Life Sciences 209 (2018) 466–471

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Protein kinase A (PKA) inhibition reduces human aortic smooth muscle cell calcification stimulated by inflammatory response and inorganic phosphate

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ARTICLE INFO

Keywords:
Calcification
Cytokine
Inflammation
Macrophage
Phosphate
Smooth muscle cell

ABSTRACT

Aims: Smooth muscle cells (SMCs) play a role in medial vascular calcification, which can be stimulated by high levels of serum phosphate and inflammatory mediators. The aim of this study was to investigate whether mitogen-activated protein kinases (MAPKs) (p38 MAPK, ERK1/2, and JNK) and protein kinase A (PKA) can participate in inorganic phosphate (Pi)- and inflammation response-stimulated SMC calcification.
Main methods: We examined the change of Pi- and/or inflammation-stimulated human aortic smooth muscle cell (HASMC) calcification in the presence and absence of inhibitors or small interfering RNAs.
Key findings: Ca levels were increased in HASMCs incubated with 1.5–3.9 mM Pi, but not with 0.9 mM Pi or compared with non-Pi-treated HASMCs. Furthermore, the addition of interferon-γ (IFN-γ) increased pro-inflammatory cytokines [interleukin (IL)-1α, IL-6, and tumor necrosis factor-α (TNF-α)] in media containing Raw 264.7 cells. Ca levels were significantly increased in HASMCs cultured in IFN-γ-treated medium, compared with non-IFN-γ-treated medium in the presence of Pi (0.9–2.4 mM). The inhibition of p38 MAPK and PKA decreased HASMC calcification stimulated by Pi and IFN-γ-treated medium, though PKA inhibition produced a more significant reduction in calcification than p38 MAPK inhibition.
Significance: These results indicate that PKA inhibition can efficiently reduce Pi- and inflammation-stimulated SMC calcification.

1. Introduction

Vascular calcification is commonly found in patients with atherosclerosis, diabetes, and chronic kidney disease (CKD) and is associated with an increased risk of cardiovascular diseases [1–3]. Most patients with cardiovascular diseases show vascular calcification, to different degrees of severity. For example, significant coronary artery calcification was identified in 65.8% of 3683 patients undergoing cardiac catheterization [4].

Vascular calcification can be divided into three types, depending on location: intimal, medial, and valvular. Intimal calcification is observed mainly in atherosclerotic regions that contain inflammation and lipid deposition. Medial calcification shows the characteristics of calcium deposition along the elastic lamellae and is associated with increased arterial stiffness. Valve calcification occurs in the aortic valve of the heart and involves an inflammatory response, as well as calcium or lipid deposition [3,5,6].

Smooth muscle cells (SMCs) are a major cell of the medial layer and are involved in medial calcification. SMC calcification can be stimulated by multiple factors, such as phosphate, transforming growth factor-β1, acetylated low density lipoprotein, endothelin-1, bone morphogenetic protein-2, Runt-related transcription factor 2 (Runx2), leptin, alkaline and phosphatase (ALP) [7,8]. During medial calcification, elevated serum phosphate levels can stimulate the transformation

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https://doi.org/10.1016/j.lfs.2018.08.051
Received 23 May 2018; Received in revised form 17 August 2018; Accepted 21 August 2018
Available online 23 August 2018
0024-3205/

of SMCs to osteoblast-like cells, which show a loss of SM markers such as SM-α-actin and SM22α, and the induction of osteogenic markers, such as ALP, osteopontin, Runx2, and osteocalcin [6,9–12]. Moreover, phosphate uptake by SMCs, through sodium-dependent phosphate co-transporters such as Pit-1 and -2, activates SMC osteochondrogenic differentiation [6,13].

Inflammatory responses can also stimulate the calcification of SMCs. For example, the addition of inflammatory cytokine tumor necrosis factor-α (TNF-α) increases the calcification of SMCs by stimulating the expression of osteochondrogenic markers such as ALP and Runx2 [14–16]. SMC calcification is also increased in a monocyte/SMC co-culture system, with or without inflammatory stimulators [(e.g., interferon-γ (IFN-γ)] [17,18]. The addition of media obtained from lipopolysaccharide-stimulated macrophages can also induce SMC calcification [19].

Protein kinase A (PKA) is activated by the binding of cyclic AMP (cAMP) to its regulatory domain and participates in the regulation of a variety of cellular functions [20]. Several studies suggested that PKA plays a key role in the inorganic phosphate (Pi)-induced calcification of SMCs [12,21,22]. However, further studies are needed to elucidate whether PKA can be involved in Pi- and inflammation-induced SMC calcification. On the other hand, there are few data available concerning the effect of mitogen-activated protein kinases (MAPKs) [p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK)] on Pi- and inflammatory response-stimulated SMC calcification.

The purpose of this study was to investigate whether Pi and inflammatory response can synergistically stimulate SMC calcification, and MAPKs (p38 MAPK, ERK1/2, and JNK) and PKA can participate in Pi- and inflammatory response-stimulated SMC calcification.

2. Materials and methods

2.1. Cell culture and Pi-mediated calcification

Human aortic smooth muscle cells (HASMCs; KS-4009; Kurabo Biomedical, Osaka, Japan) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (high glucose; Wako, Osaka, Japan). Media were supplemented with smooth muscle growth supplement (SMGS; Cascade Biologics, Tokyo, Japan), 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (all Gibco, Invitrogen Co., Grand Island, NY, USA). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Sixth passage cells were used for all experiments. To prepare the in vitro model of HASMCs calcification, cells (1×10^5 cells) were incubated in DMEM with SMGS for 24 h in a 6-well plate. At 24 h, the medium was changed to DMEM without SMGS and cells were further incubated for 24 h. After 24 h, HASMCs were induced with Pi (NaH_2PO_4; 0, 0.9, 1.5, 2.4, and 3.9 mM) for six days, with the medium being changed every two days. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; DOJINDO Laboratories, Kumamoto, Japan) according to manufacturer's instructions.

2.2. IFN-γ stimulation

Raw 264.7 cells (5×10^5; ECACC 91062702; DS Pharma Biomedical, Osaka, Japan) were maintained in DMEM medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 h of IFN-γ treatment (40 ng/ml) (Mouse IFN-γ research grade; Miltenyi Biotec, Tokyo, Japan), cells were washed with serum-free DMEM medium, and fresh DMEM containing 10% FBS was added. After incubation at 37 °C for 24 h, the media were collected and the levels of cytokines were detected using a Ready-SET-Go ELISA kit (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Non-IFN-γ-treated media (PBS-treated media) were also collected after 24 h of Raw 264.7 cell incubation and were used as controls. The collected media were stored at −80 °C and used for the calcification experiment.

2.3. HASMC calcification stimulated with Pi and IFN-γ-treated medium

HASMCs were maintained in DMEM without SMGS for 24 h in a 6-well plate. After 24 h, cells were incubated in either the IFN-γ (40 ng/ml)-treated or non-IFN-γ-treated medium with Pi (0, 0.9, 1.5, and 2.4 mM) for six days, with the medium being changed every two days. To investigate the effects of PKA and MAPKs on HASMC calcification, inhibitors of PKA (H-89), p38 MAPK (SB202190), ERK1/2 (PD184352), and JNK (SP600125) (all 10 μM, Sigma-Aldrich, St. Louis, MO) were added to HASMCs stimulated with Pi (2.4 mM) and IFN-γ (40 ng/ml)-treated medium for six days, with the medium being changed every two days. The concentrations of inhibitors were chosen based on previous studies [23,24].

2.4. RNA interference

The small interfering RNAs (siRNAs) (SMARTpool p38 MAPK and PKA) were purchased from Thermo Fisher Scientific (Lafayette, CO, USA). Each siRNA (100 nM/well) was transfected twice in a 72-h interval into HASMCs by using Lipofectamine 2000 according to manufacturer's instructions. The levels of Ca were detected at six days post transfection.

2.5. Detection of ca concentration

HASMCs were washed three times with PBS (Ca^{2+} and Mg^{2+} free) and incubated with 0.6 N HCl for 24 h. The supernatant was used for detecting Ca levels using a QuantiChrom™ calcium assay kit (BioAssay Systems, Hayward, CA, USA). Ca levels were normalized against total protein concentration. Total protein concentration was analyzed by absorbance at 595 nm using a Bio-Rad Protein Assay Dye reagent (Bio-Rad Lab., Hercules, CA, USA) according to manufacturer's instructions.

2.6. Assay of ALP activity

Intracellular ALP activity in HASMCs was measured using a LabAssay ALP kit (Wako, Tokyo, Japan), according to manufacturer's instructions. Protein concentration in the cell lysate was evaluated by using the Bio-Rad Protein Assay Dye Reagent and the ALP activity were normalized against total protein concentration.

2.7. Von Kossa staining

HASMCs were fixed with 10% formalin for 30 min and washed with distilled water (DW). The HASMCs were treated with 5% silver nitrate solution for 1 h under UV light and washed with DW. After placing the HASMCs in 5% sodium thiosulfate solution for 5 min, they were rinsed with DW. All images were obtained using standard inverted light microscopy.

2.8. Statistical analysis

All results are expressed as the mean and standard deviation. Statistically significant differences between groups were evaluated by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA).

3. Results

3.1. Pi-induced HASMC calcification

Ca levels were increased in HASMCs incubated with 1.5–3.9 mM Pi, but not in HASMCs treated with 0.9 mM Pi, compared with non-Pi-treated HASMCs (Fig. 1A). A significant reduction of cell viability was