, inflammatory signaling has

emerged as a major amplifier of phosphate-driven vascular injury [9,21-23]. Nuclear factor-kappa B (NF-kB) activation and NLR family pyrin domain containing 3 (NLRP3) inflammasome signaling enhance pro-calcific gene expression, p-Caspase-1 activation, and interleukin-1 β (IL-1β) release, thereby linking metabolic stress to vascular inflammation and calcification [24].

The nitric oxide-soluble guanylate cyclase-cyclic guanosine monophosphate pathway plays an essential role in vascular homeostasis [13,14]. Activation of soluble guanylate cyclase (sGC) increases intracellular cyclic guanosine monophosphate levels and stimulates downstream effectors, particularly protein kinase G (PKG), thereby regulating vascular tone, inflammation, fibrosis, oxidative stress, and cell survival [15]. Vericiguat is an oral sGC stimulator that directly stimulates sGC and sensitizes the enzyme to endogenous nitric oxide, resulting in enhanced cyclic guanosine monophosphate generation even under conditions of impaired nitric oxide bioavailability [17,25,26]. Clinically, vericiguat has shown benefit in patients with worsening heart failure with reduced ejection fraction and is now incorporated into contemporary therapeutic strategies for selected patients [10-12]. Beyond heart failure, experimental evidence indicates that sGC stimulation can modulate vasodilator-stimulated phosphoprotein (VASP), inhibit NF-kB/NLRP3 signaling, and exert tissue-protective and anti-inflammatory effects [18]. Vericiguat has also been reported to regulate osteoclast differentiation through VASP and NF-kB pathways, suggesting a broader role in mineralized tissue biology [16].

However, whether vericiguat protects against high phosphate-induced vascular calcification remains unclear. In the present study, we used high phosphate-treated A7r5 cells to investigate the effects of vericiguat on calcification, phenotypic switching, and inflammasome activation. We further explored the role of PKG/VASP signaling and its interaction with the NF-kB and NLRP3 pathways.

2.Materials and Methods

2.1 Cell culture and treatment

Rat thoracic aorta-derived A7r5 cells were cultured in complete growth medium under standard humidified conditions at 37 °C with 5% CO2. To establish an in vitro calcification model, cells were treated with 10 mmol/L β-GP. Vericiguat was administered at a final concentration of 100 μmol/L. To evaluate the involvement of PKG signaling, cells were pretreated with the PKG inhibitor

Rp-8-Br-PET-cGMPS at a final concentration of 10 μM before vericiguat treatment. For Alizarin Red S staining, cells were treated for 7 days. For all other experiments, including calcium content assay, Western blotting, and ELISA, cells were treated for 24 h. The experimental groups were Control, Pi, Pi + Ver, and Pi + Ver + PKGi.

2.2 Alizarin Red S staining and quantification

To assess mineralized nodule formation, A7r5 cells were cultured in the indicated media for 7 days, washed with phosphate-buffered saline, and fixed with 4% paraformaldehyde. Cells were then stained with Alizarin Red S solution to visualize calcium deposition. Representative images were captured under identical conditions. Quantitative analysis of staining intensity or calcified area was performed using an image analysis method.

2.3 Calcium content assay

Cellular calcium deposition was evaluated after 24 h of treatment using a standard calcium quantification method according to the manufacturer's instructions. Calcium levels were normalized to total protein content and expressed relative to the control group.

2.4 Western blot analysis

After 24 h of treatment, total protein was extracted from A7r5 cells using lysis buffer containing protease and phosphatase inhibitors. Protein concentration was determined using a standard protein assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated overnight at 4 °C with primary antibodies against α-SMA (Abcam, UK), SM22α (Abcam, UK), RUNX2 (Abcam, UK), BMP2 (Abcam, UK), VASP (Abcam, UK), p-VASP (Abcam, UK), IkBα (Abcam, UK), p-IkBα (Abcam, UK), NLRP3 (Cell Signaling Technology, USA), ASC (Cell Signaling Technology, USA), p-Caspase-1 (Cell Signaling Technology, USA), IL-1β (Cell Signaling Technology, USA), and GAPDH. Membranes were then incubated with appropriate secondary antibodies. Protein bands were visualized using enhanced

chemiluminescence and quantified by densitometric analysis. Protein expression levels were normalized to GAPDH or expressed as phosphorylation-to-total protein ratios where appropriate.

2.5 Enzyme-linked immunosorbent assay

After 24 h of treatment, culture supernatants were collected and centrifuged to remove debris. IL-1β concentration was measured using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

2.6 Statistical analysis

All experiments were independently repeated three times. Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism version 10.4.2. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by an appropriate post hoc test. A value of $P < 0.05$ was considered statistically significant.

3.Results

3.1 High phosphate induced calcification in A7r5 cells

To establish an in vitro calcification model, A7r5 cells were exposed to high phosphate. Alizarin Red S staining showed markedly increased mineralized nodule formation in the Pi group compared with the Control group. Quantitative analysis confirmed a significant increase in staining intensity. Consistently, calcium content was also significantly elevated after high phosphate treatment. These results indicate that high phosphate effectively induced calcification in A7r5 cells.（Figure 1）

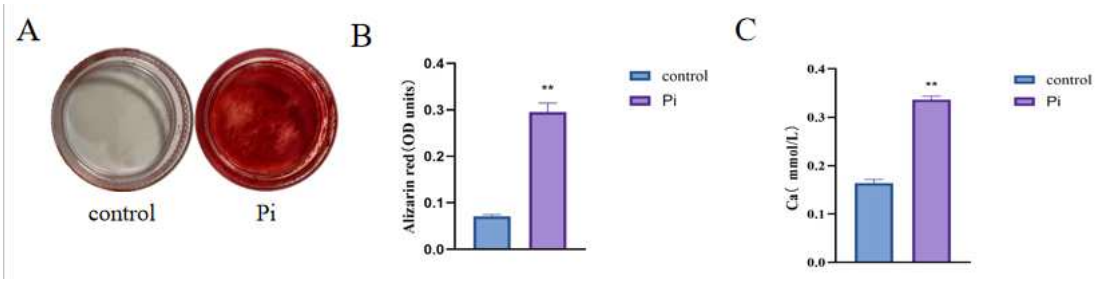


Figure 1. High phosphate promoted calcification of A7r5 vascular smooth muscle cells.

(A) Representative Alizarin Red S staining images of control and Pi-treated A7r5 cells. (B) Quantification of Alizarin Red S staining. (C) Measurement of calcium content. High-phosphate treatment significantly increased mineralized nodule formation and calcium content compared with the control group. Data are shown as mean ± SD. $^{**}P < 0.01$ vs. control.

3.2 Vericiguat attenuated high phosphate-induced calcification and phenotypic switching in A7r5 cells

To determine whether vericiguat affects phosphate-induced calcification, A7r5 cells were treated with high phosphate in the presence or absence of vericiguat. Vericiguat markedly reduced Alizarin Red S-positive calcium deposition and significantly decreased cellular calcium content relative to the Pi group.（Figure 2）

Because osteogenic transdifferentiation is central to vascular calcification, we further examined phenotype-related proteins. High phosphate reduced the expression of the contractile markers α-SMA and SM22α and increased the osteogenic markers BMP2 and RUNX2. Vericiguat substantially reversed these changes, indicating preservation of the contractile phenotype and suppression of osteogenic conversion.（Figure 2）

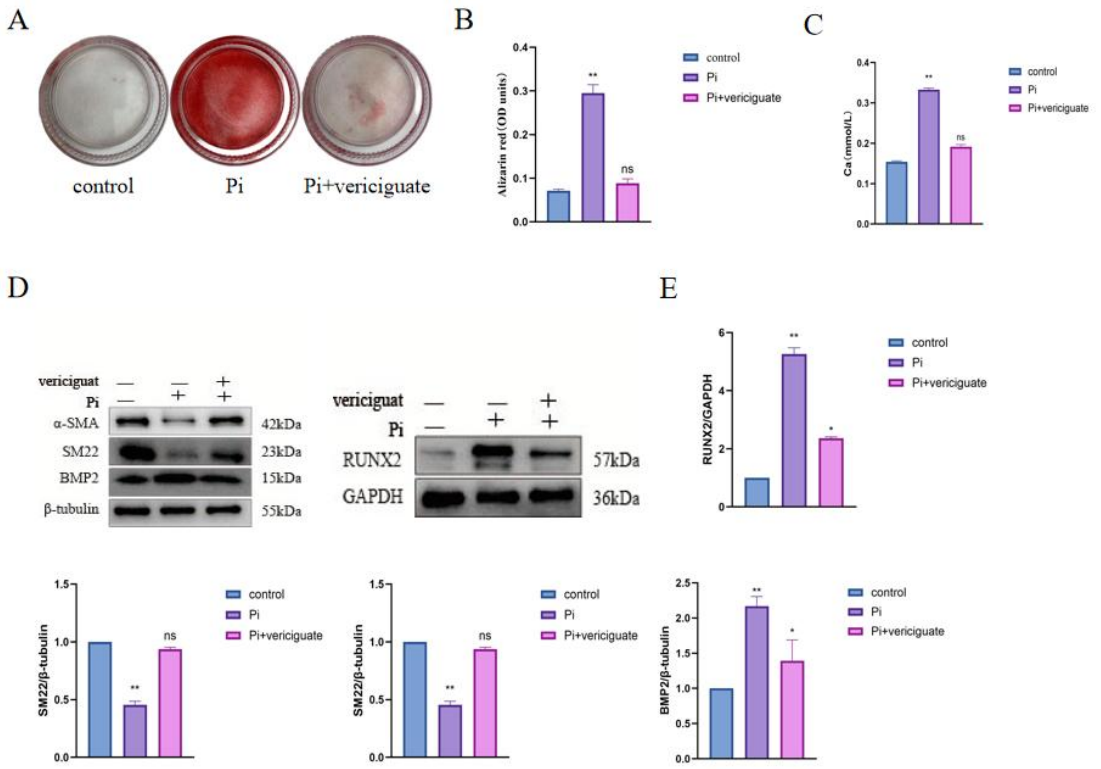


Figure 2. Vericiguat attenuated high phosphate-induced calcification and phenotypic switching in A7r5 vascular smooth muscle cells.

(A) Representative images of Alizarin Red S staining in the control, Pi, and Pi + vericiguat groups. (B) Quantitative analysis of Alizarin Red S staining. (C) Calcium content in A7r5 cells. (D) Protein expression levels of the contractile markers α-SMA and SM22, and the osteogenic markers BMP2 and RUNX2, as determined by Western blot. (E) Quantitative analysis of Western blot results. Compared with the control group, the Pi group showed significantly increased calcium deposition, calcium content, and osteogenic marker expression, along with reduced contractile marker expression. Vericiguat treatment markedly attenuated these changes. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. control; ns, not significant vs. control.

3.3 PKG inhibition partially reversed the protective effects of vericiguat on calcification and phenotypic switching

To clarify the mechanism underlying the anti-calcific effects of vericiguat, we assessed the involvement of PKG signaling using a pharmacological inhibitor. PKG inhibition partially restored high phosphate-induced mineral deposition despite the presence of vericiguat, as demonstrated by

Alizarin Red S staining and quantitative analysis. Similarly, the inhibitor attenuated the effects of vericiguat on phenotype-associated proteins, leading to lower α-SMA and SM22α expression and higher BMP2 and RUNX2 expression compared with the Pi + Ver group.（Figure 3）

We next examined downstream signaling proteins. Vericiguat increased the p-VASP (Ser239)/VASP ratio, indicating activation of PKG-dependent signaling, and reduced the p-IkBα/IkBα ratio, suggesting suppression of NF-kB pathway activation. PKG inhibition blunted the increase in p-VASP (Ser239)/VASP and partially restored the p-IkBα/IkBα ratio. These findings suggest that the PKG/VASP pathway is a major mediator of the protective effect of vericiguat and may negatively regulate NF-kB signaling under high phosphate conditions.（Figure 3）

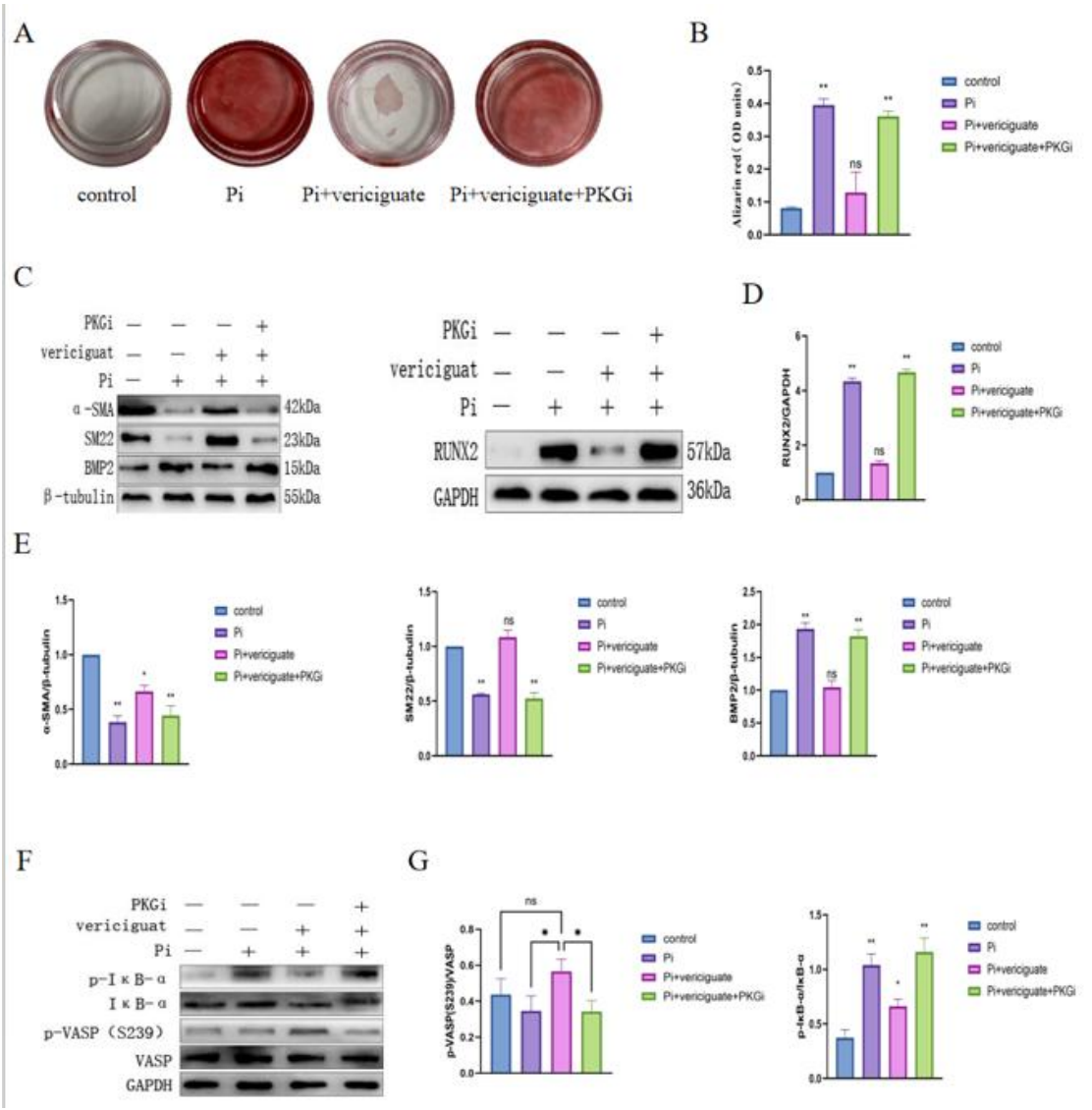


Figure 3. PKG inhibition partially reversed the protective effects of vericiguat on high phosphate-induced calcification and phenotypic switching in A7r5 vascular smooth muscle cells.

(A) Representative images of Alizarin Red S staining in the control, Pi, Pi + vericiguat, and Pi + vericiguat + PKGi groups. (B) Quantitative analysis of Alizarin Red S staining. (C) Protein expression levels of the contractile markers α-SMA and SM22, and the osteogenic markers BMP2 and RUNX2, as determined by Western blot. (D, E) Quantitative analysis of RUNX2, α-SMA, SM22, and BMP2 protein expression. (F) Western blot analysis of p-IkB-α, IkB-α, p-VASP (Ser239), and VASP expression. (G) Quantitative analysis of p-VASP/VASP and p-IkB-α/IkB-α ratios. High phosphate induced marked calcification, phenotypic switching, and NF-kappaB pathway activation, whereas vericiguat attenuated these effects. PKG inhibition partially abolished the protective effects of vericiguat, suggesting that the PKG/VASP pathway mediates the anti-calcification effect of vericiguat. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$; ns, not significant.

3.4 PKG inhibition partially abolished the inhibitory effect of vericiguat on NLRP3 inflammasome activation

Given the important role of inflammation in vascular calcification, we next evaluated NLRP3 inflammasome activation in each group. High phosphate significantly increased the expression of NLRP3, ASC, p-Caspase-1, and IL-1β in A7r5 cells and promoted IL-1β secretion into the culture supernatant. Vericiguat markedly suppressed these inflammatory changes, indicating inhibition of high phosphate-induced inflammasome activation. However, PKG inhibition partially abolished these effects, restoring inflammasome-related protein expression and increasing IL-1β release. These results suggest that vericiguat suppresses high phosphate-induced NLRP3 inflammasome activation, at least in part, through PKG-dependent signaling.（Figure 4）

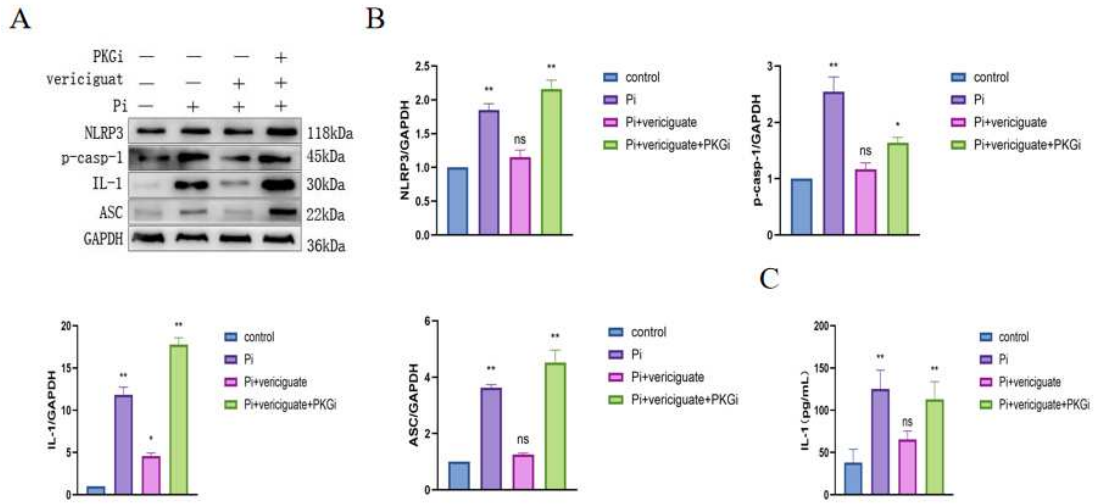


Figure 4. PKG inhibition partially abolished the inhibitory effect of vericiguat on NLRP3 inflammasome activation in high phosphate-treated A7r5 vascular smooth muscle cells.

(A) Western blot analysis of NLRP3, cleaved caspase-1, IL-1β, and ASC protein expression in the control, Pi, Pi + vericiguat, and Pi + vericiguat + PKGi groups. (B) Quantification of protein expression levels shown in (A), normalized to GAPDH. (C) Secretion of IL-1β into the culture supernatant was measured by ELISA. High-phosphate treatment significantly activated the NLRP3 inflammasome in A7r5 cells, whereas vericiguat markedly suppressed inflammasome-related protein expression and IL-1β release. These protective effects were partially reversed by PKG inhibition. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$. ns, not significant.

4.Discussion

The present study demonstrated that vericiguat attenuated high phosphate-induced calcification and osteogenic phenotypic switching in A7r5 cells. Vericiguat reduced calcium deposition, preserved the expression of contractile markers, inhibited osteogenic marker induction, and suppressed inflammatory activation. Mechanistically, these effects were associated with increased PKG/VASP signaling and reduced NF-kB/NLRP3 pathway activity. Pharmacological inhibition of PKG partially reversed the protective effects of vericiguat, indicating that PKG signaling is a key component of the underlying mechanism.

Phosphate overload is a well-established pathogenic factor in CKD-associated vascular calcification [1,5,6]. Previous studies have shown that elevated phosphate induces VSMC calcification through transporter-dependent phosphate uptake, extracellular vesicle release, osteogenic reprogramming, and calcifying matrix formation [5-7]. Our data are consistent with this framework, as high phosphate markedly increased mineral deposition and calcium content in A7r5 cells. The concomitant reduction in α-SMA and SM22α and increase in BMP2 and RUNX2 further confirm that phosphate induced a phenotypic switch from a contractile to an osteogenic state [8,19,20]. This phenotypic transition is regarded as a core event in medial vascular calcification and an important therapeutic target.

Inflammation provides an additional and clinically relevant layer to the calcification process [9]. NLRP3 inflammasome activation has been implicated in both atherosclerotic vascular injury and phosphate-induced VSMC calcification [21-23]. Once activated, the NLRP3 inflammasome promotes p-Caspase-1 activation and IL-1β maturation, thereby amplifying inflammatory injury and facilitating osteogenic signaling. In the current study, high phosphate markedly increased NLRP3, ASC, p-Caspase-1, and IL-1β expression, together with elevated IL-1β release, supporting a robust inflammasome response. These findings align with previous reports that NLRP3 activation contributes to VSMC calcification and that NF-kB signaling acts as an important upstream priming event [24].

The sGC-cyclic guanosine monophosphate-PKG pathway is a central regulator of vascular function and an attractive pharmacological target [13-14]. Vericiguat enhances cyclic guanosine monophosphate production by directly stimulating sGC and sensitizing it to endogenous nitric oxide, thereby sustaining downstream PKG signaling in disease states characterized by impaired nitric oxide bioavailability [10-12]. Although vericiguat is primarily used in heart failure, its broader vascular and anti-inflammatory actions are increasingly recognized [11,16-17]. In this study, vericiguat increased p-VASP (Ser239), a canonical indicator of PKG activation, and reduced the p-IkBα/IkBα ratio, suggesting that activation of PKG/VASP suppresses NF-kB signaling under high phosphate stress. These findings are consistent with prior evidence that sGC stimulation can restrain inflammation through a VASP/NF-kB/NLRP3 regulatory circuit [15].

A notable strength of this study is the mechanistic interrogation using PKG inhibition. The PKG inhibitor partially restored calcification, osteogenic marker expression, NF-kB activation, and inflammasome signaling, indicating that the protective actions of vericiguat are mediated in substantial

part through PKG/VASP. The reversal was partial rather than complete, suggesting that additional downstream mechanisms may also contribute. This possibility is biologically plausible because cyclic guanosine monophosphate signaling influences mitochondrial homeostasis, oxidative stress, autophagy, and innate immune responses [18].

From a translational perspective, these findings may be relevant. Patients with CKD and phosphate retention carry a high burden of vascular calcification and cardiovascular disease, yet effective targeted pharmacological strategies remain limited [2-4]. Because vericiguat is already clinically approved and has an established safety and pharmacokinetic profile [25,26], repurposing this agent for phosphate-driven vascular disease may be feasible if efficacy is confirmed in further preclinical and clinical studies. Our findings therefore provide a mechanistic rationale for exploring sGC stimulation in CKD-associated vascular calcification.

Several limitations should be acknowledged. First, the experiments were conducted in a single rat VSMC cell line, and validation in primary human VSMCs would improve translational relevance. Second, the mechanism was explored using pharmacological PKG inhibition without complementary genetic approaches. Third, upstream events linking phosphate stress to inflammasome activation, such as oxidative stress or mitochondrial dysfunction, were not directly examined. Finally, in vivo validation in CKD or vascular calcification animal models is required before clinical extrapolation.

5.Conclusions

Vericiguat significantly alleviated high phosphate-induced calcification in A7r5 cells, preserved the contractile phenotype, suppressed osteogenic transdifferentiation, and inhibited NLRP3 inflammasome activation. The protective effects of vericiguat were partially dependent on the PKG/VASP pathway and were accompanied by inhibition of NF-kB signaling. These findings support vericiguat as a potential therapeutic option for phosphate-associated vascular calcification, especially in the setting of CKD-related mineral disorder.

Funding

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Ethics Statement

Because this study was performed exclusively in the A7r5 cell line and did not involve human participants or animal experiments, ethics committee approval and informed consent were not required.

Conflict of Interest Statement

The authors declare no conflict of interest.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

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Abbreviations

α-SMA, α-smooth muscle actin

ASC, apoptosis-associated speck-like protein containing a CARD

β-GP, β-glycerophosphate

BMP2, bone morphogenetic protein 2

CKD, chronic kidney disease

ELISA, enzyme-linked immunosorbent assay

IkBα, inhibitor of nuclear factor-kappa B α

IL-1β, interleukin-1 β

NF-kB, nuclear factor-kappa B

NLRP3, NLR family pyrin domain containing 3

PBS, phosphate-buffered saline

Pi, high phosphate

PKG, protein kinase G

PKGi, PKG inhibitor

PVDF, polyvinylidene difluoride

RUNX2, runt-related transcription factor 2

sGC, soluble guanylate cyclase

SM22α, smooth muscle protein 22 α

VASP, vasodilator-stimulated phosphoprotein

VSMCs, vascular smooth muscle cells

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