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Phosphate-Induced Rat Vascular Smooth Muscle Cell Calcification and the Implication of Zinc Deficiency in A7r5 Cell Viability

Mee-Young Shin and In-Sook Kwun

Department of Food Science and Nutrition, Andong National University, Gyeongbuk 760-749, Korea

ABSTRACT: The calcification of vascular smooth muscle cells (VSMCs) is considered one of the major contributors for vascular disease. Phosphate is known as the inducer for VSMC calcification. In this study, we assessed whether phosphate affected cell viability and fetuin-A, a calcification inhibitor protein, both which are related to VSMC calcification. Also, VSMC viability by zinc level was assessed. The results showed that phosphate increased Ca and P deposition in VSMCs (A7r5 cell line, rat aorta origin). This phosphate-induced Ca and P deposition was consistent with the decreased A7r5 cell viability ($P<0.05$), which implies phosphate-induced calcification in A7r5 cells might be due to the decreased VSMC cell viability. As phosphate increased, the protein expression of fetuin-A protein was up-regulated. A7r5 cell viability decreased as the addition of cellular zinc level was decreased ($P<0.05$). The results suggested that zinc deficiency causes the decreased cell viability and it would be the future study to clarify how zinc does act for VSMC cell viability. The results suggest that the decreased VSMC viability by high P or low Zn in VSMCs may be the risk factor for vascular disease.

Keywords: rat vascular smooth muscle cell (VSMC), cell viability, fetuin-A, phosphate, zinc

INTRODUCTION

Vascular smooth muscle cell (VSMC) calcification is the major phenomenon to induce atherosclerotic vascular calcification in cardiovascular disease (CVD). One of the theories of vascular calcification has been suggested vascular smooth muscle cells are differentiated to the osteochodrogenic cells with the presence of VSMC calcification stimulating factors, such as inflammatory cytokines lipids, etc and the higher concentration of circulating nucleators, such as P and Ca (1,2). Apart from these theories, one of the potential theories for vascular calcification is cell apoptosis of vascular environment (3,4).

Vascular smooth muscle cells are the particular type of cells as smooth muscle which mainly found within the wall of blood vessels. The blood vessel is composed of the lining endothelial cells (as intima part), and underneath VSMCs are reside as the major part of the blood vessel (as media part). Vascular smooth muscle contracts or relaxes to change the volume of blood vessels, thereby this mechanism can afford to redistribute blood supply to the organ and tissues where the blood is needed. The flexibility of vascular smooth muscle cells is the major critical characteristics to regulate the volume of blood in the body (4). VSMC calcification, therefore, is a major critical event for vascular calcification and it eventually causes cardiovascular disease, such as atherosclerosis and coronary heart disease, etc due to the stiffness of blood vessel.

Inorganic phosphorus in the body, mainly in the form of phosphate, is considered as the major candidate for vascular calcification (5). There are still on-going controversial hypotheses regarding that the hypothesis of how phosphate induces vascular calcification. One proposed mechanism is that phosphate induces vascular calcification is that blood vessel cells, such as vascular smooth muscle cells, are transforming to a osteogenic-phenotype cells, which was observed as the loss of VSMC marker protein (SM α-actin, SM22α, etc.) characteristics, while the gain of osteoblast cell marker proteins (such as Runx2, osteopontin, etc) (6,7). The other potential mechanism is that phosphate induces cell death, which causes the calcification of cell matrix. It is still not clear yet whether VSMC calcification is the origin of intracellular or extracellular, otherwise due to both origins, which is the case for the secretion of matrix vesicles by living cells or cellular apoptotic bodies from the dead cells (1).

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Correspondence to In-Sook Kwun, Tel: +82-54-820-5917, E-mail: iskwun@andong.ac.kr

As phosphate-induced apoptosis or cell viability are involved in vascular calcification, it can be considered that biometal zinc can also be considered as the modulator for VSMC viability, since various cellular zinc level in general is involved in cell viability. In the present study, using rat vascular smooth muscle cell line model A7r5, that 1) whether phosphate (Na phosphate) causes Ca and P deposition, 2) whether phosphate affects cell viability and 3) whether zinc affects VSMC cell viability. We also measured whether the expression of VSMC calcification inhibitor, fetuin-A, since this molecular can inhibit vascular calcification by resolve the matrix calcification.

MATERIALS AND METHODS

A7r5 cell culture and experimental design for phosphate and zinc addition

A7r5 cells (rat aortic vascular smooth muscle cell line, ATCC CRL-1444) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin; Gibco, Grand Island, NY, USA) until confluence.

Then, the cells were cultured with the normal VSMC growth media (GM) as normal control or with inorganic phosphate (as sodium phosphate, NaP; 0～10 mM) for up to 14 days. The medium was replaced freshly every 2～3 days. Cells were also cultured with zinc level (0, 1, 15 μM $ZnCl_2$) by adding zinc back to culture media with the chelexed FBS. Chelexed FBS provided zinc-depleted serum for the cell culture for cellular zinc depletion (8).

Ca deposition by Alizarin red S staining

Alizarin red S (Sigma, St. Louis, MO, USA) staining was used to assess Ca deposition in VSMC cell layers, by which Alizarin red S dye binds with Ca ions in cell layer matrix. Cells were fixed with 2% paraformaldehyde and stained with 1% Alizarin red S (pH 4.2). The culture plates were photographed under a light microscope and assessed for the mineralized nodules which shown as red.

Phosphate deposition by von Kossa staining

von Kossa staining was used to assess the accumulation of inorganic phosphate (as phosphate ion) in cell layers. Phosphate ions normally co-precipitates Ca ions, therefore phosphate accumulation can be used for the assessment for cellular calcification. Cells were treated with 3% silver nitrate (Sigma) solution and incubated at 37°C, after that culture plates were photographed under a light microscope. Mineralized nodules were shown as dark brown deposits (Santa Cruz Biotechnology, Dallas, TX, USA).

Cell viability by MTT assay

Cell viability was determined using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay, the principle of which is measured by reduction of MTT tetrazolium compound to its formazan products by mitochondrial dehydrogenase activity in viable cells. The formazan products were measured at 570 nm.

Cellular protein assay

To assess protein concentration by the addition of inorganic phosphate, BCA assay kit (Pierce, Rockford, IL, USA) was used. The BCA assay procedure was followed as manufacturer protocol as instructed.

Western blotting for fetuin-A protein

To determine the expression of fetuin-A, an inhibitor protein of VSMC calcification, Western immunoblotting was performed after separation on a 12% SDS polyacrylamide gel and blotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), using fetuin-A antibody (Santa Cruz Biotechnology). The secondary antibody was using a donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) and the signal for immunoblotting was detected by chemiluminescence detection using ECL western blotting detection (GE Healthcare UK Ltd., Amersham Place, UK) and taking image by chemiluminescence (Bio-Rad, Hercules, CA, USA). Fetuin-A control protein of rat liver extract (Santa Cruz Biotechnology) was loaded as the detection control. The loading amount of protein was normalized with GAPDH (Santa Cruz Biotechnology).

Statistical analyses

Values for the percent of cell viability and cell protein concentration are presented as mean±SEM. Statistical significance by phosphate concentration for cell viability and cell protein concentration was analyzed using the Statistical Package for Social Sciences (SPSS) version 20 (SPSS, Inc., Chicago IL, USA). $P<0.05$ was considered to be significant.

RESULTS

Ca and P deposition increased as phosphate increased in A7r5 cells

When A7r5 cells were cultured with phosphate (0, 1, 3, 5 and 10 mM) for 14 days, Ca deposition (shown as red by Alizarin red S staining) was increased as phosphate addition increased (at 5 and 10 mM) with time-dependent manner (Fig. 1A). The pattern of this Ca deposition

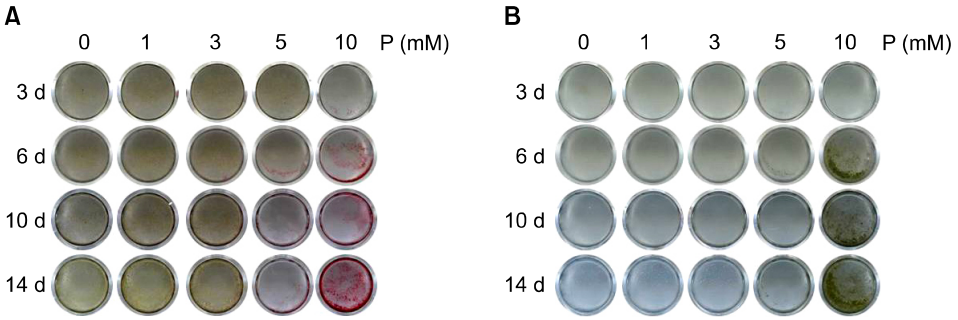

Fig. 1. Ca (A) and P (B) deposition were increased as phosphate increased in A7r5 cells. A7r5 cells were cultured with phosphate (0, 1, 3, 5 and 10 mM) up to 14 days. (A) Ca deposits were measured using Alizarin red S dye which binds with Ca in cell layer matrix. Ca deposition (as red) increased as phosphate increased. (B) P deposits was measured using von Kossa dye which binds with phosphate (as black) in cell layer matrix. Representative image of n=3.

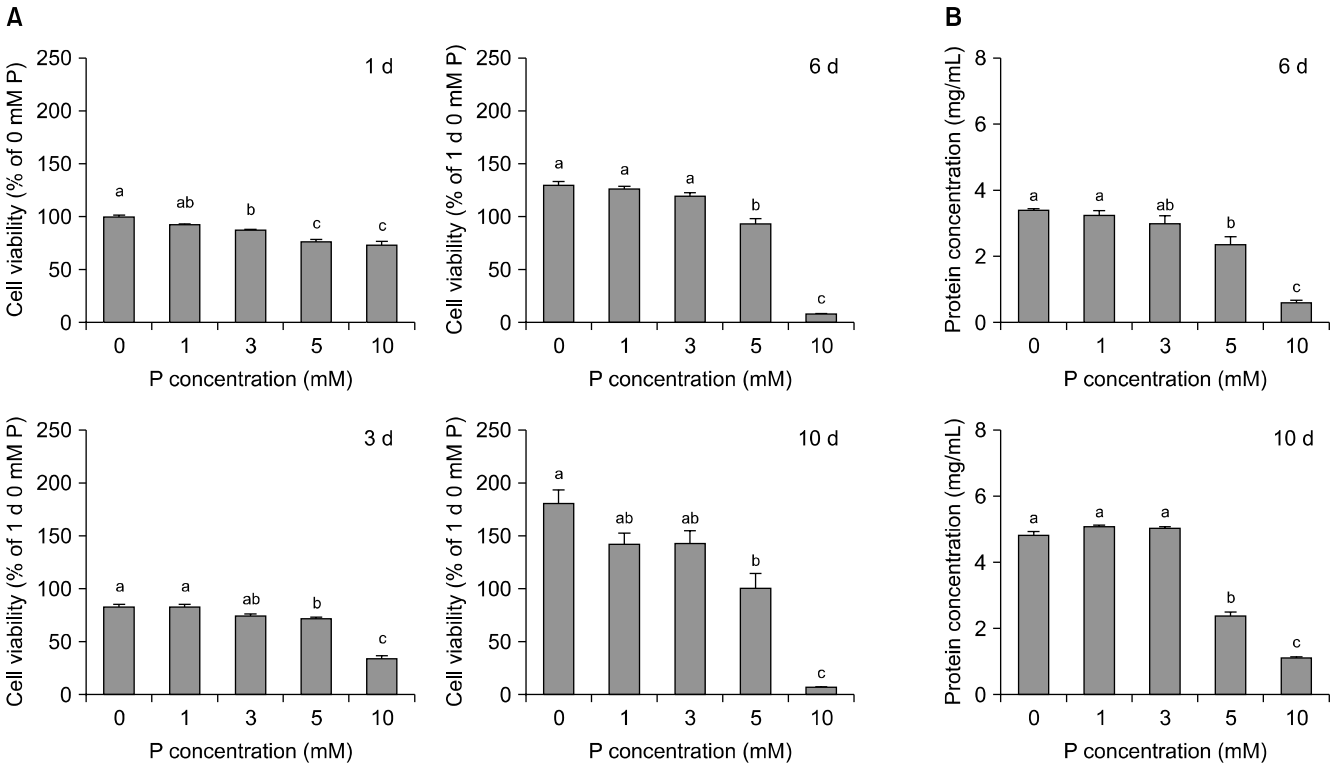

Fig. 2. Cell viability (A) and cellular protein synthesis (B) were decreased as phosphate increased in A7r5 cells. Cells were cultured with phosphate (0, 1, 3, 5 and 10 mM) up to 10 days. (A) Cell viability was measured by MTT assay and presented as % of normal growth media control of vascular smooth muscle cells (VSMC) without phosphate addition (0 mM P) in day 1. The effect of phosphate was statistically analyzed by one-way ANOVA at $P<0.05$ followed by Tukey as post hoc test, if the significance was observed. Different superscripts: Significantly different by phosphate concentration within each day. $P<0.001$ for 1 d. $P<0.05$ for 10 d. Mean±SEM (n=8) (B) Cellualr protein concentration was measured by BCA protein assay and presented as the same way in (A). Protein concentration is presented as mg protein/mL cell lysate from each well. Mean±SEM (n=3).

was also consistent with P deposition, which is shown in dark grey as silver nitrate combined with phosphate in A7r5 cell layers, by phosphate addition (Fig. 1B).

Cell viability and cellular protein synthesis were decreased, as phosphate addition was increased in A7r5 cells

Since the increasing phosphate addition to A7r5 cells, as rat vascular smooth muscle cell line, caused phosphate concentration-dependent Ca and P accumulation as shown in Fig. 1, it was determined whether cell viability and cellular protein synthesis were also affected by phosphate addition in A7r5 cells. A7r5 cell viability decreased, as the extracellular addition of phosphate increased and the pattern was consistently phosphate concentration-dependent manner as culture time went by (Fig. 2A). As cellular viability was shown inversely phosphate concentration-dependent manners, cellular protein concentration decreased as phosphate addition increased (Fig. 2B).

The expression of fetuin-A, an inhibitor protein for VSMC calcification, up-regulated as VSMC calcification condition was increased by adding phosphate

It was questioned whether the expression of VSMC calcification inhibitor protein was affected by phosphate addition, which is the condition for VSMC calcification. The protein expression of fetuin-A, as one of the VSMC calcification inhibitor proteins along with osteopontin and matrix gla-protein (MGP), etc, by external phosphate addition to A7r5 cells was up-regulated, as phosphate addition increased (Fig. 3). The detection of 10 mM P culture was not available, due to cells dying on collection.

Zinc increased VSMC viability under phosphate-added culture

A7r5 cells were cultured with zinc (GM as normal growth media, 0 and 1 μM as low Zn level and 15 μM as high Zn level) under 0, 3 and 5 mM phosphate culture condition for 5 days. Phosphate addition decreased A7r5 cell viability (in comparison of GM by phosphate level) (Fig. 4). Particularily, under 5 mM phosphate condition (at high phosphate level of calcifying condition), A7r5 cell viability showed zinc concentration-dependent manner, which implies that zinc can protect phospahte-induced VSMC viability for 5 days of culture period.

DISCUSSION

Phosphate and Ca/P deposition

Phosphate, whether is the form of inorganic (such as sodium phosphate) or organic (such as β-glycerol phosphate), is considered as the inducer of vascular calcification. In physiologically normal hard tissue, like bone, phosphate acts as the nucleator of extracellular matrix calcification which is the essential part of osteogenic calcification (9,10). The role of phosphate has gained the attention such as in soft tissue like blood vessel, since vascular calcification where pathological calcification is observed in blood vessel cellular environment (11,12).

In the present study, we tested whether inorganic phosphate induced Ca and P deposition in VSMC cells which are the most abundant cell type and therefore composed of the intima of blood vessel. Phosphate addition induced and increased Ca and P deposition, which are the characteristics of calcification (mineralization) in normal hard tissue in bone (Fig. 1).

Phosphate, VSMC calcification and VSMC viability

In the following examination of this VSMC viability by phosphate addition, cell viability was consistently decreased as external phosphate was added in phosphate concentration-dependent manner. From the present study, as phosphate externally increased to be added, VSMC Ca and P deposition increased (Fig. 1), while VSMC viability decreased (Fig. 2). This result implies that phosphate-induced VSMC calcification is due to the decreased cell viability, it might be VSMC apoptosis (1).

With the present data only, VSMC Ca and P accumulation in the layers of A7r5 cells (rat aortic vascular smooth muscle cells line), it is not clear to judge whether this Ca and P accumulation in VSMCs was originated from intracellular microcalcifications within vascular smooth muscle cells or, extracellular Ca and P accumulation, as cells die, it causes to Ca and P deposition in extracellular matrix. From the decreased VSMC viability by phosphate addition from the present data, it can be

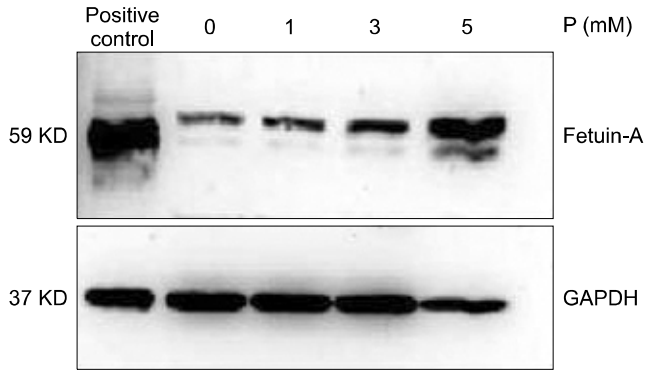

Fig. 3. The expression of fetuin-A, an inhibitor protein for VSMC calcification, upregulated as extracellular phosphate added in A7r5 cells. A7r5 cells were cultured with phosphate (0, 1, 3, 5 and 10 mM) for 10 days. Cell collection from 10 mM P culture was not available at collection day, since the cells were died. Cellular fetuin-A protein expression was measured using Western blotting. Molecular weight of fetuin-A: 59 kDa. Positive control: rat liver extract for the detection by Western blotting. GAPDH was used as the internal loading control.

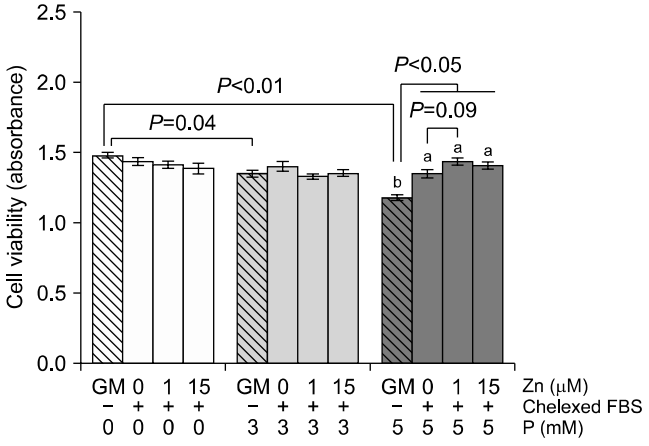

Fig. 4. Zinc increased VSMC viability under phosphate-added culture condition. A7r5 cells were cultured with various level of zinc addition (normal growth media, 0 and 1 (as low Zn level) and 15 M (as high Zn level) under 0, 3 and 5 mM phosphate condition for 5 days. VSMC viability was lower in high phosphate addition (5 mM P), with the zinc cencentration-dependent cell viability pattern. Mean±SEM (n=8).

assumed that cells die first by phosphate, then making Ca position, but still well-regulated experiments are needed to answer for this phosphate-induced VSMC calcification.

Zinc deficiency and its VSMC viability

Biometal zinc affects VSMC viability in this study as extrcellular zinc level decreased, cell viability decreased. Recently, the implication of zinc deficiency in VSMC integration is supposed from the work of transcriptomic study using (13). The mechanism for VSMC apoptosis by zinc is also suggested that the apoptotic cell bodies and debris are accumulated at extracellular matrix to be calcified (1). In their study, zinc deficiency increased cell apoptosis by decreasing the phosphorylation of Bcl-2-associated death promoter (BAD) protein, a member of Bcl family proteins, by which the phosphorylation of BAD which makes the cell being stable from the cell apoptosis (14). It would be the future concern to clarify how zinc deficiency-induced apoptosis is involved in VSMC integration to protect vascular disease.

Phosphate-induced calcification and fetuin-A expression

It is wondered whether phosphate affects the VSMC calcification inhibitor protein synthesis such as fetuin-A as an inhibitor proteins of VSMC calcification. Fetuin-A is blood protein being secreted into the bloodstream mainly made by liver cells as well as VSMCs in body system (15-17). This protein is regarded as a potent inhibitor of systemic calcification, since knocking out the gene for fetuin-A in mice showed insulin irregulation and widespread calcification (ectopic mineralization) in system (18). The results from the present study showed that the fetuin-A protein expression in A7r5 cells increased as external phosphate addition increased (as the calcifying condition was increased with the addition of phosphate). It seems from the present protein detection data using Western blotting that, as phosphate increased in VSMC culture, which is the culture condition for calcifying culture condition, fetuin-A synthesis up-regulates to inhibit calcification of VSMCs. This seems fetuin-A synthesis responses the negative feedback to protect VSMC calcification with more VSMC calcification inhibitor protein in cellular condition.

Taken all together, it can be suggested in conclusion that phosphate induces Ca and P deposition in VSMCs which can be the sign for vascular calcification and this phosphate-induced VSMC calcification seems to be paralleled with the decreased VSMC viability. Again, zinc deficiency in VSMCs would be the potential condition where VSMC viability can be occurred for the formation of vascular calcification. It would be of interests to identify how zinc regulates vascular calcification in the future study.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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