Original Article

Trichostatin A, an HDAC Class I/II Inhibitor, Promotes Pi-Induced Vascular Calcification Via Up-Regulation of the Expression of Alkaline Phosphatase

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Aim: Vascular calcification, a major complication of chronic kidney disease (CKD), refers to the mineralization of vascular smooth muscle cells (VSMCs), resulting from a phenotypic change towards osteoblast-like cells. Histone deacetylase inhibitors (HDIs), potential therapeutic agents for CKD, are known to promote the differentiation and mineralization of osteoblasts. In this study, we aimed to determine the effects of an HDI on the phenotypic change of VSMCs and the development of vascular calcification.

Methods: The effect of trichostatin A (TSA), an HDI, on human aortic smooth muscle cells (HASMCs) was determined. The mineralization of HASMCs was induced by inorganic phosphorus (Pi), and was confirmed by quantitation of Ca levels and by von Kossa staining. Furthermore, we examined the effect of alkaline phosphatase (ALP) suppression using siRNA on Pi-induced vascular calcification in the presence or absence of TSA.

Results: TSA increased the expression and activity of ALP in HASMCs at a concentration which showed an inhibitory effect of histone deacetylase (HDAC) activity but not on cell viability. Moreover, TSA promoted the Pi-induced mineralization of HASMCs. In addition, both phosphonoformic acid (PFA), which is a sodium-dependent phosphate transporter inhibitor, and suppression of ALP expression by siRNA markedly inhibited the TSA-promoted mineralization of HASMCs.

Conclusion: These data show that inhibition of HDAC activity promotes Pi-induced vascular calcification via the up-regulation of ALP expression. Taken together, HDIs may increase the risk of vascular calcification in CKD patients.

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Key words: Vascular calcification, Vascular smooth muscle cells, Trichostatin A, Alkaline phosphatase, Inorganic phosphorus

Introduction

Chronic kidney disease (CKD) is a risk factor for end-stage renal disease (ESRD), a devastating disorder that requires dialysis or renal transplantation, and is a worldwide health problem with high cost, increasing

prevalence and poor outcome. Many clinical and epidemiological studies have suggested that cardiovascular disease (CVD) is the leading cause of mortality in CKD patients¹⁻³), and that cardiovascular mortality is responsible for around 50% of all deaths of dialysis (CKD stage 5D) patients⁴). Vascular calcification, a major complication for CKD patients, refers to the deposition of hydroxyapatite in cardiovascular tissue and decreases the distensibility of the aorta followed by an increased risk of CVD. In addition, it is well known that vascular calcification is an independent predictor of subsequent cardiovascular mortality in dialysis patients^{5, 6}). Thus, the prevention and treat-

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ment of vascular calcification are important from the viewpoint of convalescence improvement of CKD patients.

Currently, there is strong evidence that the serum phosphorus level is an independent risk factor for cardiovascular events and mortality in CKD^{7, 8)}. Several studies have indicated that high serum phosphorus induces a phenotypic change of vascular smooth muscle cells (VSMCs) towards osteoblast-like cells followed by the development of vascular calcification⁹⁻¹²⁾. Furthermore, other risk factors for CVD, such as TNF- α , promote the Pi-induced osteoblast differentiation of VSMCs and exacerbate vascular calcification^{13, 14)}. Therefore, it is important to understand the mechanisms of this differentiation, and regulation of this differentiation should be a useful approach to prevent vascular calcification.

To date, histone deacetylases (HDACs) have been shown to regulate the differentiation of several cell types¹⁵⁻¹⁷⁾. HDAC is a family of enzymes that remove acetyl groups from histone tails and promote chromatin condensation, followed by transcriptional repression¹⁸⁻²⁰⁾. Recently, the association of HDACs with osteoblast differentiation has indicated that the down-regulation of HDAC1 and HDAC7 promotes osteoblast differentiation with osteogenic gene expression²¹⁻²³⁾. Moreover, it was reported that histone deacetylase inhibitors (HDIs), such as trichostatin A (TSA) and sodium butyrate (NaB), increase osteogenic gene expression and promote osteoblast differentiation^{24, 25)}; therefore, we hypothesized that HDIs also affect VSMC differentiation, resulting in the development of vascular calcification. In this study, we aimed to determine the effect of TSA, a representative HDI, on osteogenic gene expression of VSMCs and the development of vascular calcification.

Methods

HASMC Culture and RNA Interference

Human aortic smooth muscle cells (HASMCs) were obtained from Kurabo Industries (Osaka, Japan). Cells were routinely cultured in growth medium with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with sodium pyruvate, penicillin and streptomycin, all from Life Technologies (Carlsbad, CA, USA).

HASMCs were transfected with 5 nM small interfering RNA (siRNA; Dharmacon, Chicago, IL, USA) for alkaline phosphatase (ALP; siGENOME SMARTpool, Human ALPL) or control (Ctrl; ON-TARGETplus Non-Targeting Pool) using Lipofectamine RNAiMax (Life Technologies) according to

Table 1. Primer sequences for RT-qPCR

Gene	Oligonucleotide sequence
ALP	
Forward	5'- GGGACTGGTACTCAGACAACG -3'
Reverse	5'- CCCATGATCACGTCAATGTC -3'
Runx2	
Forward	5'- TTACTTACACCCCGCCAGTC -3'
Reverse	5'- TATGGAGTGCTGCTGGTCTG -3'
Pit-1	
Forward	5'- TGCCTAATGGGAACCTTGTC -3'
Reverse	5'- TCTTTGTACAGGCCGGAATC -3'
GAPDH	
Forward	5'- CTTTTCGCTCGCCAGCCGAG -3'
Reverse	5'- ACCAGGCGCCCAATACGACC -3'

the manufacturer's instructions. HASMCs were treated simultaneously with these siRNAs at the start of inorganic phosphate (Pi) treatment.

Induction of HASMC Calcification

HASMCs (at passage 4 to 15) were seeded at a density of 2.7×10^4 cells/cm². After 2 days, cells were treated with a pathological concentration of Pi in calcification medium for 6 days. To set up the calcification medium, a mixed solution of Na₂HPO₄ and NaH₂PO₄, whose pH was adjusted to 7.4, was added to the growth medium to a final dose of up to 3.0 mM. Moreover, cells were treated with trichostatin A (TSA; Sigma, St. Louis, MO, USA), an HDAC class I/II inhibitor, and/or phosphonoformic acid (PFA; Sigma), an sodium-dependent phosphate transporter inhibitor, while being cultured in the calcification medium.

HDAC Activity Assay

HDAC class I/II activity was measured using the HDAC-Glo I/II Assay (Promega, Fitchburg, WI, USA). HASMCs were cultured with TSA treatment for 6 days. At the end of the culture period, HDAC-Glo I/II Buffer and Serum-free DMEM without phenol red were added to each well, and then incubated at 37°C for 4 hr. After incubation, HDAC-Glo I/II Reagent was added to each well followed by incubation at room temperature for 1 hr. HDAC class I/II activity was assessed in a Wallac ARVO SX 1420 multi-label counter (Perkin Elmer, Waltham, MA, USA) by measuring the luminescence.

Cell Viability Assay

Cell viability was measured with a Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). HASMCs were cultured with TSA treatment for 6

Fig. 1. Effect of TSA on HDAC class I/II activity and the viability of HASMCs.

HASMCs were treated with TSA for 6 days at the indicated concentration (0, 10, 100, 500 nM). A, The effect of TSA on HDAC class I/II activity was examined using the HDAC-Glo I/II Assay. B, The effect of TSA on cell viability was examined using a Cell Counting Kit-8. All data (means $\pm$ SD) are expressed as a relative ratio to the value obtained with the control (0 nM TSA); * p < 0.05 vs control (n = 3 each).

days. At the end of the culture period, 10 μ L Cell Counting Kit-8 solution was added to each well and incubated at 37°C for 0.5 hr. Cell viability was assessed in a MTP-450Lab microplate reader (Corona Electric, Katsuta, Japan) by measuring the absorbance at a wavelength of 450 nm.

RT-qPCR

Total RNA was isolated from cultured HASMCs using RNeasy RT (Molecular Research Center, Cincinnati, OH, USA). One microgram of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega). Real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA), according to the manufacturer's instructions. Sequences of forward and reverse primers are described in **Table 1**. mRNA expression was quantified by the standard curve method according to the manufacturer's protocol and was normalized to GAPDH.

Alkaline Phosphatase (ALP) Staining

To determine ALP activity, ALP staining was performed using a TRAP/ALP stain kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Briefly, after fixing with 4% paraformaldehyde and permeabilizing with EtOH/acetone (50%:50% v/v), ALP was stained by incubation with an ALP pre-mixed substrate mixture. ALP was visualized as blue-violet under a microscope.

Evaluation of HASMC Calcification

For quantitation of Ca levels, cells were decalcified with 0.6 N HCl for 24 hr. The calcium content of the HCl supernatant was determined by the methyl xylene blue (MXB) method using Wako Calcium E Test (Wako Pure Chemical Industries). After decalcification, the cells were washed with phosphate-buffered saline (PBS) and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). Protein contents were measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The calcium content of the cell layer was normalized to the protein content.

Deposition of hydroxyapatite was visualized by von Kossa staining. Briefly, after fixing with 4% paraformaldehyde, hydroxyapatite deposits were stained with 5% silver nitrate and then with 5% sodium thiosulfate. Deposition of hydroxyapatite was visualized as dark brown under a microscope.

Statistical Analysis

One-way ANOVA with Tukey-Kramer's post-hoc test was used to compare multiple groups. An unpaired t test was performed for statistical analysis of 2 groups. All results are expressed as the means $\pm$ SD, and p < 0.05 was considered significant.

Results

Effect of TSA on HDAC Class I/II Activity and the Viability of HASMCs

Since HDIs are well known to induce the down-regulation of HDAC activity as well as the apoptosis

Fig. 2. TSA increases mRNA expression and activity of ALP in HASMCs.

HASMCs were treated with 100 nM TSA for 6 days. A, Total RNA was extracted from HASMCs and RT-qPCR was performed. Data (means $\pm$ SD) are expressed as the ratio of each mRNA/GAPDH mRNA, relative to the control (0 nM TSA) assigned a value of 1.0; * p < 0.05 vs control. (n = 3 each). B, ALP activity was assessed by ALP staining using a TRAP/ALP stain kit, and was visualized as blue-violet under a microscope. Magnification $\times$ 40.

of several types of cells, we first confirmed the effects of TSA on HDAC activity and the viability of HASMCs. HASMCs were cultured in the presence or absence of TSA (0 to 500 nM) for 6 days following by analyses of HDAC class I/II activity and cell viability. The results showed that HDAC class I/II activity was significantly decreased by TSA treatment in a dose-dependent manner (**Fig. 1A**). Moreover, the cell viability assay showed no significant differences among the groups except for the 500 nM TSA treatment

group (**Fig. 1B**). On the basis of these results, we used 100 nM TSA, which shows an inhibitory effect on HDAC class I/II activity but no reduction of cell viability of HASMCs, for subsequent experiments.

TSA Increases the Expression and Activity of ALP in HASMCs

Because HDIs have been shown to increase osteogenic gene expression in osteoblasts and promote osteoblast maturation²⁴, we assessed the effect of TSA

Fig. 3. TSA promotes Pi-induced vascular calcification.

Vascular calcification was induced in HASMCs with 3.0 mM Pi and 100 nM TSA for 6 days. A, Calcium levels of the cultures were determined by the methyl xlenol blue (MXB) method using Wako Calcium E Test (Wako), and are normalized to the volume of total cellular protein. Value are expressed as means $\pm$ SD; * p < 0.05 (n = 3 each). B, Mineralization of HASMCs was confirmed by von Kossa staining, and was visualized as dark brown under a microscope. Magnification $\times$ 100. Bar, 100 μ m

Supplemental Fig. 1.

HASMCs were induced to undergo vascular calcification with 3.0 mM Pi and 100 nM TSA for 1-6 days. Total RNA was extracted from HASMCs and RT-qPCR was performed. Data (means $\pm$ SD) are expressed as the ratio of ALP mRNA / GAPDH mRNA, relative to the non-treatment group (Pi-/TSA-) assigned a value of 1.0; * p < 0.05 (n = 3 each).

on the expression of an osteogenic gene that is closely related to vascular calcification in HASMCs. RT-qPCR results showed that TSA significantly increased the expression of ALP mRNA in HASMCs cultured for 6 days (**Fig. 2A**). On the other hand, TSA treatment slightly increased the expression of runt-related transcription factor 2 (Runx2) and type III sodium-

dependent phosphate co-transporter (Pit-1) in HASMCs. Moreover, TSA increased ALP activity in HASMCs confirmed by ALP staining (**Fig. 2B**). These results suggest that TSA partially converts the phenotype of HASMCs towards osteoblast-like cells, including the up-regulation of ALP expression.

Fig. 4. PFA inhibited Pi-induced vascular calcification in the presence or absence of TSA.

Vascular calcification was induced in HASMCs with 3.0 mM Pi, 100 nM TSA and 100 µM PFA for 6 days. A, Calcium levels of the cultures were determined by the methyl xylene blue (MXB) method using Wako Calcium E Test (Wako), and are normalized to the volume of total cellular protein. Value are expressed as the means $\pm$ SD; * p < 0.05 (n = 3 each). B, Mineralization of HASMCs was confirmed by von Kossa staining, and was visualized as dark brown under a microscope. Magnification $\times$ 100. Bar, 100 µm

Supplemental Fig. 2. TSA promotes Pi-induced vascular calcification.

HASMCs were induced to undergo vascular calcification with 3.0 mM Pi and 100 nM TSA for 6 days. Total protein was extracted from HASMCs and a Western blot assay was performed using cleaved PARP and GAPDH antibody. Positive control: total protein from HASMCs treated with tunicamycin (5 µg/mL) for 48 hr.

TSA Promotes the Pi-Induced Vascular Calcification of HASMCs

We next examined whether TSA treatment promotes the Pi-induced vascular calcification of HASMCs. As shown in **Fig. 3A**, Ca levels of HASMCs were increased by excessive Pi stimulation (3.0 mM), and this increased Ca level was significantly promoted by TSA treatment. Moreover, Pi-induced hydroxyapatite deposition was significantly increased by TSA treatment (**Fig. 3B**). Similar to **Fig. 2A**, TSA increased ALP expression of HASMCs under both normal and high phosphorus conditions (**Supplemental Fig. 1**). Additionally, PFA, which is a sodium-dependent phosphate transporter inhibitor, completely blocked

Pi-induced vascular calcification in the presence or absence of TSA (**Fig. 4A** and **B**). Although it is well known that apoptosis is a regulator of vascular calcification^{26, 27}, we did not detect the expression of cleaved PARP, which is an apoptosis marker, in TSA-promoted vascular calcification (**Supplemental Fig. 2**).

Suppression of ALP Expression Inhibits TSA-Promoted Vascular Calcification

Because ALP is an osteoblast differentiation marker required for bone matrix mineralization²⁸, we examined whether the up-regulation of ALP expression plays an important role in TSA-promoted vascular calcification using ALP siRNA. RT-qPCR results

Fig. 5. Suppression of ALP expression inhibits TSA-promoted vascular calcification.

Vascular calcification was induced in HASMCs with 3.0 mM Pi and 100 nM TSA with or without the knockdown of ALP expression for 6 days. A, Total RNA was extracted from HASMCs and RT-qPCR was performed. Data (means $\pm$ SD) are expressed as the ratio of ALP mRNA/GAPDH mRNA, relative to the only Pi-stimulated group assigned a value of 1.0; * p < 0.05, # p < 0.05 (n = 3 each). B, Calcium levels of the cultures were determined by the methyl xlenol blue (MXB) method using Wako Calcium E Test (Wako), and are normalized to the volume of total cellular protein. Value are expressed as the means $\pm$ SD; * p < 0.05, # p < 0.05 (n = 3 each). C, Mineralization of HASMCs was confirmed by von Kossa staining, and was visualized as dark brown under a microscope. Magnification $\times$ 100. Bar, 100 μ m

showed that transfection of ALP siRNA diminished ALP expression in HASMCs treated with or without TSA (**Fig. 5A**). Next, we induced vascular calcification with both Pi stimulation and TSA treatment with or without knockdown of ALP expression. Quantitation of Ca levels and von Kossa staining showed that suppression of ALP expression inhibited TSA-promoted mineralization of HASMCs (**Fig. 5B** and **C**). Moreover, the mineralization of HASMCs was also significantly decreased by the suppression of ALP expression in the absence of TSA. These results suggest that the

up-regulation of ALP expression plays an important role in the progression of vascular calcification.

Discussion

The purpose of this study was to clarify the effects of HDIs on the development of vascular calcification. The results demonstrate the effects of TSA on VSMCs, which play a central role in the development of vascular calcification. First, our results show that TSA treatment increases the expression and activity of

ALP in HASMCs. Next, excessive Pi stimulation induces the mineralization of HASMCs, promoted by TSA treatment. Finally, both PFA treatment and the suppression of ALP expression by siRNA transfection retard the TSA-promoted mineralization of HASMCs. These results suggest that TSA enhances the mineralization of HASMCs with Pi stimulation via the up-regulation of ALP expression.

Recent studies reported that HDIs increase the expression of osteogenic genes such as ALP and Runx2 in osteoblast lineage cells and promote osteoblast maturation^{22, 24}. Similarly, our results show that TSA increases ALP and Runx2 expression in HASMCs, suggesting that TSA induces phenotypic changes of HASMCs towards osteoblast-like cells. In general, acetylation of histone lysine residues correlates with transcriptional activation and the level of histone acetylation is regulated by histone acetyltransferase (HAT) and HDACs. Thus, HDIs induce histone hyperacetylation followed by transcriptional activation of several genes; however, our preliminary data indicate that TSA does not induce histone acetylation in the ALP promoter region. In an osteoblast cell line, Runx2 is known to regulate ALP promoter activity²⁹. The activity of several transcription factors is known to be associated with the level of their acetylation, and HDAC inhibition regulates these activities followed by the transcription of downstream genes³⁰⁻³³. Indeed, the acetylation and activity of Runx2 are promoted by HDAC inhibition in C2C12 myoblast cells³⁴. Although we did not check the acetylation level of Runx2 and other transcription factors, our results suggest that TSA induces the acetylation of several transcription factors, including Runx2, followed by the up-regulation of ALP expression. Additional studies will be necessary to understand how HDIs increase ALP expression in HASMCs.

Recently, several studies reported that overexpression of ALP enhances mineral deposition of non-osteogenic cells in the presence of β -glycerophosphate³⁵, and that an ALP inhibitor suppresses Pi-induced vascular calcification³⁶. Accordingly, our results show that TSA promotes the mineralization of HASMCs with increased ALP expression, and that suppression of ALP expression results in the reduction of HASMC mineralization in response to excessive Pi stimulation with or without TSA. Moreover, there is no statistical difference in Ca deposition with or without TSA under the suppression of ALP, suggesting that ALP plays an essential role in TSA-promoted vascular calcification under a high Pi condition.

In this study, we also confirm that TSA slightly increases the expression of Pit-1, which is a type III

sodium-dependent phosphate co-transporter in HASMCs. Several studies have demonstrated that elevated Pi levels induce the calcification of VSMCs, and that excessive Pi uptake through Pit-1 plays a crucial role in vascular calcification³⁷. Indeed, suppression of Pit-1 using siRNA and PFA blocked Pi-induced calcification of VSMCs and aortic tissue^{38, 39}. Consistent with these reports, our results show that PFA treatment inhibits Pi-induced vascular calcification with or without TSA, and that there is no statistical difference in Ca deposition between Pi+/TSA- and Pi+/TSA+ with PFA treatment. Therefore, excessive Pi uptake through Pit-1 may be a contributing factor to TSA-promoted vascular calcification under high phosphorus conditions. Further studies will be necessary to clarify the effect of TSA on Pi uptake through Pit-1 in vascular calcification.

Recently, Takemura *et al.* have demonstrated that down-regulation of sirtuin 1 (SIRT1), which is a class III HDAC, with cellular aging and/or siRNA transfection promotes Pi-induced vascular calcification⁴⁰. They also showed that the acetylation level of histone is increased with excessive Pi stimulation, indicating one possibility that there is some interaction between histone acetylation and inorganic phosphorus in vascular calcification of HASMCs. Although we did not test the acetylation level of histone, TSA may induce histone hyperacetylation by inhibiting HDAC activity. Moreover, TSA accelerated the mineralization of HASMCs in high phosphate media but did not induce mineralization of HASMCs in normal phosphate media. Therefore, we consider that histone hyperacetylation is indirectly associated with the development of Pi-induced vascular calcification.

Recently, many studies have suggested that epigenetic modification, such as histone acetylation and DNA methylation, is associated with the development of several diseases, including cancer and psychiatric disorders; therefore, regulation of the modification by HDIs and other reagents is expected as a new target of therapy in these diseases⁴²⁻⁴⁵. The association of HDAC with renal fibrosis, which is a common pathology in CKD patients⁴¹, has also been indicated by the high expression level of HDAC1 and 2 observed in kidneys injured by ureteral obstructions, and by the finding that administration of HDIs attenuates renal fibroblast activation and renal fibrosis in mouse and rat models⁴⁶⁻⁴⁸; therefore, HDIs are thought to be useful therapeutic agents for CKD patients. Considering that HDIs are administered systemically to CKD patients, it is necessary to clarify their effects on representative complications such as vascular calcification, which is a predictor of subsequent cardiovascular mor-

tality in CKD patients⁴⁻⁶). Although the effect of HDIs on the development and progression of CVD remains controversial, a recent study showed that TSA exacerbates atherosclerosis in LDLR-deficient mice⁴⁹). Additionally, our present study shows that TSA promotes vascular calcification at a high phosphorus level while not at a normal phosphorus level. It has been reported that serum phosphorus is elevated in CKD advanced-stage 4/5 but not early-stage 1-3^{50, 51}). Therefore, HDIs may not affect the risk of cardiovascular disease in the early CKD stage, but may increase the risk in the advanced CKD stage; in other words, HDIs are thought to be valuable for CKD patients in the early stage, but may not be desirable for CKD patients in the advanced stage. Further study from the viewpoint of epigenetic modification may lead to the establishment of new therapies for vascular calcification in CKD patients.

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Conflicts of Interest

None.

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