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Phosphatidylinositol 3-kinase/Akt Pathway Regulates Inflammatory Mediators-induced Calcification of Human Vascular Smooth Muscle Cells

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Abstract

Background

Vascular calcification is a clinically significant component of atherosclerosis and may be promoted by inflammatory stimuli. Phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is involved in the regulation of cell metabolism, survival, migration, and inflammatory responses in various cell types.

Methods

In this study, we investigated the roles of PI3K/Akt signaling pathway in human vascular smooth muscle cell (HVSMC) calcification induced by the inflammatory mediators (IM) including interferon- γ , tumor necrosis factor- α , oncostatin M, and 1 α ,25-dihydroxyvitamin D₃.

Results

Pharmacological inhibition of PI3K with wortmannin dose-dependently increased IM-induced HVSMC calcification. IM-induced expression of alkaline phosphatase (ALP) in HVSMC was also augmented by wortmannin, while wortmannin did not induce apoptosis of HVSMCs in the presence or absence of IM. Moreover, wortmannin inhibited Akt activation in HVSMC by short-term exposure to IM. Overexpression of wild-type or dominant-negative forms of Akt significantly attenuated or enhanced IM-induced ALP expression in HVSMC, respectively. Furthermore, suppression of Akt with siRNA significantly intensified IM-induced ALP expression in HVSMC.

Conclusions

These data suggest that PI3K/Akt pathway may play an inhibitory role in IM-induced HVSMC calcification through regulating ALP expression.

Key Words: Atherosclerosis; Calcification; Osteoblast; Inflammation

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Introduction

Vascular calcification is a common and clinically significant component of atherosclerosis. Coronary artery calcification is pathognomic of coronary atherosclerosis and is an independent marker of cardiovascular risk providing incremental prognostic value over traditional and emerging risk markers¹. The quantitative measurement of coronary calcium has been demonstrated to be a good predictor of cardiovascular events including acute myocardial infarction, sudden death, and coronary revascularization²⁻⁵.

Atherosclerosis is a chronic vascular inflammatory process. Calcified plaque lesions contain not only various components associated with bone mineralization, including osteoblast-like vascular cells, matrix vesicles, alkaline phosphatase (ALP), bone morphogenetic proteins, osteocalcin, and osteopontin, but also inflammatory cells such as macrophages and lymphocytes⁶. Therefore, atherosclerotic calcification may be an osteogenic process associated with chronic vascular inflammation.

Recent studies suggest that inflammatory process may be involved in vascular calcification. Monocytes/macrophages and proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) can enhance in vitro calcification of vascular cells⁷. We have also demonstrated the important roles of macrophages in vascular calcification by using a coculture of human vascular smooth muscle cells (HVSMCs) with THP-1 macrophages and developed an in vitro model of vascular calcification induced by inflammatory mediators such as interferon- γ (IFN- γ), 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), TNF- α , and oncostatin M (OSM)⁸.

Phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B, PKB) signaling pathway is crucial to widely divergent physiological processes that include cell cycle progression, differentiation, transcription, translation, and apoptosis^{9,10}. Serine/threonine kinase Akt/PKB is a crucial kinase in this pathway. A disturbed activation of PI3K/Akt pathway has been associated with the development of diseases such as cancer and diabetes mellitus. Recently, the role of Akt1 in atherogenesis in vivo has been clarified¹¹. The genetic ablation of Akt1 on an apoE knockout background increased aortic plaque lesion formation and promoted coronary atherosclerosis through enhancing expression of proinflammatory genes and endothelial cell and macrophage apoptosis. Moreover, disruption of Akt1 in mice led to low-turnover osteopenia associated with osteoblast dysfunctions including the increased susceptibility to apoptosis and the decreased transcriptional activity of runt-related transcription factor 2 (Runx2)¹². Therefore, it is likely that PI3K/Akt signaling pathway may play an important role in the development of atherosclerotic calcification, an osteogenic process associated with atherosclerosis.

In this study, we investigated the roles of PI3K/Akt signaling pathway in HVSMC calcification induced by inflammatory mediators (IM) including IFN- γ , 1,25(OH)₂D₃, TNF- α , and OSM. We found that PI3K/Akt pathway plays an inhibitory role in IM-induced HVSMC calcification through regulating ALP expression, suggesting that this pathway may exert protective functions against the development of atherosclerotic calcification.

Methods

Reagents

Media and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). 1,25(OH)₂D₃ and β -glycerophosphate (β -GP) were obtained from Sigma (St. Louis, MO).

Recombinant IFN- γ , TNF- α , and OSM were purchased from Genzyme/Techne (Cambridge, MA). Wortmannin was obtained from Calbiochem (La Jolla, CA). Anti-Akt and anti-phospho Akt (Ser473) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti- α -smooth muscle mouse monoclonal antibody was obtained from Sigma (St. Louis, MO). Unless otherwise mentioned, all other reagents were obtained from Wako (Osaka, Japan).

Cell Culture

Primary HVSMCs derived from neonatal umbilical arteries were obtained from Cell Systems (Kirkland, WA), maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS containing 100 U/mL of penicillin and 100 μ g/mL of streptomycin, and passaged weekly using trypsin-EDTA (Sigma, St. Louis, MO).

Induction of HVSMC Calcification by Inflammatory Mediators

HVSMCs were cultured for the indicated period of time with 10 mmol/L β -GP in the presence of IFN- γ (100 ng/mL), 1,25(OH) $_2$ D $_3$ (10^{-7} mol/L), TNF- α (1.0 ng/mL), and OSM (10 ng/mL) as previously described⁸. Calcium deposits of cell layer were assessed by *o*-cresolphthalein complexone method (Calcium C-test Wako) and normalized by protein content as previously described⁸.

ALP Assay

After the cells were washed twice with PBS, the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were assayed for ALP activity as described previously⁸. One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

Western Blot Analysis

HVSMC lysates were prepared and analyzed as previously described⁸. Anti-phospho-Ser473Akt, anti-Akt, and anti- α -smooth muscle actin antibodies were used at dilutions with 1:1000, 1:1000, and 1:500, respectively.

Real Time Quantitative Reverse Transcription Polymerase Chain Reaction (real time qRT-PCR)

Real time qRT-PCR was performed using the 5' fluorogenic nuclease assay and an ABI Prism 7000 (Applied Biosystems, Foster City, California, USA) to determine the relative abundance of assayed mRNAs. Samples were normalized by determining the relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The 5' terminus of fluorogenic probes was labeled with FAM (6-carboxy-fluorescein) or VIC and the 3' terminus contained the quenching dye TAMRA (6-carboxytetramethyl-rhodamine). Taqman primer-probe sets (Taqman Assay-on-Demand Gene Expression Products) for human ALP and GAPDH were obtained from Applied Biosystems. PCR reactions contained Universal Master Mix (Applied Biosystems), specific primers, Taqman probe, and cDNA. Two-step PCR cycling was carried out as follows: 50°C 2 min for 1 cycle, 95°C 10 min for 1 cycle, and 95°C 15 sec, 60°C 1 min for 40 cycles. The relative levels of mRNA of a specific gene were calculated using standard curve generated from cDNA of SaOS2 cells.

Plasmids, siRNA and Nucleofections

The dominant negative Akt plasmid was a gift from Dr. J.R.Woodget (University of Toronto,

Toronto, Canada). The wild-type mouse AKT1 expression plasmid was prepared as described¹³⁾. Akt1/2/3 siRNAs were obtained from New England Biolabs (London, England). HVSMCs were resuspended in a nucleofector solution, mixed with Plasmids or siRNA in the Amaxa cuvette, and directly placed in the nucleofector device. HVSMCs were treated with the Program U-25. The program used has been optimized and recommended by the manufacturer. Cell suspension was removed immediately from the cuvette by adding prewarmed medium with the Amaxa pipette. Cell suspension was added to a 6-well plate containing prewarmed medium and incubated at 37°C for 24 hr. The cells were used for the experiments 24 hr after nucleofection.

Evaluation of apoptosis

The Cell Death Detection ELISA Kit (Roche Applied Science, Mannheim, Germany) was used to detect mono-oligonucleosomes (histone-associated DNA fragments) as an indicator of apoptosis. Briefly, cytoplasmic lysates were transferred to a streptavidin-coated plate supplied by the manufacturer. A mixture of biotin-labeled anti-histone and peroxidase-labeled anti-DNA antibodies were added to cell lysates and incubated for 2 hr. The complex was then simultaneously conjugated to form an immune complex on the plate, which then was read for optical density at 405 nm with a reference wavelength at 490 nm.

Statistics

In certain experiments, data were analyzed for statistical significance by ANOVA with post hoc analysis (Fisher's protected least significant test). These analyses were performed with the assistance of a computer program (StatView version 5.0, Abacus Concepts, Berkeley, CA).

Results

To clarify the potential involvement of PI3K/Akt signaling pathway in vascular calcification, we first examined the effects of a specific inhibitor of PI3K, wortmannin on IM-induced calcification of HVSMCs. Wortmannin significantly increased HVSMC calcification in a dose-dependent manner as compared with the control cultures treated with IM (Fig. 1). Since IM-induced HVSMC calcification is dependent on the induction of ALP in HVSMCs by IM as previously demonstrated, we next examined the effects of wortmannin on ALP expression in HVSMCs. Wortmannin significantly augmented ALP activities and its mRNA expression in HVSMC in a dose-dependent manner as compared with the IM-treated cultures (Figs. 2A and 2B). Since the inhibitory role of PI3K/Akt in apoptosis associated with vascular calcification has been indicated¹⁸⁾, we examined whether wortmannin induces apoptosis. Wortmannin did not induce apoptosis of HVSMCs in the presence or absence of IM as evidenced by ELISA quantification of histone-associated DNA fragments (Fig. 2C).

Since one of the major downstream targets of PI3K is the Akt kinase, we next examined if IM can induce phosphorylation of Akt in HVSMCs in a short time-course (0-120 min). Activation of Akt was detected at 15 min and peaked within 30 min after the addition of IM. Moreover, 0.1 μ M of wortmannin inhibited activation of Akt by IM (Fig 3). However, phosphorylation of Akt was not detected in HVSMCs by the 72 hr-exposure of IM in the presence of 10% FCS (data not shown). These data suggest that PI3K/Akt pathway plays an important role in inducing ALP and subsequent in vitro calcification of HVSMCs by IM.

To confirm the role of Akt in acquisition of calcifying phenotype of HVSMCs by IM, we next investigated the effects of modulation of Akt function by nucleofection of either wild type Akt1 or

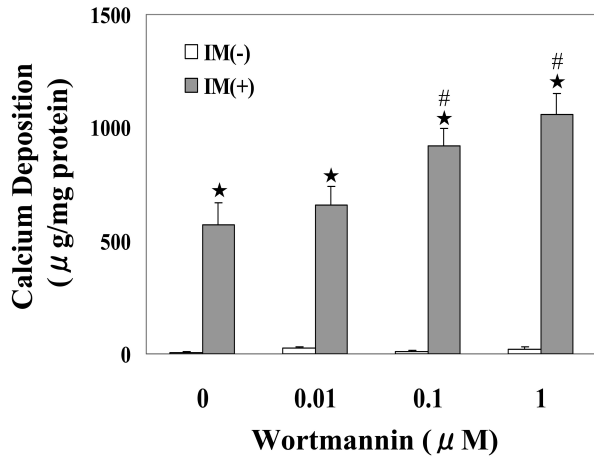


Figure 1. Effects of wortmannin on in vitro calcification of HVSMCs. HVSMCs were treated for 10 days with wortmannin at the indicated concentrations. HVSMCs were cultured in the media containing 10 mmol/L β -glycerophosphate in the presence or absence of the inflammatory mediators (100 ng/mL of IFN- γ 10^{-7} mol/L of $1,25(\text{OH})_2\text{D}_3$, 1.0 ng/mL of TNF- α , and 10 ng/mL OSM) (IM) as described. Calcium deposition was quantified by *o*-cresolphthalein complexone methods. The data are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM.

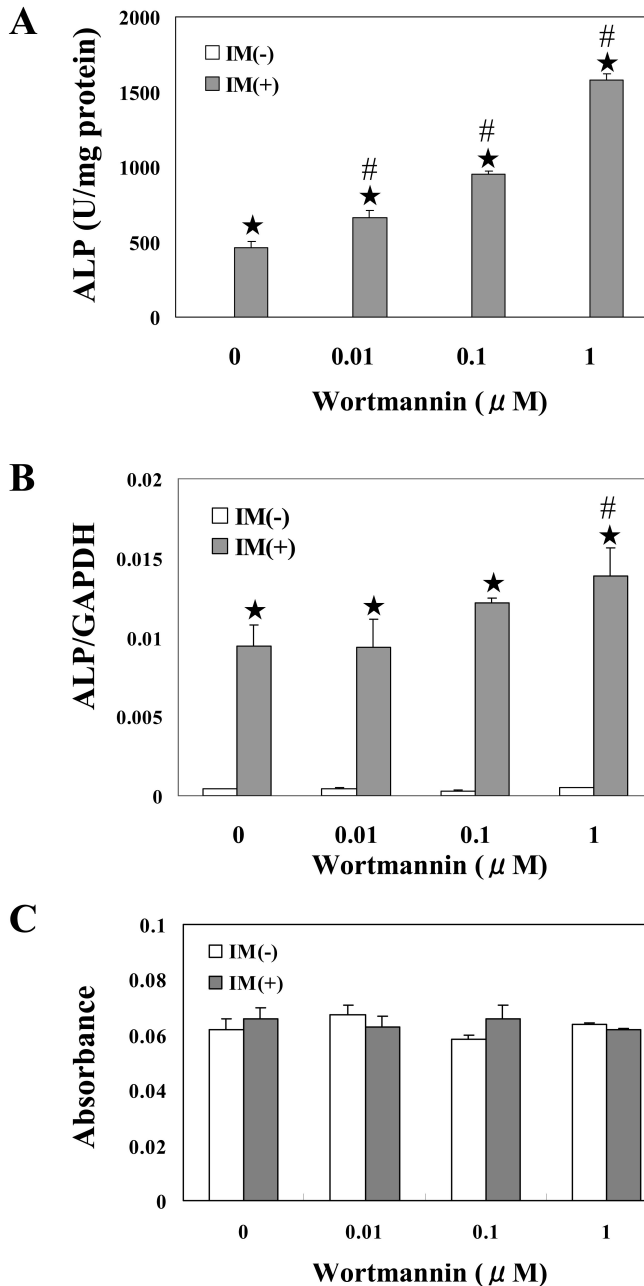


Figure 2. A, Effects of wortmannin on ALP activities in HVSMCs. HVSMCs were incubated for 3 days in the presence or absence of the inflammatory mediators (IM) with wortmannin at the indicated concentrations. ALP activities were measured, normalized to cellular protein contents, and are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM. B, Real time quantitative RT-PCR analysis of mRNA expression of ALP in HVSMCs. HVSMCs were cultured for 24 hr under the indicated conditions and total RNA was harvested and utilized for the experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM. C, Effects of wortmannin on apoptosis of HVSMCs. HVSMCs were treated with the indicated reagents for 72 hr and then the levels of mono-oligo nucleosome fragments was quantified using Cell Death Detection ELISA Kit. The data are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively.

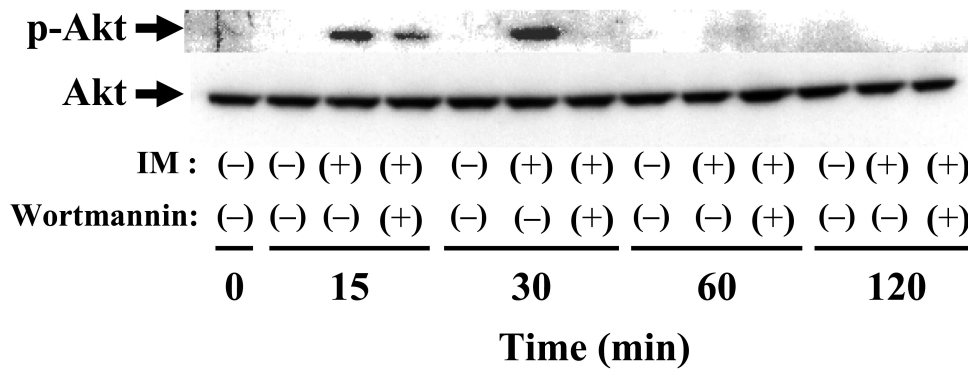


Figure 3. Activation of Akt by the inflammatory mediators (IM) in HVSMCs. HVSMCs were cultured with DMEM containing 0.2% FCS for 48 hr and then incubated in the presence or absence of IM and wortmannin for the indicated time periods. Cell lysates were analyzed with Western blot using antibodies recognizing phospho-Akt (p-Akt) and total Akt (Akt) as described. Equal total Akt expression was monitored by Western blot using an antibody specifically recognizing total Akt. (–) and (+) indicate the absence and presence of the reagents, respectively.

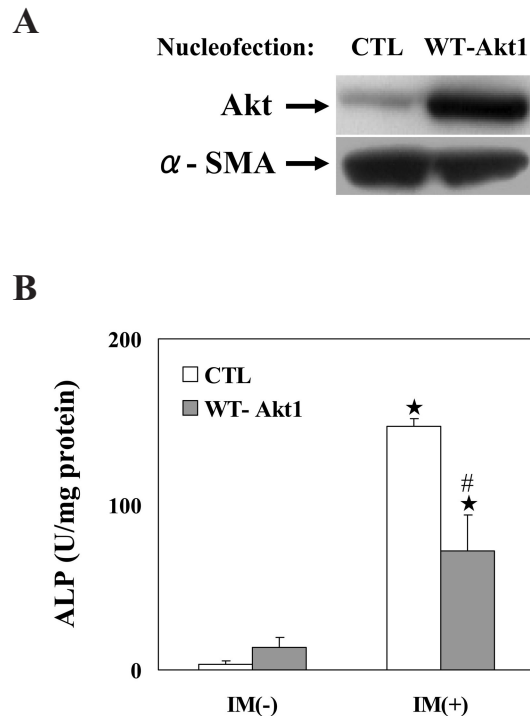


Figure 4. Effects of Akt overexpression on ALP activities in HVSMCs. HVSMCs were nucleofected with control (CTL) and wild-type Akt1 (WT-Akt1) plasmids as described. A, Forty-eight hours after nucleofection, the cells were harvested and used for western blot analysis of Akt and α -smooth muscle actin (α -SMA) expression. B, Forty-eight hours after nucleofection with control (CTL) and wild-type Akt1 (WT-Akt1) plasmids, the cells were incubated for an additional 48 hr in the presence or absence of IM and then ALP activities were quantified as described. The data are presented as mean \pm SEM (n=3). IM (–) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM.

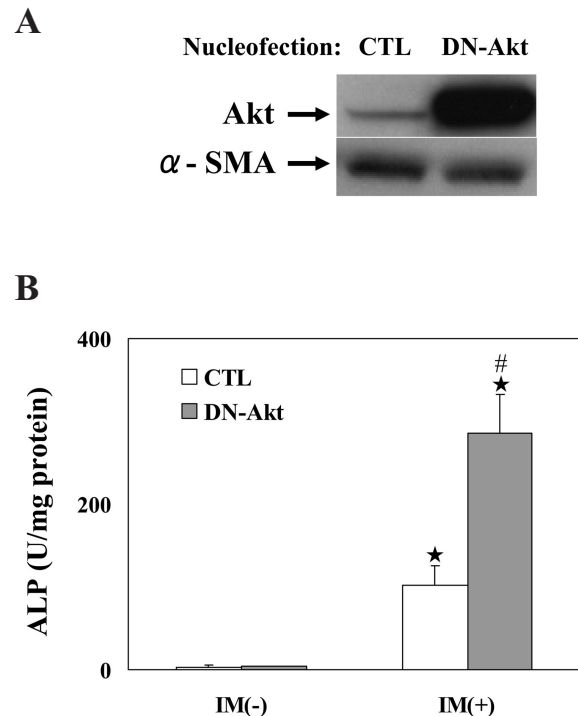


Figure 5. Effects of dominant negative Akt on ALP activities in HVSMCs. HVSMCs were nucleofected with control (CTL) and dominant negative Akt (DN-Akt) plasmids as described. A, Forty-eight hours after nucleofection, the cells were harvested for western blot analysis of Akt and α -smooth muscle actin (α -SMA) expression. B, Forty-eight hours after nucleofection, the cells were incubated for an additional 48 hr in the presence or absence of IM and then ALP activities were quantified as described above. The data are presented as mean \pm SEM (n=3). IM (–) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM.

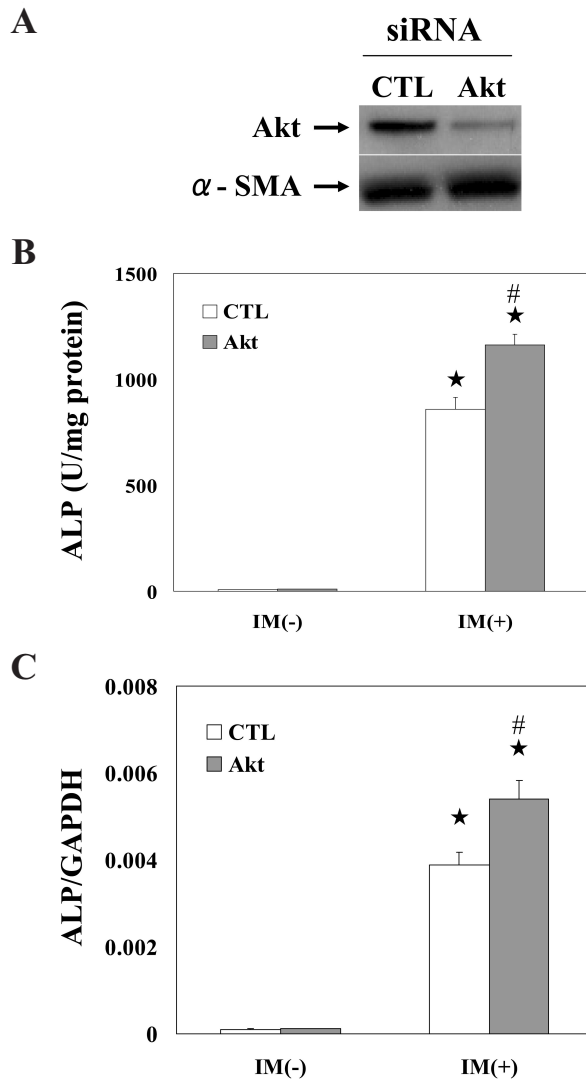


Figure 6. Effects of Akt siRNA on ALP expression in HVSMCs. HVSMCs were nucleofected with control (CTL) and Akt siRNA as described. **A**, Forty-eight hours after nucleofection, the cells were harvested for western blot analysis of Akt and α -smooth muscle actin (α -SMA) expression. **B**, Twenty-four hours after nucleofection, the cells were incubated for an additional 24 hr in the presence or absence of IM and then ALP activities were quantified as described above. The data are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the control cultures without IM. #p<0.05 versus the control cultures with IM. **C**, Twenty-four hours after nucleofection, the cells were incubated for an additional 24 hr in the presence or absence of IM and total RNA was harvested and utilized for Real time RT-PCR. Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean \pm SEM (n=3). IM (-) and IM (+) indicate the absence and presence of IM in culture media, respectively. *p<0.05 versus the IM (-) cultures with control siRNA. #p<0.05 versus the IM (+) cultures with control siRNA.

dominant-negative Akt on expression of ALP in HVSMCs.

Nucleofection of wild type Akt1 by electroporation significantly attenuated IM-induced ALP expression compared with that of HVSMCs nucleofected with control vector (Fig. 4). On the other hand, dominant-negative Akt significantly enhanced ALP induction by IM compared with that of the cultures nucleofected with control vector (Fig. 5).

To further confirm the role of Akt in IM-induced ALP expression in HVSMCs, functional gene silencing of Akt was achieved using the ShortCut™ siRNA Mix as described in Materials and Methods. HVSMCs were nucleofected with siRNA against Akt1/2/3 or with a scrambled non-targeting siRNA control. Twenty four hours after nucleofection, the cells were stimulated with IM for 24 hr. Western blot analysis revealed significant reduction of Akt expression in HVSMCs transfected with Akt siRNA. Nucleofection with Akt siRNA significantly augmented IM-induced ALP expression compared with that with control siRNA (Fig. 6). These data suggest that PI3K/Akt pathway plays an important role in regulation of ALP expression in HVSMCs.

Discussion

In this study, we have investigated the roles of PI3K/Akt pathway on vascular calcification

induced by IM. Inhibition of PI3K by a specific inhibitor wortmannin increased IM-induced in vitro calcification and expression of ALP. Nucleofection with wild-type or dominant-negative forms of Akt significantly attenuated or enhanced IM-induced ALP expression in HVSMCs, respectively. Furthermore, suppression of Akt with siRNA significantly intensified IM-induced ALP expression in HVSMC. These data indicate that PI3K/Akt pathway may inhibit IM-induced HVSMC calcification through regulating ALP expression, suggesting an inhibitory role of PI3K/Akt signaling pathway in inflammation-induced vascular calcification such as atherosclerotic calcification.

The recent studies have clarified the two major mechanisms of vascular calcification: osteogenic differentiation and apoptosis of vascular smooth muscle cells^{6,14,15}. Vascular smooth muscle cells undergo phenotypic changes into osteoblast-like cells in response to inflammatory stimuli or oxidative stress that induce the transcription factors specific for osteogenic differentiation including Msx2, Runx2, and osterix in VSMCs. These transcription factors stimulate the expression of osteoblast-specific marker genes including ALP, osteopontin, and osteocalcin. On the other hand, apoptotic pathways in vascular smooth muscle cells are activated by various factors including mechanical stress, vasoactive substances (angiotensin II, nitric oxide), oxidized low density lipoprotein (LDL), and inflammatory mediators (TNF- α , IFN- γ , interleukin-1 β). Moreover, inorganic phosphate has been demonstrated to induce apoptosis and calcification of human vascular smooth muscle cells through Gas6/Axl pathways¹⁶.

This study indicates the inhibitory role of PI3K/Akt signaling pathway in inflammation-induced vascular calcification. The previous studies have suggested the different roles of PI3K/Akt signaling pathway in vascular calcification. The stimulatory role of PI3K/Akt signaling has been recently demonstrated in oxidative stress (H₂O₂)-induced osteogenic differentiation in mouse VSMCs¹⁷. Pharmacological inhibition of PI3K/Akt suppresses H₂O₂-induced Runx2 expression and in vitro calcification. We have also examined the role of Runx2 in IM-induced HVSMC calcification in this study. Runx2 was not induced by the treatment with IM in HVSMCs (data not shown), suggesting that our in vitro model is not dependent on Runx2 in acquisition of calcifying phenotype of HVSMCs and that activation of PI3K/Akt pathway by different stimuli may exert different effects on osteogenic differentiation. Moreover, the inhibitory role of PI3K/Akt in apoptosis associated with vascular calcification has been indicated¹⁸. PI3K/Akt signaling functions as a downstream pathway of Gas6/Axl and inhibits apoptosis of VSMC and its in vitro calcification. In this study, wortmannin did not affect apoptosis of HVSMCs in the presence or absence of IM as evidenced by ELISA quantification of histone-associated DNA fragments¹⁹. Therefore, the major role of PI3K/Akt pathway in our model is not regulating cell survival/apoptosis.

In this study, we clarified the role of PI3K/Akt signaling pathway in regulating inflammation-induced ALP expression. ALP, a phenotypic marker of osteoblasts, is thought to be essential for vascular calcification and has been demonstrated to be upregulated by proinflammatory cytokines such as TNF- α ^{8,20}. However, the molecular mechanisms of inflammation-induced ALP gene expression in VSMCs remain to be clarified. It has been reported that forkhead transcription factor FOXO1 regulates ALP gene promoter activity in osteoblastic cells such as MC3T3-E1, SaOS2, and UMR106 cells²¹. Since FOXO transcription factors including FOXO1 are important down-stream targets PI3K/Akt signaling pathway, PI3K/Akt may regulate ALP

expression in HVSMCs through FOXO1 phosphorylation²²⁾. In the preliminary study, we found that IM induces FOXO1 expression in HVSMCs in association with upregulation of ALP gene (data not shown).

As shown in this study, IM simultaneously activated both the stimulatory and inhibitory signaling pathways for expression of ALP in HVSMCs. Our data suggest that IM-induced activation of PI3K/Akt signaling pathway may play a negative regulatory role in acquisition of calcifying phenotype of HVSMCs. Recently, the negative regulatory role of PI3K/Akt pathway in inflammatory responses has been clarified²³⁾. Lipopolysaccharide (LPS) stimulation of monocytes/macrophages induces the expression of genes encoding proinflammatory cytokines such as TNF- α and IL-6 and the procoagulant protein, tissue factor. Induction of these genes is mediated by various signaling pathways, including mitogen-activated protein kinases and several transcription factors, including Egr-1, AP-1, ATF-2, and NF- κ B. PI3K/Akt signaling pathway is also activated in macrophages by LPS. Activation of this pathway negatively regulates LPS induction of TF and inflammatory cytokines in macrophages by inhibiting activation of the MAPK pathways and the transcription factors ATF-2 and Egr-1. Therefore, PI3K/Akt signaling pathway may negatively regulate inflammation-induced vascular calcification such as atherosclerotic calcification.

The physiological role of Akt signaling in the regulation of bone metabolism has been recently clarified¹²⁾. There are three Akt family members, Akt1/PKB α , Akt2/PKB β , and Akt3/PKB γ . Among these Akt isoforms, Akt1 is the major Akt isoform in bone and the deficiency of this isoform causes low turnover osteopenia. Akt1 deficiency causes decreased bone formation through enhancing susceptibility to apoptosis of osteoblasts via FoxO3a and impairing Runx2-dependent differentiation and function of osteoblasts. Moreover, Akt1 ablation also impairs bone resorption via dysfunctions of osteoclasts and osteoblasts. Therefore, Akt1 functions as a crucial regulator of osteoblasts and osteoclasts by promoting their differentiation and survival to maintain bone mass and turnover, suggesting that the role of Akt in osteogenesis in vascular calcification as shown in this study may be different from that in bona fide skeletal tissues.

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