Study of vascular smooth muscle cell calcification induced by hyperphosphate and intervented by phosphonoformic acid

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Abstract

Objective: To evaluate the effects of different concentrations of phosphate on calcium deposition and osteocalcin level in cultured bovine aortic smooth muscle cell, investigate the mechanism of hyperphosphatemia to evoke calcification of vascular smooth muscle cell and observe the effects of phosphonoformic acid(PFA) in different concentrations on vascular calcification. **Methods:** The bovine aortic smooth muscle cells (BASMC) were cultured.Calcium deposition and the expression of osteocalcin of BASMC in different concentrations of phosphate (1.5 mmol/L and 2.0 mmol/L) and PFA were determined by o-cresolphthalein complexone and radioimmunity methods, respectively. Osteocalcin mRNA expressions were determined by RT-PCR. **Results:** After six or nine days of BASMC cultured, the calcium deposition in Pi 2.0 mmol/L group was more than that in Pi 1.5 mmol/L group[$(77.187 \pm 11.692)\mu g/(mg \cdot protein)$ vs$(25.768 \pm 1.750)\mu g/(mg \cdot protein)$, $P < 0.01$ and$(125.399 \pm 16.677)\mu g/(mg \cdot protein)$ vs$(29.046 \pm 2.635)\mu g/(mg \cdot protein)$, $P < 0.01$ respectively]. The calcium deposition was dependent on time and dosage of phosphate treatment. After 72 h culture the osteocalcin in Pi 2.0 mmol/L group was more than that in Pi 1.5 mmol/L group[in supernatant,$(1.503 \pm 10^{-2} \pm 2.601 \times 10^{-3})ng/(\mu g \cdot protein)$ vs$(2.981 \times 10^{-3} \pm 8.382 \times 10^{-4})ng/(\mu g \cdot protein)$, $P < 0.001$], the same was found in osteocalcin mRNA expression[OC/GAPDH, (1.906 ± 0.132) vs(0.748 ± 0.037)，$P < 0.001$]. Compared to Pi 1.5mmol/L group,bovine smooth muscle cells(BSMC) cultured in media containing Pi 2.0 mmol/L phosphate levels increased calcium deposition[On day 6,$(77.187 \pm 11.692)\mu g/(mg \cdot protein)$ vs $(25.768 \pm 1.750)\mu g/(mg \cdot protein)$, $P < 0.001$]. Elevated phosphate treatment of BSMCs also enhanced the expression of the osteoblastic differentiation marker osteocalcin[On day 3, Pi 2.0 mmol/L group vs Pi 1.5mmol/L group,$(1.503 \times 10^{-2} \pm 2.601 \times 10^{-3})ng/(\mu g \cdot protein)$ vs$(2.981 \times 10^{-3} \pm 8.382 \times 10^{-4})ng/(\mu g \cdot protein)$, $P < 0.001$]. PFA decreased ciacium deposition and osteocalcin expression statistically[Pi 2.0 mmol/L+PFA1.0 mmol/L group vs Pi 2.0mmol/L group , ciacium deposition, $(37.729 \pm 5.899)\mu g/(mg \cdot protein)$ vs $(77.187 \pm 11.692)\mu g/(mg \cdot protein)$, $P < 0.001$]; Osteocalcin in supernatant, $(4.529 \times 10^{-3} \pm 1.250 \times 10^{-3})ng/(\mu g \cdot protein)$ vs$(1.503 \times 10^{-2} \pm 2.601 \times 10^{-3})$ ng/$(\mu g \cdot protein)$, $P < 0.001$; osteocalcin mRNA expression, OC/GAPDH, (0.642 ± 0.092) vs (1.89 ± 0.165), $P < 0.01$]. **Conclusion:** Hyperphosphate may directly promote calcium deposition and the osteocalcin expression of BASMCs. It may be a new explanation for the phenomenon of vascular calcification in hyperphosphatemic conditions. Hyperphosphatemia is an independent factor to stimulate vascular calcification. PFA can inhibit calcium deposition and osteocalcin expression induced by elevated phosphate.PFA may be a new medicine to treat vascular calcification induced by elevated phosphate.

Key words: vascular smooth muscle cell; phosphate; calcification; osteocalcin; phosphonoformic acid

INTRODUCTION

The number of patients with end-stage renal disease (ESRD) undergoing chronic renal replacement therapy is increasing worldwide, which is not only a major medical problem but also a social and economical one, so cost-effective care is important in this field. One of the unfortunate medical problems that many ESRD patients hare is dislay vascular disease, which is a major cause of mortality from ischaemic heart disease, cerebral vascular disease and arteriosclerosis obliterans.Vascular calcification is often observed in ESRD, even in young

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patients, and it is related to an increased mortality risk in ESRD patients. Identification of the factors that cause vascular calcification is therefore important[1]. Hyperphosphatemia is commonly observed in renal disease, especially in ESRD patients. Elevated serum phosphorus is a major risk factor for vascular calcification and cardiovascular mortality in these patients[2-3]. Kestenbaum observed associations between elevated serum phosphate levels and the risk for mortality and MI among ESRD patients, independent of renal function and other known confounding factors. Surprisingly, the association between higher phosphate levels and mortality risk was present among patients with absolute serum phosphate levels in the high-normal range[4]. It is meaningful to explain the mechanism of vascular calcification induced by hyperphosphatemia and take active measures to treat vascular calcification, with these objectives we designed and carried out this study. The aim of this study is to observe the effect of phosphate on calcium deposition and level of osteocalcin(OC) the effect of phosphonoformic acid(PFA) on calcification induced by elevated inorganic phosphate in cultured bovine aortic smooth muscle cell(BASMC), and offer an experimental basis for studying mechanism and treatment of vascular calcification in urmia.

MATERIAS AND METHODS

Reagents

DMEM(high glucose, 4.5 g/L of glucose) and Trypsin were purchased from Gibco. Calcium Kit was purchased from Sigma(USA). BCA Kit and osteocalcin RIA Kit were obtained from Jietai Biotechnology(Shanghai) and Xiehe Biotechnology(Tianjin) Co.Ltd. respectively. BcaBEST RNA PCR Kit Ver.1.1 and DNA Marker were purchased from TaKaRa Biotechnology(Dalian) Co.Ltd. Phosphonoformic acid was provided by Tianqing Pharmaceutical Factory(China).

Cell culture

Bovine vascular smooth muscle cells were obtained by an experimental method as previously described[5]. Medial tissue was separated from segments of bovin aorta. Small pieces of tissue(1-2 mm³) were placed in 100ml culture bottles and cultured for several weeks in Dulbecco's modified Eagle's medium(DMEM)(high glucose, 4.5 g/L of glucose) containing 15% FBS, and 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2. Cells that had migrated from the experiments were collected and maintained in the growing medium. The cells up to passage 5-6 were used for experiments. Purity of cultures was assessed by positive immunost-aining for α-actin and negtive immunostaining for keratin,

proving their smooth muscle origin.

Induction of calcification and PFA's intervention

BASMCs were routinely subcultured in growth medium. At confluence, the cells were switched to different medium, Pi 1.5 mmol/L, Pi 2.0 mmol/L, Pi 2.5 mmol/L, Pi 2.0 mmol/L+PFA0.25 mmol/L, Pi 2.0 mmol/L +PFA0.5 mmol/L, Pi 2.0 mmol/L+ PFA1.0 mmol/L.All media contained 1.8 mmol/L calcium.The medium was replaced with fresh medium every 2 days. For time-course experiments, the first day of culture in different media was defined as day 0.

Quantification of calcium deposition

Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complexone method. After decalcification, the cells were washed three times with D-Hanks and solubilized with 0.1mol/L NaOH/0.1% SDS.The protein content was measured with a BCA protein assay kit. The calcium content of the cell layer was normalized to protein content.

Osteocalcin determination by RIA

The secretion of OC by BASMC was assessed by measuring OC content of the culture supernatant with a radioimmunity assay kit.The supernatant was collected after the fresh medium containing 15% FBS was incubated for 72 h with BVSMC in 6-well plates.The data were normalized by the protein content of the cell layer.

RNA isolation and reverse transcription-PCR analysis(RT-PCR)

Total RNA was isolated from BASMC by extraction with acid guandium thiocyanate-phenol-chloroform. RNA was reverse-transcribed,and for PCR amplification. 2.5 μl of diluted cDNA was used in 20 μl reactions. The cycling parameters were 94℃ for 45 s, 55℃ for 45 s, and extension at 72℃ for 90 s for 30 cycles. A 10 μl aliquot of each reaction was electrophoresed through 2% agarose gel,and the DNA was visualized by ethidium bromide under UV light transillumination.The oligonucleotide primers for bovine OC were 5′-GGC GCT ACC TGG ACC ACT G -3′ (forward) and 5′-GCC GTA GAA GCG CCG ATA G-3′ (reverse). Control primer to bovine GAPDH, 5′-TTC CGC GTC CCC ACT CCC AAC-3′ (forward), 5′-GTG GCG GAG ATG GGG CAG GAC T-3′ (reverse). OC primer was designed according to bovine osteocalcin mRNA rank in GenBank.

Statistics

Data were analyzed for statistical significance by ANOVA with post hoc Scheffé's F analysis, unless otherwise stated. These analyses were performed with

the assistance of a computer program(SPSS 10.0 for windows).

RESULTS

Pi dose and time dependently induces calcium deposition in BASMC cultures

As shown in *Fig 1*, the effect of Pi on calcification of BASMC cultures was examined.In Pi 1.5 mmol/L groups, BASMC accumulated very little calcium mineral. In contrast, in Pi 2.0 mmol/L groups, calcium deposition dramatically increased in a time-dpendent manner[on day 9, Pi 2.0 mmol/L groups verse Pi 1.5 mmol/L groups:(125.399 ± 16.677) verse(29.046 ± 2.635) µg/mg protein, mean $\pm$ SEM, $n = 6$]. Furthermore, the
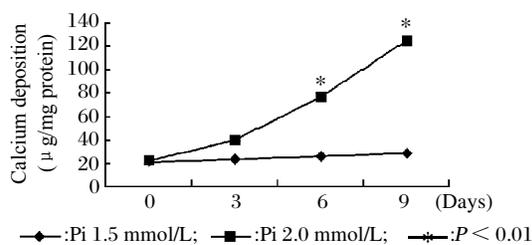

Fig 1 Effect of Pi on BASMC calcification

Phosphate elevated and PFA decreased osteocalcin level in supernatant of BASMC

The OC content in supernatant was collected after the fresh medium containing 15% FBS was incubated for 72 h with BVSMC in 6-well plates.Compared with Pi 1.5 mmol/L groups$[(2.981 \times 10^{-3} \pm 8.382 \times 10^{-4})$ ng/(µg · protein)], the levels of OC in Pi 2.0 mmol/L groups$[(1.503 \times 10^{-2} \pm 2.601 \times 10^{-3})$ng/(µg · protein)] and Pi 2.5 mmol/L groups$[(1.659 \times 10^{2} \pm 4.411 \times 10^{3})$ ng/(µg·protein)] were statistically elevated$(P < 0.001)$, but had no difference between Pi 2.0 mmol/L groups and Pi 2.5 mmol/L groups statistically. PFA decreased OC level statistically[Pi 2.0 mmol/L +PFA1.0 mmol/L groups:$(4.529 \times 10^{-3} \pm 1.250 \times 10^{-3}$)ng/(µg · protein), compared to Pi 2.0 mmol/L groups, $P < 0.001$]($Tab\ 1$).

Phosphate induced and PFA inhibited osteocalcin mRNA expression in BASMC

Compared with Pi 1.5 mmol/L groups,OC mRNA expression was increased statistically in Pi 2.0 mmol/L groups and Pi 2.5 mmol/L groups.PFA decreased Oc mRNA expression effectively$(P < 0.01;$ *Fig 3~6*).

DISCUSSION

Phosphorus is essential for multiple and diverse biological functions, including cellular signal transduction, mineral metabolism, and energy exchange. Serum phosphorus primarily occurs in the form of

effect of Pi was dose-dependent[on day 6, Pi 2.0 mmol/L groups verse Pi 1.5 mmol/L groups: (77.187 ± 11.692) verse(25.768 ± 1.750) µ g/mg protein, mean $\pm$ SEM, $n = 6$]. These results indicate that BASMC cultures are susceptible to calcification when cultured in media containing Pi concentrations typically observed in ESRD patients with hyperphosphatemia.

PFA inhibited calcium deposition in BASMC cultures

As shown in *Fig 2*, elevated phosphate promoted calcium deposition significantly$(P < 0.01)$, but PFA could inhibit calcium deposition statistically$(P < 0.01)$ in BASMC culture.
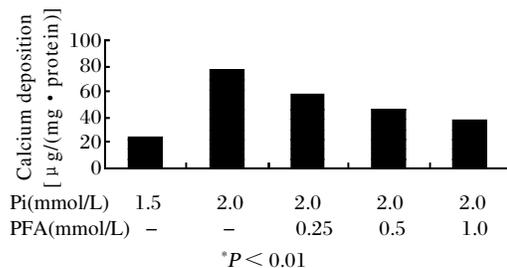

Fig 2 Efect of PFA on caicium deposition

inorganic phosphate, which is maintained within the physiological range by regulation of dietary absorption, bone formation, and renal excretion, as well as equilibration with intracellular stores[6]. A decline in renal function leads to phosphate retention, elevated parathyroid hormone(PTH) levels, and low 1,25-dihydroxy vitamin D levels. Phosphate excess may also influence mortality and cardiovascular risk by increasing circulating PTH or decreasing 1,25-dihydroxy vitamin D

Tab 1 Osteocalcin level in supernatant of BASMC

$(\overline{x} \pm s)$

Groups(mmol/L)	Osteocalcin[ng/(µg · protein)]
Pi 1.5	$2.981 \times 10^{-3} \pm 8.382 \times 10^{-4*}$
Pi 2.0	$1.503 \times 10^{-2} \pm 2.601 \times 10^{-3}$
Pi 2.5	$1.659 \times 10^{-2} \pm 4.411 \times 10^{-3}$
Pi 2.0+PFA 0.25	$1.114 \times 10^{-2} \pm 3.506 \times 10^{-3\diamond}$
Pi 2.0+PFA 0.5	$6.867 \times 10^{-3} \pm 1.619 \times 10^{-3*}$
Pi 2.0+PFA 1.0	$4.529 \times 10^{-3} \pm 1.250 \times 10^{-3*}$

Compared with Pi 2.0 mmol/L, $^*P < 0.001$, $^\diamond P < 0.05$.
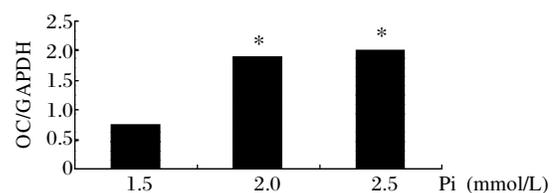
Compared with Pi 1.5 mmol/L, $^*P < 0.01$.

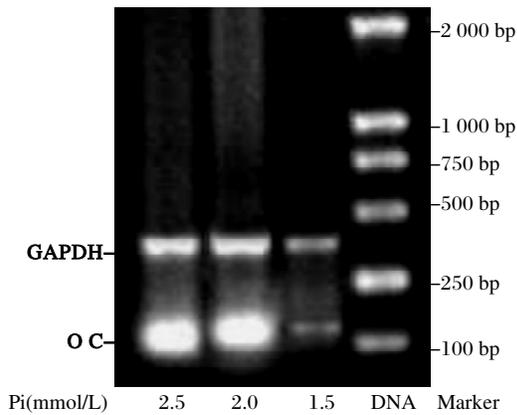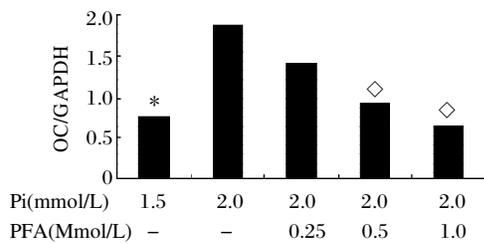**Fig 3** OC mRNA expression in different Pi concentrations of BVSMCs

Fig 4   OC mRNA expression in different Pi concentrations of BVSMCs

Compared with Pi 2.0 mmol/L, *P< 0.001, ◇ P< 0.01.

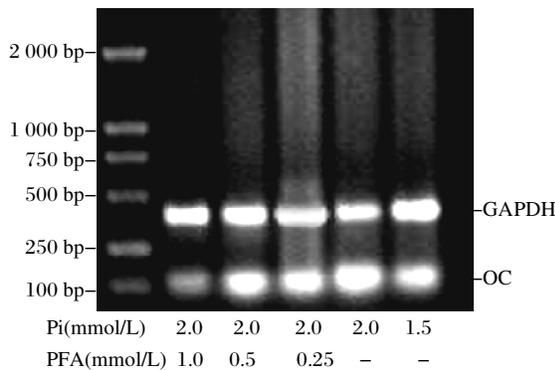***Fig 5***   Effect of PFA on OC mRNA expression in BASMC culture

Fig 6   Effect of PFA on OC mRNA expression in BASMC culture

levels. Elevated serum phosphate levels are associated with higher PTH levels among CKD patients, and hyperparathyroidism is associated with cardiovascular disease in states of abnormal and normal renal function[4,7-8]. Calcification has been positively correlated with coronary atherosclerotic plaque burden,increased risk of myocardial infarction, and plaque instability. These findings may explain that vascular medial calcification in large arteries leads to increased stiffness and therefore decreases compliance of these vessels[9-10]. These mechanical changes are associated with increased arterial pulse wave velocity and pulse pressure, and lead to impaired arterial distensibility, increased afterload favoring left ventricular hypertrophy, and compromised

coronary perfusion[11-12]. Thus, intimal and medial calcifications may contribute to the morbidity and mortality associated with cardiovascular disease[2]. In this study, Pi levels regulated the propensity of BVSMC cultures to calcify. Pi increased BVSMC calcification in a time- and dose-dependent manner(Pi<0.01 respectively).

It is becoming increasingly clear that vascular calcification is an actively regulated process that may be initiated by a number of different, nonmutually exclusive mechanisms[13-16]. These mechanisms have been extensively reviewed elsewhere and include:① loss of mineral inhibiting factors; ②induction of bone formation; ③cell death; and ④ circulating nucleational complexes (ie, aggregates of calcium phosphate and proteins released from remodeling bone that may initiate ectopic mineralization). Abnormalities in mineral metabolisms that enhance the calcium $\times$ phosphate product(Ca$\times$P) may further exacerbate vascular calcification initiated by any of these mechanisms. Recent evidences indicate that bone matrix proteins such as osteopontin, matrix Gla protein(MGP), and osteocalcin are expressed in calcified atherosclerotic lesions, and that calcium-regulating hormones such as vitamin D_3 and parathyroid hormone-related protein regulate vascular calcification in vitro(vascular calcification models based on cultured aortic smooth muscle cells). These findings suggest that vascular calcification is an actively regulated process similar to osteogenesis, and that bone-associated proteins may be involved in the development of vascular calcification[17].

In this study, compared with Pi 1.5 mmol/L groups, levels of OC in Pi 2.0 mmol/L groups and Pi 2.5 mmol/L groups was increased in BASMC cultures. Osteocalcin (also called BGP),composed of 49-50 aminos and three ɤ-Gla, molecular weight 5.6-6.5 kDa, is synthesized by dependent vitamin K and CO_2 enzyme compands. OC is combined with calcium in hydroxyapatite(HA) dependent negative charge existed in ɤ-Gla and promotes conversion from non-crystal calcium phosphate to HA.OC which increases bone mineral content and bone strength.OC is a major noncollagenous protein found in bone matrix and regarded as pathognomonic in osteoblastic terminal differentiation. The presence of OC suggests a role of a cell expressing an osteoblastic phenotype as a central component in vascular calcofication etiology[18].

Jono[19] speculates that under conditions of high extracellular phosphate or enhanced cellular sodium-dependent phosphate cotransporter(NPC) levels, intracellular levels of Pi are increased via the action of Pit-1[20-21]. This may lead to mechanisms initiating promineralizing metabolic processes within the cell. The first mechanisms may increase elaboration of an extra-cellular ma-

trix that is prone to mineralize. They found that elevated phosphate levels stimulated expression of both Cbfa-1 and its downstream transcriptional target, osteocalcin, in cultured cells. Similarly, elevated phosphate level was previously shown to induce osteopontin expression in bovine aortic SMCs. Cbfa-1 is an osteoblast-specific transcription factor required for osteoblast differe-ntiation, bone matrix gene expression, and consequently, bone mineralization. Cbfa-1 has been previously shown to directly regulate the expression of the major compo-nents of bone matrix including col-lagen type I, osteocalcin, and osteopontin[14].Thus, it is likely that phosphate-signal increases in Cbfa-1 gene expression in BVSMCs leading to enhanced transcrip-tion and se-cretion of an osteoid-like extracellular matrix that con-tributes to enhanced calcification under hyperphos-phatemic conditions.

Preventing hyperphosphatemia and elevated calcium x phosphorus product in renal failure ameliorates not only the progression of secondary hyperparathyroidism and bone disease but also the morbidity and mortality resulting from vascular calcification[22]. In this study, PFA could decrease calcium deposition and OC expres-sion in BASMC cultured.PFA is an antiviral agent, a specific, competitive inhibitor of sodium-dependent phosphate cotransporter(NPC)[14,18]. PFA can induce phosphaturia both after parenteral and oral administration. Furthermore, it can inhibit intestinal phosphate absorption when administered orally. PFA blunts the adaptive increase in intestinal and renal Na (+)-P(i) cotransport which accompanies dietary phos-phorus restriction. PFA is shown to inhibit hydroxya-patite crystal formation and calcium-phosphate precipi-tation when tested in in-vitro systems[23]. These properties, and the low toxicity of PFA, point to poten-tial new applications for PFA and some of its analogs in chronic renal insufficiency[14], but need to be further studied.

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