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# Selenium Suppressed Hydrogen Peroxide-Induced Vascular Smooth Muscle Cells Calcification Through Inhibiting Oxidative Stress and ERK Activation

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## ABSTRACT

Atherosclerosis is frequently associated with vascular calcification. Increasing evidences underline that the essential micronutrient selenium may prevent atherosclerosis, but the role of selenium in vascular calcification remains unknown. In this study, we assessed the effect of sodium selenite ($Na_2SeO_3$) on $H_2O_2$-enhanced vascular smooth muscle cells (VSMCs) calcification and examined the involvement of extracellular signal-regulated kinase (ERK) signaling pathway. Hydrogen peroxide enhanced vascular calcification by inducing osteoblastic differentiation of VSMCs, as showed by up-regulating the mRNA expression of type I collagen, osteocalcin, and Runx2, a key transcription factor for osteoblastic differentiation, increasing alkaline phosphatase activity, and calcium deposition. These effects of $H_2O_2$ were suppressed by pretreatment of the cells with selenite (0.1–1 μM) for 24 h. In addition, $H_2O_2$ activated the phosphorylation of ERK1/2 and inhibition of $H_2O_2$-activated ERK signaling by MEK inhibitor PD98059 blocked the effect of $H_2O_2$ on osteoblastic differentiation of VSMCs. Furthermore, $H_2O_2$ induced oxidative stress in calcifying VSMCs, as evidenced by the increase of intracellular reactive oxygen species production and malondialdehyde level, and the decrease of total protein thiols content and the activity of antioxidant selenoenzyme glutathione peroxidases. Selenite pretreatment also attenuated $H_2O_2$-induced oxidative stress and ERK activation. These results suggested that selenite suppressed $H_2O_2$-enhanced osteoblastic differentiation and calcification of VSMCs through inhibiting oxidative stress and ERK activation, indicating a potential preventive role for selenium in vascular calcification. J. Cell. Biochem. 111: 1556–1564, 2010. 

**KEY WORDS:** SELENIUM; VASCULAR CALCIFICATION; VASCULAR SMOOTH MUSCLE CELLS; ERK; OXIDATIVE STRESS

Vascular calcification is a prominent feature of atherosclerosis, and has emerged as a powerful predictor of cardiovascular morbidity and mortality [Abedin et al., 2004]. Accumulating evidence supports the concept that vascular calcification is an active, regulated process similar to osteogenesis, instead of a passive precipitation of hydroxyapatite on the arterial wall. During this process, the differentiated vascular smooth muscle cells (VSMCs) undergo trans-differentiation, and subsequently osteoblastic transition that results in vascular calcification [Trion and van der Laarse, 2004].

Oxidative stress describes a condition occurring when the generation of reactive oxygen species (ROS) in a system exceeds the system's ability to neutralize and eliminate them. The imbalance can result from a lack of antioxidant capacity caused by disturbance in production, distribution, or by an overabundance of ROS from endogenous sources or environmental stressors. If not regulated properly, excess ROS can damage a cell's lipids, proteins or DNA, inhibiting normal function. Increasing evidences suggest that oxidative stress has been implicated in the pathogenesis of vascular calcification [Parhami et al., 1997; Mody et al., 2001; Valabhji et al., 2001; Liu et al., 2004; Zoccali et al., 2004; Tang et al., 2006; Byon et al., 2008]. Many factors that have been linked to an increased prevalence of vascular calcification are associated with elevated oxidative stress, including hypercholesterolemia, diabetes mellitus, and dialysis-dependent end stage renal disease [Valabhji et al., 2001; Zoccali et al., 2004; Tang et al., 2006]. Minimally modified oxidized low density lipoprotein (LDL) and different lipid peroxidation products induced VSMCs to undergo osteoblastic differentiation [Parhami et al., 1997]. Our previous experiments demonstrated that oxysterol cholestane-3β, 5α, 6β-triol increased intracellular ROS and enhanced VSMCs calcification, and that these effects were attenuated by antioxidant vitamin C plus vitamin E [Liu

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et al., 2004]. Moreover, exogenous hydrogen peroxide ($H_2O_2$) was reported to enhance osteoblastic differentiation and calcification of VSMCs [Mody et al., 2001; Byon et al., 2008].

The trace mineral selenium (Se) is an essential nutrient of fundamental importance to human biology. The biologic function of Se is primarily implemented through its incorporation into selenoproteins. Selenoproteins with known functions play a critical role in a variety of biologic processes, and several of them are involved in antioxidant defense [Rayman, 2000]. For example, four glutathione peroxidases (GPx) protected cells against peroxidative damage by reducing $H_2O_2$, free fatty acid hydroperoxides, and phospholipid hydroperoxides. Interest in the role of Se in the cardiovascular system was roused in particular by the epidemiologic finding of an inverse correlation between Se status and risks of cardiovascular diseases [Rayman, 2000]. Experimental data supported that dietary Se supplementation might be protective against atherosclerosis [Thomas et al., 1993; Huang et al., 2002; Wu et al., 2003; Tang and Huang, 2004; Wu and Huang, 2004, 2006; Tang et al., 2005; Espinola-Klein et al., 2007; Torzewski et al., 2007]. Se-dependent GPx played an important role in cellular defense against oxidized LDL-induced lethal damage to endothelial cells, presumably by detoxifying lipid hydroperoxides [Thomas et al., 1993]. Deficiency or decreased activity of GPx1 accelerated the progression of atherosclerosis [Espinola-Klein et al., 2007; Torzewski et al., 2007]. Our previous reports showed that an inverse relationship existed between dietary Se and antioxidant capacity of rat vascular tissues [Wu et al., 2003; Wu and Huang, 2004] and Se inhibited oxysterol-induced VSMCs apoptosis [Tang and Huang, 2004; Tang et al., 2005] and ECs death [Wu and Huang, 2006] in vitro, as well as vascular damage in vivo [Huang et al., 2002], by protecting cells from oxidative stress. All these studies indicated that Se supplementation might prevent atherosclerosis, but the underlying mechanism has not yet been fully elucidated. Considering that atherosclerosis is closely associated with vascular calcification and oxidative stress plays a critical role in the pathogenesis of vascular calcification, we hypothesized that Se might also exert a protective role against vascular calcification by inhibiting oxidative stress. Therefore, in the present study, the effect of sodium selenite ($Na_2SeO_3$) on $H_2O_2$-enhanced VSMCs calcification was examined. We found that $Na_2SeO_3$ pretreatment suppressed $H_2O_2$-enhanced vascular calcification by inhibiting osteoblastic differentiation of VSMCs. In addition, we demonstrated that $Na_2SeO_3$ exerted its effect by inhibiting oxidative stress and extracellular signal-regulated kinase (ERK) activation. Taken together these results demonstrate for the first time the preventive effect of Se on vascular calcification, which may provide more insights about the inhibitory mechanism of Se on the development of atherosclerosis.

## MATERIALS AND METHODS

### CHEMICALS

Dulbecco modified Eagle's medium (DMEM), newborn calf serum (NCS), and Trizol were purchased from Invitrogen. Sodium β-glycerophosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ascorbic acid, type I collagenase, bovine serum albumin (BSA), *p*-nitrophenyl phosphate, *p*-nitrophenol, 2,7-dichlorofluorescein diacetate (2,7-DCFH-DA), PD98059, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), aprotinin, leupeptin, thiobarbituric acid (TBA), and dithiobis 2-nitrobenzoic acid (DTNB) were obtained from Sigma–Aldrich. M-MLV reverse transcriptase was obtained from Promega. SYBR Green PCR Master Mix kit was purchased from Toyobo. Antibodies against phosphorylated ERK1/2, β-actin, and the corresponding secondary antibodies were obtained from Santa Cruz. The enhanced chemiluminescence (ECL) kit was purchased from Pierce.

### CELL CULTURE AND IN VITRO CALCIFICATION

VSMCs were isolated from rat aortic media and identified as described by Smith and Brock [1983]. The growing media used was DMEM (high glucose, 4.5 g/L) containing 15% NCS and 1 mM sodium pyruvate. The cells between passages 3 and 7 were used for all experiments. VSMCs calcification was induced by the method of Shioi et al. [1995]. Briefly, VSMCs were cultured in growing medium. After confluence, the cells were switched to DMEM containing 15% NCS, 10 mM sodium pyruvate, 10 mM sodium β-glycerophosphate, $10^{-7}$ M insulin, 50 μg/ml ascorbic acid, 100 U/ml penicillin, and 100 μg/ml streptomycin (calcifying media) for 11 days. The medium was replaced with fresh one every 2 or 3 days. After 11 days, the cells became calcifying, as characterized by the multilayer nodules undergoing calcification detected by von Kossa staining as described in our previous study [Liu et al., 2004]. They were used as calcifying VSMCs for the following experiments.

Calcifying VSMCs were treated with different concentrations of $H_2O_2$ for indicated time in calcifying media. In some cases, cells were pretreated with 0.1 and 1 μM $Na_2SeO_3$ for 24 h or 1 μM PD98059, a specific inhibitor of MAPK kinase MEK, for 2 h before exposure to $H_2O_2$.

### CELL VIABILITY ASSAY

Cell viability was evaluated using the MTT assay [Denizot and Lang, 1986]. In which, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance at 570 nm of the resulting solution was measured. The data were from a representative of at least three experiments shown as a percentage of control ± SD.

### QUANTIFICATION OF CALCIUM DEPOSITION

Quantification of calcium deposition in extracellular matrix was performed as described by Wada et al. [1999]. Briefly, calcifying VSMCs were decalcified with 0.6 M HCl for 24 h. The calcium content of HCl supernatant was determined by atomic absorption spectroscopy (AA-300, Perkin Elmer). After decalcification, the cells were washed three times with PBS and solubilized with 0.1 M NaOH/0.1% SDS, and total protein content was determined with Lowry method [Lowry et al., 1951] using BSA as a standard. The calcium content of the cell layer was normalized to protein content.

### QUANTITATIVE REAL-TIME PCR

The expression of bone-related gene markers was determined by real-time PCR. Total RNA was isolated from calcifying VSMCs using Trizol and reverse-transcribed into cDNA. The specific primers for

rat type I collagen (Col I), osteocalcin (OC), Runx2, and GAPDH were designed as described by Byon et al. [2008]. SYBR Green-based real-time PCR was performed on a DNA Engine Opticon 2 (MJ Research, Boston, MA) using the SYBR Green PCR Master Mix kit, according to the vendor's protocol. GAPDH was used as a housekeeping gene for internal control. Expression levels of Col I, OC, and Runx2 gene were normalized to the GAPDH mRNA level.

### ALKALINE PHOSPHATASE ACTIVITY ASSAY

Calcifying VSMCs after treatment were washed three times with PBS, and the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Alkaline phosphatase (ALP) activity in supernatants was measured by spectrophotometry. Briefly, lysate samples (120 μl) were added to 100 μl substrate mixture consisting of 15 mM *p*-nitrophenyl phosphate in 0.2 M sodium bicarbonate buffer at pH 10.1 and incubated at 37°C for 30 min. The reaction was stopped by adding 50 μl of 1 M NaOH. The absorbance was measured at 410 nm. A reaction standard curve was obtained using *p*-nitrophenol. One unit of ALP activity was defined as the activity producing 1 nmol of *p*-nitrophenol within 30 min. ALP activity was normalized to total protein content of the cell layer.

### WESTERN BLOT ANALYSIS

Whole cell extracts from calcifying VSMCs were prepared in lysis buffer (50 mM HEPES, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.4% SDS, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 1 mM DTT, 1 mM $Na_3VO_4$, and 1 mM NaF). After centrifugation at 3,000 g for 20 min, the supernatants were collected for Western blot analysis. The total protein concentration of the supernatants was determined with Lowry method [Lowry et al., 1951]. Equal amounts of protein were electrophoresed on 10% SDS–PAGE and transferred onto nitrocellulose membranes. Non-specific binding of the membrane was blocked with 5% BSA in TBS-T (20 mM Tris–HCl, pH 7.5, containing 137 mM NaCl, and 0.1% Tween 20). The membrane was incubated with monoclonal mouse antibody for phosphorylated ERK1/2. After the membrane was washed in TBS-T, it was incubated with anti-mouse IgG conjugated to horseradish peroxidase. The blots were developed using ECL detection system. Polyclonal rabbit anti-β-actin was used as a loading control.

### MEASUREMENT OF INTRACELLULAR ROS

ROS levels in calcifying VSMCs were estimated using a fluorescence probe 2,7-DCFH-DA as described by Mody et al. [2001]. Calcifying VSMCs cultured in petri dish were loaded with 5 μg/ml 2,7-DCFH-DA for 20 min at room temperature in modified Kreb's Ringer buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 1.2 mM $KH_2PO_4$, 5 mM $NaHCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$, and 1.0 mM $CaCl_2$). Fluorescent signal was recorded using a confocal fluorescence microscope (FV500, Olympus). ROS level was quantified by measurement of fluorescence intensity of eight fields per dish with software provided with the microscopy system. The data were from a representative of three experiments shown as the mean ± SD of fluorescence intensity.

### DETERMINATION OF CELLULAR GPX ACTIVITIES, LIPID PEROXIDATION, AND PROTEIN THIOLS CONTENTS

Cellular GPx activity was measured according to the method of Hafeman et al. [1974]. One activity unit of GPx was defined as a decrease of 1 μmol/min in the GSH concentration after the decrease in the GSH concentration of the non-enzymatic reaction was subtracted and was expressed in unit per milligram protein. Cellular oxidative damage was evaluated by the contents of lipid peroxidation and total protein thiols. Malondialdehyde (MDA), an end product of lipid peroxidation, was measured to estimate the level of lipid peroxidation in calcifying VSMCs using the TBA method [Ohkawa et al., 1979] and expressed as nanomoles per milligram protein. Cellular total protein thiols were determined using DTNB as described by Ellman [1959]. The contents of total protein thiols were expressed as nanomoles per milligram protein using GSH as a standard.

### STATISTICAL ANALYSIS

Results from a representative of three independent experiments are shown as mean ± SD. Means were compared by one-way ANOVA, with comparison of different groups by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered significant.

## RESULTS

### SODIUM SELENITE ATTENUATED $H_2O_2$-INDUCED CELL INJURY

As demonstrated by MTT assay, $Na_2SeO_3$ alone at 0.1 μM significantly increased the viability of calcifying VSMCs after treatment for 4 and 8 days, but had no effect after treatment for 12 days. The cell viability had no change after treatment with 1 μM $Na_2SeO_3$ alone for 4 and 8 days, but was significantly reduced after treatment for 12 days (Fig. 1A). These data suggested that low dose (0.1 μM) of $Na_2SeO_3$ was relatively safe to cells under our experimental condition, and long time treatment with high dose (1 μM) of $Na_2SeO_3$ might be toxic. This result follows Weinberg curve which has been used to describe the relationship between the biologic effects and the concentrations of trace elements. Thus, 0.1 μM $Na_2SeO_3$ was more often used in the following experiments to test the positive role of $Na_2SeO_3$ against $H_2O_2$-induced effects. However, further in vivo studies are needed to demonstrate experimentally if $Na_2SeO_3$ at this concentration is toxic or not.

The effect of $H_2O_2$ on the viability of calcifying VSMCs was concentration- and time-dependent (Fig. 1B). No significant change in cell viability was observed after treatment with 0.2 mM $H_2O_2$ even for 9 days (data not shown). Hydrogen peroxide at 0.5 and 1 mM had no significant effect on cell viability after 3 days treatment, but induced cell injury after 7 days treatment, as demonstrated by the decrease of cell viability to 88.4% ($P < 0.05$) and 64.2% ($P < 0.01$) of control, respectively. The cytotoxicity of $H_2O_2$ was significantly suppressed by pretreatment with 0.1 μM $Na_2SeO_3$ for 24 h (Fig. 1B).

### SODIUM SELENITE INHIBITED $H_2O_2$-EHANCED VSMCS CALCIFICATION

The effect of $Na_2SeO_3$ on $H_2O_2$-enhanced VSMCs calcification was evaluated based on calcium deposition in extracellular matrix

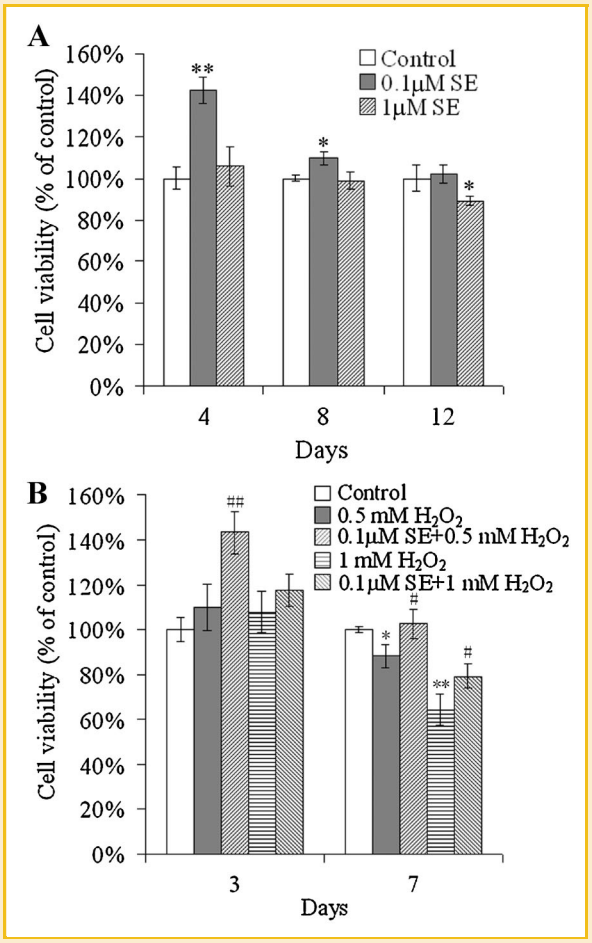


Fig. 1. Effect of $H_2O_2$ on the viability of calcifying VSMCs and the influence of $Na_2SeO_3$ (SE). A: Cells were treated with 0.1 and 1 μM $Na_2SeO_3$ alone for 4, 8, and 12 days. B: Cells were treated with indicated dose of $H_2O_2$ alone for 3 and 7 days or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. The cell viability was evaluated using the MTT assay (mean ± SD, n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with $H_2O_2$ alone.

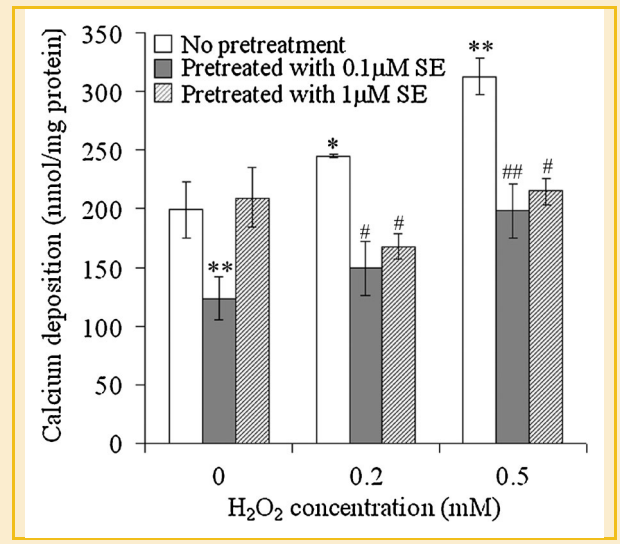


Fig. 2. Effect of $Na_2SeO_3$ on $H_2O_2$-enhanced VSMCs calcification. Calcifying VSMCs were treated with indicated dose of $H_2O_2$ alone for 9 days or pretreated with 0.1 and 1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$ with media change every 2 or 3 days. In vitro VSMC calcification was evaluated based on calcium deposition in extracellular matrix determined by atomic absorption spectroscopy (mean ± SD, n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with $H_2O_2$ alone.

(Fig. 2). Sodium selenite alone at 0.1 μM significantly inhibited β-glycerophosphate-induced VSMCs calcification, while no obvious change was observed at 1 μM. Hydrogen peroxide treatment promoted VSMCs calcification in a dose-dependent manner. In calcifying VSMCs exposed to 0.2 and 0.5 mM $H_2O_2$ for 9 days, the calcium deposition were increased 23.0% and 56.8%, respectively, compared with control group. The effect of $H_2O_2$ on calcium deposition was significantly inhibited by $Na_2SeO_3$ pretreatment. For example, in calcifying VSMCs pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to 0.5 mM $H_2O_2$, the calcium deposition was decreased to the level of control cells.

## SODIUM SELENITE SUPPRESSED $H_2O_2$-PROMOTED OSTEOBLASTIC DIFFERENTIATION OF VSMCS

Previous studies had shown that $H_2O_2$ enhanced vascular calcification through promoting osteoblastic differentiation of VSMCs [Mody et al., 2001; Byon et al., 2008]. Osteoblastic differentiation of VSMCs is characterized by the expression of multiple bone-related molecules including ALP, Col I, and OC. In addition, Runx2, also known as Cbfa1/Osf2/AML3/PEBP2aA, is a key transcription factor that was up-regulated during osteoblastic differentiation of VSMCs [Byon et al., 2008; Nakahara et al., 2010]. Therefore, we determined the effect of $Na_2SeO_3$ on $H_2O_2$-promoted osteoblastic differentiation of VSMCs by examining the expression of Runx2, Col I, and OC, as well as ALP activity.

Quantitative real-time PCR was first performed, which confirmed increased expression of Runx2, Col I, and OC after calcifying VSMCs were exposed to 1 mM $H_2O_2$ for 3 days (fold increase: Runx2 = 1.3 ± 0.3, Col I = 5.0 ± 1.4, and OC = 0.4 ± 0.2, compared with control, Fig. 3). Increased expressions of Runx2, Col I and OC by $H_2O_2$ were significantly attenuated in calcifying VSMCs pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$.

We next determined the activity of ALP in calcifying VSMCs treated with $H_2O_2$ alone or pretreated with $Na_2SeO_3$ before exposure to $H_2O_2$. The data showed that $H_2O_2$ treatment resulted in a significant increase in ALP activity in a time- and dose-dependent manner, which was markedly inhibited by $Na_2SeO_3$ (0.1 and 1 μM) pretreatment (Fig. 4). The inhibitory effect of $Na_2SeO_3$ at the dose of 0.1 μM was much stronger, which was consistent with the result of calcium deposition in extracellular matrix (Fig. 2).

## ERK-DEPENDENT PATHWAY REGULATED THE $H_2O_2$-INDUCED OSTEOBLASTIC DIFFERENTIATION OF VSMCS AND THE INFLUENCE OF $NA_2SEO_3$

The growing list of literatures points to the importance of ERK activation in osteoblastic differentiation of VSMCs regardless of the stimuli [Lovdahl et al., 2000; Ding et al., 2006; Bear et al., 2008; You

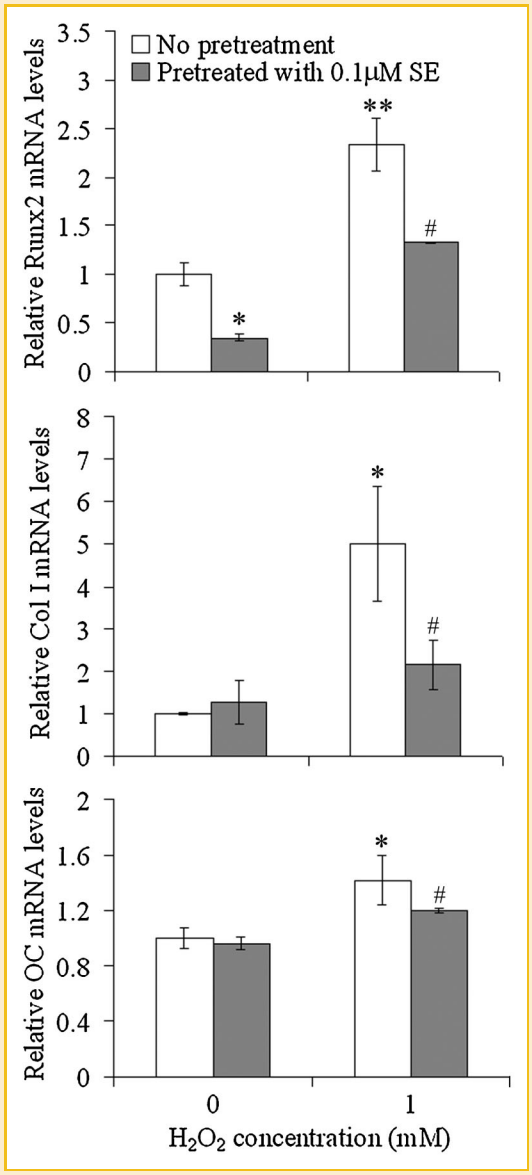


Fig. 3. Effect of $Na_2SeO_3$ on $H_2O_2$-induced expression of bone-related markers. Calcifying VSMCs were exposed to 1 mM $H_2O_2$ alone for 3 days or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. Expressions of bone-related molecules Runx2, Col I, and OC were determined by real-time PCR. GAPDH was measured as an internal control. The data were shown as mean ± SE (n = 3) of relative expression level. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, compared with cells treated with $H_2O_2$ alone.

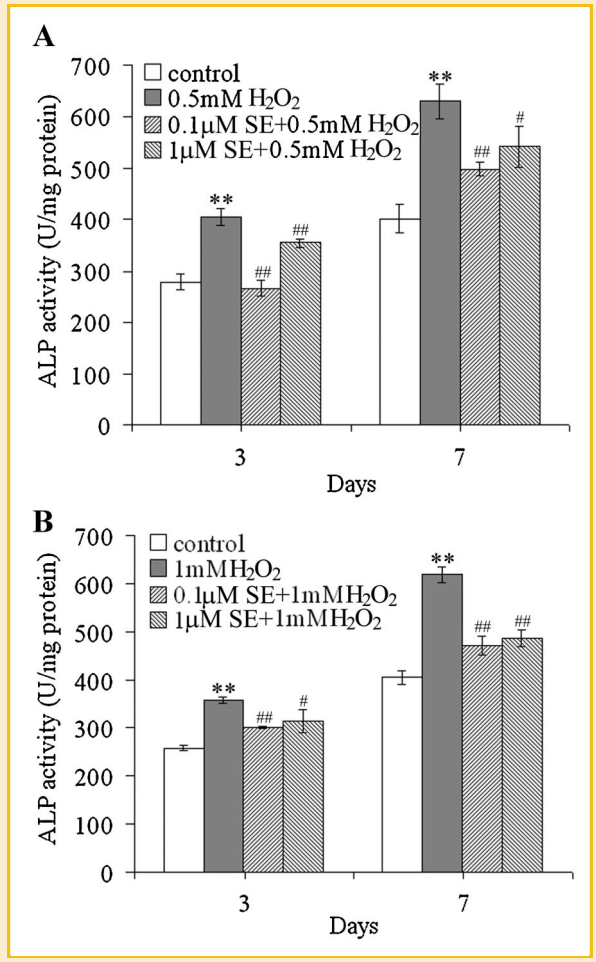


Fig. 4. Effect of $Na_2SeO_3$ on $H_2O_2$-promoted increase in ALP activity in calcifying VSMCs. Cells were treated with indicated dose of $H_2O_2$ alone for 3 and 7 days or pretreated with 0.1 and 1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. ALP activity was measured by spectrophotometry (mean ± SD, n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with $H_2O_2$ alone.

et al., 2009; Nakahara et al., 2010]. Thus, in this work, calcifying VSMCs were treated with $H_2O_2$ alone or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$, and whole cell extracts were prepared for Western blot analysis for phosphorylated ERK1/2. The data showed that treatment of calcifying VSMCs with 1 mM $H_2O_2$ alone resulted in the phosphorylation of ERK1/2, which occurred rapidly and remained high for up to 4 h (Fig. 5A). Sodium selenite alone at 0.1 μM did not affect the level of ERK phosphorylation (data not shown), but markedly inhibited $H_2O_2$-induced ERK activation (Fig. 5A). The role of ERK in $H_2O_2$-enhanced osteoblastic differentiation of VSMCs was further evaluated by using specific MEK1 inhibitor PD98059. Pretreatment with 1 μM PD98059 for 2 h completely blocked the induction of Runx2 expression and ALP activity by $H_2O_2$ (Fig. 5B), suggesting that $H_2O_2$-enhanced osteoblastic differentiation of VSMCs was dependent on ERK activation. Taken together, our present data indicated that $Na_2SeO_3$ suppressed $H_2O_2$-induced osteoblastic differentiation of VSMCs probably through blocking ERK pathway.

### SODIUM SELENITE ATTENUATED $H_2O_2$-INDUCED OXIDATIVE STRESS IN CALCIFYING VSMCS

The effect of $Na_2SeO_3$ on $H_2O_2$-induced oxidative stress in calcifying VSMCs was observed by examining intracellular ROS generation, the contents of MDA and total protein thiols, as well as the activity of GPx, a very important antioxidant enzyme in cellular defense system (Fig. 6). The intracellular ROS generation (Fig. 6A) and MDA content (Fig. 6B) were significantly increased in $H_2O_2$-treated cells. In contrast, protein thiols content (Fig. 6C) and GPx activity (Fig. 6D)

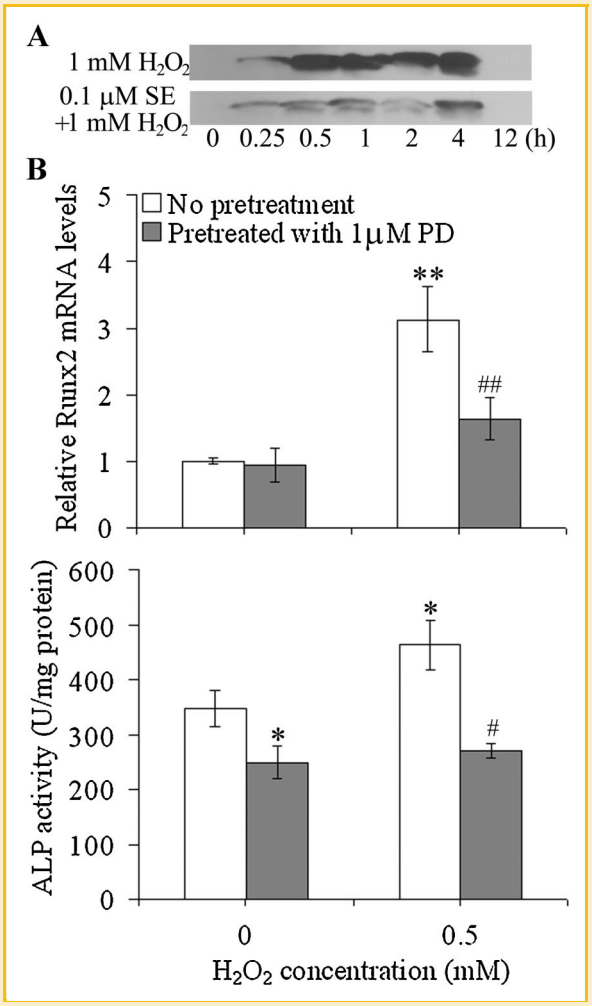


Fig. 5. ERK-dependent pathway regulated $H_2O_2$-induced osteoblastic differentiation of VSMCs and the influence of $Na_2SeO_3$. A: Time-course of $H_2O_2$-induced ERK1/2 phosphorylation in calcifying VSMCs and the influence of $Na_2SeO_3$. Calcifying VSMCs were treated with 1 mM $H_2O_2$ alone for indicated time or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. The phosphorylation of ERK was determined by Western blot analysis. B: The effect of MEK1 inhibitor PD98059 (PD) on the induction of Runx2 expression and ALP activity by $H_2O_2$. Calcifying VSMCs were treated with 0.5 mM $H_2O_2$ alone for 7 days or pretreated with 1 μM PD98059 for 2 h before exposure to $H_2O_2$. Expression of Runx2 was determined by real-time PCR (mean ± SD, n = 3) and ALP activity was measured by spectrophotometry (mean ± SD, n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with $H_2O_2$ alone.

were significantly decreased after $H_2O_2$ treatment. These results confirmed that $H_2O_2$ induced oxidative stress in calcifying VSMCs. Pretreatment with 0.1 μM $Na_2SeO_3$ for 24 h significantly reversed the effects of $H_2O_2$ on intracellular ROS generation, the contents of MDA and protein thiols. Moreover, compared with cells treated with $H_2O_2$ alone, GPx activity was increased 84.4% ($P < 0.05$) in the cells pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. Sodium selenite treatment alone also markedly increased GPx activity. These results reflected that $Na_2SeO_3$ effectively suppressed $H_2O_2$-induced oxidative stress in calcifying VSMCs through increasing cellular levels of antioxidant capacity such as the activity of GPx.

## DISCUSSION

Knowledge on import and management of the essential nutritional trace element Se at the cell biologic, biochemical, and molecular biologic level has increased considerably in recent years [Reeves and Hoffmann, 2009]. Accumulated lines of evidence indicated that dietary Se supplementation might prevent atherosclerosis by protecting cells from oxidative stress [Thomas et al., 1993; Huang et al., 2002; Wu et al., 2003; Tang and Huang, 2004; Wu and Huang, 2004, 2006; Tang et al., 2005; Espinola-Klein et al., 2007; Torzewski et al., 2007], but there were no reports about the effect of Se on vascular calcification. In the present study, the effect of $Na_2SeO_3$ on $H_2O_2$-induced VSMCs calcification and the related mechanism were investigated. The results showed that $Na_2SeO_3$ suppressed $H_2O_2$-enhanced vascular calcification by inhibiting osteoblastic differentiation of VSMCs; this effect was associated with the inhibition of oxidative stress and ERK activation.

Osteoblastic differentiation of VSMCs is critical in the development of calcification in atherosclerotic lesions and is characterized by the expression of multiple bone-related molecules including ALP, Col I, and OC [Trion and van der Laarse, 2004]. During osteoblastic differentiation these molecules are expressed at different phases and reflect different aspects of osteoblast function and mineral formation. ALP and Col I are early markers, and OC is a late marker. In addition, Runx2 is a key transcription factor that has been shown to induce ALP activity and the expression of osteoblast-specific genes such as OC, Col I [Byon et al., 2008]. In the present work, $H_2O_2$ (0.2–1 mM) was found to dose- and time-dependently enhance vascular calcification through inducing osteoblastic differentiation of VSMCs, as demonstrated by the increase of the mRNA expression of Runx2, Col I, and OC, ALP activity, as well as calcium deposition in extracellular matrix (Figs. 2–4). This observation was in agreement with the previous works reported by Byon et al. [2008] and Mody et al. [2001]. Our data showed that $Na_2SeO_3$ pretreatment (0.1–1 μM) for 24 h before $H_2O_2$ exposure suppressed $H_2O_2$-enhanced osteoblastic differentiation and calcification of VSMCs, as evidenced by: (1) decreased the calcium deposition of calcifying VSMCs, suggesting that $Na_2SeO_3$ could inhibit VSMCs calcification (Fig. 2); (2) reduced the mRNA expressions of Col I and OC, and ALP activity in calcifying VSMCs (Figs. 3 and 4), indicating that $Na_2SeO_3$ inhibited osteoblastic differentiation of VSMCs; and (3) down-regulated the mRNA expression of Runx2 (Fig. 3), implying that $Na_2SeO_3$ inhibited osteoblastic differentiation of VSMCs by reducing Runx2 transactivity. Our in vitro evidence suggested that Se supplementation might be a valuable approach to limit the development of vascular calcification. However, further studies are needed to test this hypothesis by using in vivo animal model of vascular calcification. It should be noted that the relationship between the biologic effects and the concentrations of Se also follows Weinberg's principle [Yang, 1987], thus it was reasonable that the inhibitory effect

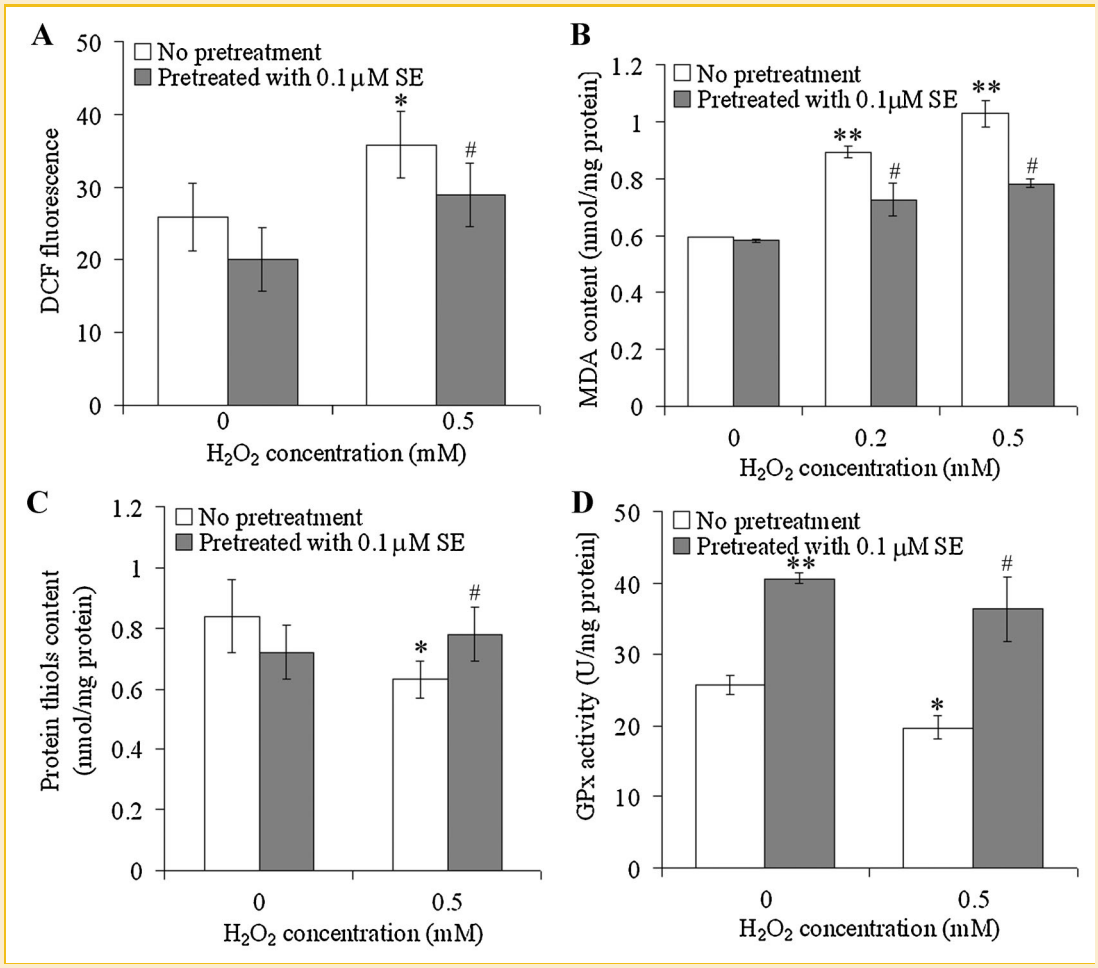


Fig. 6. Effects of $Na_2SeO_3$ on $H_2O_2$-induced oxidative stress in calcifying VSMCs. A: Cells were treated with 0.5 mM $H_2O_2$ alone for 2 h or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$. The intracellular ROS generation was measured by DCF fluorescence (mean ± SD, n = 8). B–D: Cells were treated with indicated dose of $H_2O_2$ alone for 7 days or pretreated with 0.1 μM $Na_2SeO_3$ for 24 h before exposure to $H_2O_2$, and then MDA content (B), cellular total protein thiols (C), and GPx activity (D) were measured, respectively (mean ± SD, n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with $H_2O_2$ alone.

of $Na_2SeO_3$ at 0.1 μM on osteoblastic differentiation and calcification of VSMCs enhanced by $H_2O_2$ was much stronger than $Na_2SeO_3$ at 1 μM, as observed in the present work.

The mechanism by which $Na_2SeO_3$ inhibited $H_2O_2$-enhanced vascular calcification remained to be investigated. Hydrogen peroxide is a cell-permeable ROS that has emerged as a main source of oxidative stress. Previous studies reported that oxidative stress induced by exogenous $H_2O_2$ enhanced osteoblastic differentiation of VSMCs [Mody et al., 2001; Byon et al., 2008]. In this work, oxidative stress induced by exogenous $H_2O_2$ (0.2–0.5 mM) was visualized by the increase of intracellular ROS production, and the decrease of the activity of GPx, an endogenous scavenger of $H_2O_2$ (Fig. 6A and D). In addition, the excess ROS induced by $H_2O_2$ caused obviously oxidative damage of cellular lipids and proteins, as evidenced by the increased MDA level and the decreased protein thiols content (Fig. 6B and C). Several selenoproteins, such as GPx, TR, have been characterized as antioxidant enzymes, serving to mitigate oxidative damage caused by ROS. Our previous studies suggested that VSMCs could take up $Na_2SeO_3$ and incorporate Se into selenoproteins, such as GPx. Increasing activity of selenoproteins in VSMCs could protect markedly cells against oxidative stress [Tang and Huang, 2004; Tang et al., 2005]. Consistent with these results, our present data demonstrated that GPx activity and protein thiols content were markedly increased, while the intracellular ROS production and MDA level were significantly decreased in calcifying VSMCs pretreated with 0.1 μM $Na_2SeO_3$ before exposure to $H_2O_2$, compared with the cells treated with $H_2O_2$ alone (Fig. 6). These results suggested that $Na_2SeO_3$ at appropriate concentrations protected cells from $H_2O_2$-mediated oxidative injury by increasing the antioxidation of selenoenzymes, and thus suppressed osteoblastic differentiation and calcification of VSMCs. These results were supported by previous studies showing that $H_2O_2$-enhanced osteoblastic differentiation of vascular cells was blocked by exogenous antioxidants pyrrolidinedithiocarbamate (PDTC), a thiol-containing antioxidant, and Trolox, a hydrophilic vitamin E analog [Mody et al., 2001], and vitamin E antagonized acceleration

of vascular calcification by hypercholesterolemia in rats [Tang et al., 2006].

Oxidative damage of cellular lipids and proteins induced by $H_2O_2$ might result in the change of signal transduction pathways. The present study found that the ERK pathway was activated following $H_2O_2$ treatment, and inhibition of ERK pathway by MEK1 inhibitor PD98059 completely blocked $H_2O_2$-induced Runx2 expression and ALP activity (Fig. 5), indicating that $H_2O_2$-mediated promotion of osteoblastic differentiation of VSMCs was dependent on ERK activation. Pretreatment with $Na_2SeO_3$ before exposure to $H_2O_2$ was shown to attenuate ERK phosphorylation (Fig. 5A). Furthermore, oxidative damage of cellular lipids and proteins induced by $H_2O_2$ in calcifying VSMCs was significantly inhibited by $Na_2SeO_3$ pretreatment (Fig. 6), as discussed above. Taken together, our results suggested that $Na_2SeO_3$ pretreatment suppressed $H_2O_2$-enhanced osteoblastic differentiation of VSMCs probably through inhibiting oxidative stress, and subsequent ERK activation. The present result about the role of ERK pathway in $H_2O_2$-enhanced osteoblastic differentiation of VSMCs was in agreement with previous studies demonstrating that ERK phosphorylation was associated with osteoblastic differentiation of VSMCs enhanced by oxidized LDL, fibroblast growth factor-2 (FGF-2), fibronectin, advanced oxidation protein products, and injury [Lovdahl et al., 2000; Ding et al., 2006; Bear et al., 2008; You et al., 2009; Nakahara et al., 2010]. Moreover, Nakahara et al. [2010] reported that FGF-2 induced $H_2O_2$ production and antioxidant *N*-acetyl-L-cysteine attenuated FGF-2-induced ERK activation and the osteoblastic marker osteopontin expression, suggesting that $H_2O_2$ are required for ERK activation and osteoblastic differentiation by FGF-2 in VSMCs. However, Byon et al. [2008] obtained a different result that $H_2O_2$-activated ERK signaling was not required for $H_2O_2$-induced VSMCs calcification. Therefore, further researches are needed to confirm the role of ERK pathway in $H_2O_2$-enhanced vascular calcification.

In summary, our study demonstrated that $Na_2SeO_3$ not only attenuated the intracellular oxidative stress and ERK activation induced by $H_2O_2$, but also blocked $H_2O_2$-induced osteoblastic differentiation and calcium deposition in calcifying VSMCs. Considering the important role of oxidative stress and ERK signal in $H_2O_2$-enhanced vascular calcification, we concluded that $Na_2SeO_3$ suppressed $H_2O_2$-enhanced VSMCs calcification by inhibiting oxidative stress and ERK activation. These results indicate a potential preventive role for Se in vascular calcification, suggesting that vascular calcification may be another target of Se action in anti-atherosclerosis. However, our conclusion is just drawn from in vitro experiment. Due to the potential side effects of Se, the safety using Se as preventive drug for vascular calcification needs to be further addressed in animal experiments, especially in human experiments.

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