Original Paper

High Phosphate-Induced Calcification of Vascular Smooth Muscle Cells is Associated with the TLR4/NF- κ B Signaling Pathway

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Key Words

High phosphate • Vascular calcification • VSMCs • TLR4 • NF- κ B

Abstract

Background/Aims: Hyperphosphatemia is one of the most notable features of chronic kidney disease (CKD). Numerous epidemiological and clinical studies have found that high serum phosphate concentrations are associated with calcification in the coronary arteries. However, the mechanisms underlying the vascular calcification induced by high phosphate have not been understood fully. **Methods:** Vascular smooth muscle cells (VSMCs) were cultured in high-phosphate media to induce vascular calcification, which was detected by Alizarin red S staining. Gene expression and protein levels of differentiation markers were determined by real-time RT-PCR and western blotting, respectively. Protein levels of phosphorylated NF- κ B and TLR4 were detected by western blotting, and the role of NF- κ B/TLR4 was further confirmed by using an NF- κ B inhibitor or TLR4 siRNA. **Results:** Our results showed that high-phosphate media induced obvious calcification of VSMCs. Simultaneously, VSMC differentiation was confirmed by the increased expression of bone morphogenetic protein-2 and Runt-related transcription factor 2 and decreased expression of the VSMC-specific marker SM22 α , which was accompanied by the increased expression of inflammatory cytokines. Moreover, a significant upregulation of TLR4 and phosphorylated NF- κ B was also detected in VSMCs with high-phosphate media. In contrast, VSMC calcification and the increased expression of inflammatory cytokines were markedly attenuated by pretreatment with TLR4 siRNA and pyrrolidine dithiocarbamic acid, an NF- κ B inhibitor. **Conclusion:** These data suggest that high-phosphate conditions directly induce vascular calcification via the activation of TLR4/NF- κ B signaling in VSMCs. Moreover, inhibition of the TLR4/NF- κ B signaling pathway might be a key intervention to prevent vascular calcification in patients with CKD.

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Introduction

The morbidity and mortality of chronic kidney disease (CKD) are increasing worldwide [1]. Eventually, many patients with CKD deteriorate into end-stage renal disease. The complications of CKD can affect many other organs besides the kidney. Cardiovascular disease (CVD), which accounts for more than 50% of total CKD mortality, is the most prevalent and important factor among all of the complications observed in CKD patients [2-3]. Notably, vascular calcification (VC) is not only an important pathological change in CKD but also an independent risk factor for the morbidity and mortality of cardiovascular diseases [4-6]. Therefore, the prevention of VC is important in the clinical setting.

VC often occurs with aging, but also occurs in some diseases, including CKD, diabetes [7], and chronic heart failure [8]. Among them, CKD-induced VC often occurs in the medial layer through an active gene-controlled process. It has already been found that phosphate induces VC of vascular smooth muscle cells (VSMCs) *in vitro* [9]. The differentiation of VSMCs into osteoblast-like cells is considered to be the key step of VC in the medial layer. Recently, vascular inflammation and medial calcification were found to be present in the early stages of CKD [10]. Thus, it is important to explore the mechanisms underlying the differentiation of VSMCs into osteoblast-like cells, which will provide new ideas and novel targets for the clinical prevention of VC.

Impaired kidney function reduces urinary phosphate excretion. Hyperphosphatemia is a key pathological feature of CKD [11-13]. Although hyperphosphatemia often occurs in stage 4 or 5 CKD, the increase of serum phosphate concentration begins earlier. Epidemiological studies have confirmed a link between serum phosphorus and adverse outcomes in CKD patients [14]. Moreover, high plasma phosphate was found to be a risk factor for the decline in renal function and mortality in chronic hemodialysis patients [15, 16]. In order to attenuate the severity of hyperphosphatemia, dietary phosphate restriction and oral phosphate binders are first-line treatments for CKD stage 5D patients [17-19]. More recently, tenapanor, a small-molecule inhibitor of sodium/hydrogen exchanger isoform 3, was found to reduce serum phosphate concentrations significantly in patients receiving hemodialysis [20]. All of these data suggest that hyperphosphatemia in CKD patients is a critical cardiovascular risk factor.

Conversely, elevated inflammatory responses were found in CKD patients with VC. Studies have also shown that inflammatory responses and inflammatory factors such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) could accelerate the differentiation of VSMCs into osteoblast-like cells [21, 22]. Agharazii et al. demonstrated that inflammatory cytokines and reactive oxygen species can function as mediators of CKD-related VC in a rat model fed with a high calcium-phosphate diet [23]. These findings suggest that hyperphosphatemia and chronic inflammation are closely related to the differentiation of VSMCs into osteoblast-like cells. In the present study, we first examined the direct effect of high phosphate on the calcification of VSMCs and subsequently identified the important role of toll-like receptor 4 (TLR4)/nuclear factor- κ B (NF- κ B) signaling in this process.

Materials and Methods

Materials

Primary antibodies against Runt-related transcription factor 2 (Runx2), smooth muscle protein 22- α (SM22 α), bone morphogenetic protein-2 (BMP2), and TLR4 were purchased from Abcam (Cambridge, UK), and an antibody against NF- κ B was purchased from Cell Signaling Technology (Beverly, MA, USA). Lipofectamine Reagent 2000 and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). Small interfering RNA duplex (siRNA) was synthesized by Santa Cruz Biotechnology (Shanghai, China). IL-1 β , IL-6, and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were obtained from Beyotime Biotechnology

(Nantong, China). All primers were obtained from Shanghai Sangon Biotechnology (Shanghai, China). The materials for cell culture were obtained from Thermo Fisher Scientific (Shanghai, China).

Cell culture and induction of calcification

VSMCs were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (HyClone, Logan, UT, USA). For the induction of calcification, the cells were cultured in DMEM containing a 2.6 mM Na₂HPO₄/NaH₂PO₄ solution for 5 days and then analyzed further. Media were replaced every second day.

ELISA

The culture medium was collected and centrifuged at 1,000 $\times$ g for 5 min. The supernatant was collected and the content of IL-1 β , IL-6, and TNF- α in the supernatant was measured by commercial ELISA kits, according to the manufacturer's instructions.

Alizarin red S staining

VSMCs in the logarithmic growth phase were seeded on a coverslip in a 12-well plate and treated as desired. After removal of the media, the cells were washed with phosphate-buffered saline, fixed with 4% formaldehyde, and stained with Alizarin red S for 30 min. Photographs were taken using a digital microscope.

Western blotting

VSMCs were harvested and total proteins were isolated and quantified by the BCA method. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and transferred onto a nitrocellulose membrane. After blocking in 3% nonfat milk for 30 min, the membrane was incubated with primary antibodies against TLR4 (Abcam), NF- κ B (Cell Signaling), phosphorylated (p)-NF- κ B (Cell Signaling), Runx2 (Abcam), BMP2 (Abcam), SM22 α (Abcam), and β -actin (Abcam), followed by incubation with corresponding horseradish peroxidase-labeled secondary antibodies for 1 h. The bands were visualized by using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA). The expression level of each protein was normalized by that of β -actin.

Transfection of siRNA

VSMCs were transfected with specific siRNA against TLR4 or control siRNA (Santa Cruz) using Lipofectamine 2000 according to the manufacturer's instructions. Then, the cells were allowed to recover and further cultured in high-phosphate media for 5 days.

Real-time RT-PCR

Total RNA was extracted from the samples with the TRIzol reagent. RNA (0.5 μ g) was reverse transcribed to cDNA according to the manufacturer's directions (Roche [China] Ltd., Shanghai, China). For real-time RT-PCR, amplification was performed with a 10 μ L final reaction mixture consisting of 0.5 μ L reverse transcription reaction mixture, 0.1 μ M each primer pair (Table 1), and 1 $\times$ PCR mixture of SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), using the Bio-Rad iCycler system (Bio-Rad). The PCR conditions were: initial denaturation at 95°C for 5 min; and 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The dissociation curves were analyzed to ensure a single peak and no off-target amplification was detectable on an agarose gel. Gene expression was normalized to the corresponding level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and was presented as fold change relative to that of control.

Statistical analysis

Experiments were repeated at least three times. SPSS 19.0 (SPSS Japan, Tokyo, Japan) was used for statistical analysis, which was performed using an unpaired, 2-tailed Student's t test and one-way analysis of variance with Tukey's multiple comparison test. A value of $p < 0.05$ was considered significant.

Table 1. Sequences of real-time RT-PCR primers

Gene Name	Sequence	Size (bp)
IL-1 β	F: GATGATGACGACCTGCTAGTGT	307
	R: TCTTCTTTGGGTATTGTTTGG	
IL-6	F: CAATGGCAATTCTGATTGTATG	216
	R: ATGACTCTGGCTTTGTCTTTC	
TNF- α	F: CACCACGCTCTTCTGTCTACTG	150
	R: TGGGCTACGGGCTTGTCACTCG	
GAPDH	F: TGACTTCAACAGCAACTC	110
	R: TGTAGCCATATTCATTGTCA	

Fig. 1. High phosphate induces the osteoblast differentiation and calcification of VSMCs. VSMCs were cultured in high-phosphate media for 5 days and stained with Alizarin red S (A). The protein expression of SM22 α , BMP2, and RUNX2 was detected by western blotting (B). β -Actin protein expression was determined as a control for equal loading. The experiments were repeated at least three times. NP: normal phosphate medium; HP: high-phosphate medium. Scale bar: 200 μ m. ** p <0.01.

Results

High phosphate induces osteoblast differentiation and calcification of VSMCs

The normal physiological concentration of phosphate is approximately 1.3 mM; thus, 2.6 mM phosphate is used to mimic the high-phosphate condition. After culture in high-phosphate media (containing 2.6 mM phosphate) for 5 days, significant calcification was induced in VSMCs as determined by Alizarin red S staining (Fig. 1A). Moreover, the protein expression of Runx2 and BMP2, which were used as osteoblast markers, was significantly increased in the high-phosphate condition (Fig. 1B). However, the protein level of SM22 α , a specific VSMC marker, was significantly decreased.

High phosphate induces the increased expression of IL-1 β , IL-6, and TNF- α

Previous studies have shown that chronic inflammation is closely related to the differentiation of VSMCs into osteoblast-like cells [24]. We wondered whether high phosphate could induce the expression of inflammatory cytokines. To this end, the mRNA levels of IL-1 β , IL-6, and TNF- α were detected by real-time RT-PCR. As shown in Fig. 2A, gene expression of IL-1 β , IL-6, and TNF- α was significantly upregulated by high phosphate. Moreover, the protein levels of IL-1 β , IL-6, and TNF- α in the culture medium were also significantly increased (Fig. 2B). These data clearly indicated that high phosphate induced the increased expression of inflammatory cytokines in VSMCs.

NF- κ B is activated by high phosphate in VSMCs

Next, we investigated the mechanism underlying the effect of high phosphate. Many studies have demonstrated that the TLR4/NF- κ B pathway is involved in chronic inflammation

[25]. To test whether NF- κ B is activated by high phosphate, the protein levels of p-NF- κ B were detected by western blotting. The results showed that the expression of p-NF- κ B was significantly increased (Fig. 3A). Moreover, this activation of NF- κ B could be attenuated by pyrrolidine dithiocarbamic acid (PDTC), a specific inhibitor of the NF- κ B pathway (Fig. 3B). Simultaneously, the calcification of VSMCs induced by high phosphate

Fig. 2. High phosphate induces the increased expression of IL-1 β , IL-6, and TNF- α . VSMCs were cultured in high-phosphate media for 5 days. The cells and culture media were harvested and analyzed by real-time RT-PCR (A) and ELISA (B), respectively. NP: normal phosphate medium; HP: high-phosphate medium. * p <0.05, ** p <0.01.

Fig. 3. NF- κ B is activated in VSMCs by high phosphate. VSMCs were cultured in high-phosphate media for 5 days and total proteins were detected by western blotting (A). Then, VSMCs were pretreated with or without PDTC (20 μ M) and further cultured in high-phosphate media for 5 days. Total proteins were isolated for western blotting (B and D) and the cells were stained with Alizarin red S (C). RNA was isolated from similarly treated VSMCs for real-time RT-PCR (E). β -Actin protein and GAPDH mRNA expression was determined as a control for equal loading. The experiments were repeated at least three times. NP: normal phosphate medium; HP: high-phosphate medium. Scale bar: 200 μ m. * p <0.05, ** p <0.01.

Fig. 4. Knockdown of TLR4 attenuates high phosphate-induced NF- κ B activation. VSMCs were cultured in high-phosphate media for 5 days and total proteins were detected by western blotting (A). For TLR4 knockdown, VSMCs were transfected with TLR4 siRNA or control siRNA and then further cultured in high-phosphate media for 5 days. RNA was detected by real-time RT-PCR (B) and total proteins were detected by western blotting to determine the expression of TLR4 (C) and p-NF- κ B (D). β -Actin protein expression was determined as a control for equal loading. The experiments were repeated at least three times. NP: normal phosphate medium; HP: high-phosphate medium. * $p < 0.05$, ** $p < 0.01$.

was also partially reversed by PDTC (Fig. 3C). More importantly, the altered protein expression of Runx2, BMP2, and SM22 α was significantly reversed by PDTC (Fig. 3D). In addition, the expression of IL-1 β , IL-6, and TNF- α by high phosphate was significantly attenuated by PDTC (Fig. 3E). These data suggested that high phosphate induced the osteoblast differentiation and calcification of VSMCs by activation of the NF- κ B pathway.

Knockdown of TLR4 attenuates high phosphate-induced NF- κ B activation

Many upstream signals can lead to NF- κ B activation. TLR4 is one of the most notable features that is involved in chronic inflammation. We wondered whether TLR4 was associated with the high phosphate-induced VC and differentiation of VSMCs. As shown in Fig. 4A, the protein expression of TLR4 was upregulated by high phosphate. Then, its expression was knocked down by siRNA, as detected by real-time RT-PCR and western blotting (Fig. 4B–C). To test the effect of TLR4 siRNA on NF- κ B activation, the protein level of p-NF- κ B was

Fig. 5. Knockdown of TLR4 attenuates the high phosphate-induced osteoblast differentiation and calcification of VSMCs. VSMCs were transfected with TLR4 siRNA or control siRNA and then further cultured in high-phosphate media for 5 days. Then, the cells were stained with Alizarin red S (A) and total proteins were detected by western blotting to determine the protein expression of SM22 α , BMP2, and RUNX2 (B), and the density of bands was analyzed by Quantity One (C). β -Actin protein was determined as a control for equal loading. The experiments were repeated at least three times. NP: normal phosphate medium; HP: high-phosphate medium. Scale bar: 200 μ m. * p <0.05, ** p <0.01.

detected by western blotting. The results showed that the elevated p-NF- κ B level induced by high phosphate was significantly downregulated by TLR4 siRNA, compared with that of control siRNA (Fig. 4D).

Knockdown of TLR4 attenuates high phosphate-induced VSMC calcification and differentiation

Finally, we considered the effects of TLR4 siRNA on the osteoblast differentiation and calcification of VSMCs induced by high phosphate. Similar to the effect of PDTC, calcification of VSMCs was significantly attenuated by TLR4 siRNA, compared with that of control siRNA (Fig. 5A). Moreover, the expression of osteoblast-associated markers and SM22 α in high phosphate-induced cells was also reversed by TLR4 siRNA (Fig. 5B–C). These data clearly indicated that TLR4/NF- κ B signaling plays an important role in the high phosphate-induced osteoblast differentiation and calcification of VSMCs.

Discussion

CVD, which accounts for more than 50% of total CKD mortality, is the most prevalent and important factor among all of the complications observed in patients with CKD [26].

With the decrease of renal function, serum phosphate increases gradually; thus, most CKD patients suffer from high serum phosphate levels. One of the most serious effects of high serum phosphate is the induction of VC. Although many genetic and environmental factors contribute to VC, high serum phosphate is one of the most notable factors associated with VC in CKD patients. And some chemicals were tested for treatment of VC induced by high phosphate [27, 28]. However, the mechanisms underlying VC are not fully understood. It has been verified that nanocrystals of basic calcium phosphate are the active form of elevated intracellular phosphate, and the formation of soluble calciprotein particles by other proteins such as fetuin-A can inhibit crystal growth and aggregation [29-31]. In the present study, we focused on the functions of phosphate regardless of basic calcium phosphate and found that high phosphate could activate the TLR4/NF- κ B signaling pathway, accompanied by the enhanced expression of inflammatory cytokines.

Medial VC is found frequently in CKD and diabetic patients [32], which is accompanied by changes of the cell phenotype-differentiation of VSMCs into an osteoblast-like phenotype. The increased gene expression of Runx2 and BMP2 is considered to be an important molecular marker of VC [33, 34]. In the present work, the expression of Runx2 and BMP2 was significantly induced by high phosphate, which accounted for the calcification of VSMCs.

Chronic inflammation plays an important role in the development of CVD as well as VC [35]. TLR4/NF- κ B is a classic signaling pathway related to the inflammatory response. NF- κ B is a transcription factor that is known to regulate the expression of inflammatory cytokines [36]. NF- κ B activation has been found in many chronic inflammatory responses such as rheumatoid arthritis and inflammatory bowel disease [37]. NF- κ B is activated continuously in patients with CKD and in animal models. For example, the NF- κ B pathway plays a central role in uric acid-induced inflammation for kidney injury in hyperuricemic rodents [38]. Here, NF- κ B activation was found in high phosphate-treated VSMCs, which was significantly attenuated by an NF- κ B inhibitor. More importantly, this NF- κ B activation was accompanied by the increased expression of inflammatory cytokines. This clearly indicated that NF- κ B plays a central role in the high phosphate-induced osteoblast differentiation and calcification of VSMCs. This was consistent with other findings in a cell model of VSMC calcification [39].

What is the upstream signal that leads to the activation of NF- κ B? Previous reports have shown that TLR4 can activate NF- κ B and plays an important role in chronic inflammation. There are two different pathways for the activation of NF- κ B: myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent. Exposure of endothelial cells to uremic sera induces TLR4 expression, which is paralleled with the induction of MyD88 and the activation of NF- κ B [40]. In the present study, TLR4 expression was induced by high phosphate, while TLR4 siRNA attenuated its induction. However, we are not certain whether the activation of NF- κ B is MyD88-dependent. In fact, other factors might contribute to VC by activating TLR4/NF- κ B signaling. A report indicated that TLR4/NF- κ B activation is also involved in the Ox-LDL-induced calcification of human VSMCs [41]. Accordingly, our study clearly revealed that high phosphate, as the most important factor inducing VC in CKD patients, triggered calcification of VSMCs through the activation of TLR4/NF- κ B signaling.

What is the meaning of TLR4/NF- κ B activation by high phosphate? *In vitro* studies and animal models suggest that inflammatory cytokines can promote VC and the differentiation of VSMCs. For example, Tintut et al. found that TNF- α can promote the calcification of vascular cells via the cAMP pathway *in vitro* [42]. In a rat model of CKD-related VC, the gene expression of IL-1 β , IL-6, and TNF- α was also increased in the thoracic aorta [43]. The current study also showed that the inflammatory response might play an important role in VC. Similarly, our data also showed that IL-1 β , IL-6, and TNF- α were induced by high phosphate, which contributed to the calcification of VSMCs, while inhibition of the production of cytokines was accompanied by the alleviation of VC.

Conclusion

In summary, our study found that high phosphate could upregulate TLR4 expression and activate the NF-κB signaling pathway, and thus give rise to the production of inflammatory cytokines, which subsequently initiated the differentiation of VSMCs into osteoblast-like cells and induced calcification. This may be an important mechanism of VC in CKD patients. Moreover, inhibiting the activation of the TLR4/NF-κB signaling pathway might be a key intervention to prevent the development of VC in patients with CKD.

Disclosure statement

The authors declare that there are no conflicts of interest.

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