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Chapter 3

***In Vitro* Calcification of Neonatal Rat Vascular Smooth Muscle Cells**

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Submitted

Abstract

Background Vascular calcification is a prominent feature of atherosclerosis and associated with an increased risk of myocardial infarction. Recent data indicate that vascular calcification is an organized process in which vascular smooth muscle cells (VSMCs) are the principal component.

Methods To study the process of VSMC calcification we developed and characterized an *in vitro* model of VSMC calcification. Neonatal rat VSMCs were isolated by outgrowth from aortic explants and characterized by immunohistochemistry. VSMC calcification was induced by incubating the cells with a particular calcification medium containing 10 mmol/L β -glycerophosphate, 8 mmol/L CaCl_2 , 10 mmol/L sodium pyruvate, 1 $\mu\text{mol/L}$ insulin, 50 $\mu\text{g/mL}$ ascorbic acid and 100 nmol/L dexamethazone. Calcification of neonatal rat VSMCs was examined by quantitation of calcium deposition using the o-cresolphthalein method. Osteopontin expression was assessed by Western blotting.

Results Incubation of VSMCs in calcification medium resulted in deposition of calcium salts within 21 days. Calcium deposition was dependent upon the extracellular concentration of organic phosphate ($p=0.01$), calcium ions ($p=0.01$) and ascorbic acid ($p=0.05$), and was observed in the presence of dexamethazone and insulin. Osteopontin expression was dependent upon the calcium and β -glycerophosphate concentration in the calcification medium.

Conclusion This *in vitro* model of VSMC calcification allows the study of arterial wall calcification as well as modifying factors, including pharmacotherapeutics.

Keywords: calcification, atherosclerosis, vascular smooth muscle cells, *in vitro* model

Introduction

Atherosclerosis is the predominant cause of myocardial infarction, stroke and peripheral vascular disease and is the major cause of mortality in the Western hemisphere ¹. The presence of calcium deposits in the vessel wall is indicative of advanced atherosclerosis, and the extent of coronary calcification ("calcium score") has been found to add prognostic significance to conventional risk factors for coronary artery disease ^{2,3}. Vascular calcification refers to the deposition of calcium phosphate salts, most often in the form of hydroxyapatite, in the vessel wall. Calcification of the vessel wall and in heart valves is associated with ageing, diabetes and uraemia ⁴⁻⁶.

Previously arterial calcification was considered to be a degenerative, end-stage process of vascular disease. However, recent data indicate that vascular calcification is an organized process in which vascular smooth muscle cells (VSMCs) are the principal component ⁷. Calcified vascular tissues have been shown to contain proteins that are associated with bone formation such as bone morphogenetic proteins ^{8,9}, osteonectin, osteocalcin and matrix Gla protein (MGP), indicating that calcification is an organized and regulated process similar to mineralization in bone tissue. Also supporting this hypothesis is the presence of matrix vesicle-like structures in atherosclerotic plaque ¹⁰. Matrix vesicles, which are commonly found in cartilage and bone, are membrane-bound structures that bud-off from chondrocytes and osteoblasts, and are thought to serve as nucleation sites for calcium crystal growth ¹¹.

Although calcification in atherosclerotic lesions has been described in morphological terms, the molecular and cellular mechanisms involved in the calcification process are still largely unknown. We used an *in vitro* cell model for research in this area of interest. There are two major advantages of using cultured cells, the first advantage being the control of experimental variables and the second being the reliable, manipulatable, and consistent source of relatively large quantities of biological material generally needed for cellular and molecular studies.

Boström et al. have previously developed a model system using cells isolated from the arterial wall of bovine and human origin, which undergo spontaneous calcification in cell culture. These VSMC-derived calcifying vascular cells (CVC) were found to form calcium-mineral containing nodules *in vitro*. TGF- β and 25-hydroxycholesterol both increased the rate of mineralised nodule formation by CVC in this model system ¹².

We adapted the incubation protocol described by Shioi *et al.* ¹³ to develop an *in vitro* model of vascular calcification using neonatal rat VSMCs, isolated by outgrowth from aortic explants. The purpose of this investigation was to identify the factors, and their concentrations, that are mandatory to induce calcification of neonatal rat VSMCs *in vitro*.

Materials and methods

Cell culture

VSMCs were obtained from segments of aortas explanted from 2-day old Wistar rats. The aortic segments were obtained aseptically and cut open longitudinally. The endothelium was removed by gently rubbing the luminal side of the aortas over the surface of a tissue culture dish (Falcon). Subsequently the aortas were placed, lumen side down, on the bottom of a tissue culture flask (Greiner) and allowed to adhere for approximately 3 h. Thereafter the tissues were immersed in growth medium. VSMCs were isolated by outgrowth from the aortic explants. The standard growth medium for the VSMCs was Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), penicillin (100 U/mL) and streptomycin (100 µg/mL) (both supplied by BioWhittaker Europe). Seven days later the aortic tissues were removed and the VSMCs that had grown out of the aortic tissue were detached by trypsinisation. The detached cells were resuspended in growth medium and seeded in tissue culture flasks (Greiner), 6-well plates or on cover slips.

In vitro calcification of VSMCs

Calcification of VSMC cultures was induced by the method of Shioi *et al.*¹³ with minor modifications. When confluent, the incubation medium of VSMCs was switched from growth medium to calcification medium. Calcification medium consists of DMEM (high glucose, 4.5 g/L) containing 15% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), 8 mmol/L CaCl₂, 10 mmol/L sodium pyruvate, 1 µmol/L insulin, 50 µg/mL ascorbic acid, 10 mmol/L β-glycerophosphate and 100 nmol/L dexamethazone. The medium was replaced with fresh medium every 2-3 days.

Assesment of calcium deposition

Detection of calcification: The presence of calcification was assayed by the Von Kossa method, adopted from Proudfoot *et al.*¹⁴. Cells were grown in 6-well plates and treated with calcification medium for 21 days as described in the preceding paragraphs. Subsequently, cells were washed with PBS containing 1% FBS and then fixed in 1% formaldehyde in PBS for 1 hour on ice. The cells were washed in distilled water and exposed to 5% aqueous AgNO₃ for 60 min. The cells were then exposed to 2.5% sodium thiosulfate for 5 min. Black color represents staining positive of calcification.

Quantification of calcium deposition: Neonatal VSMCs were grown in 12-well plates for 21 days in the presence of calcification medium as described in the previous paragraphs. The

VSMCs were decalcified with 0.6 N HCl for 24 hours. The calcium content of the supernatants was determined by spectrophotometer using the o-cresolphthalein method (Roche Diagnostics). After decalcification the cells were washed with PBS and scraped from the culture plate. The protein content was measured using BCA protein assay reagent (Pierce). The calcium content of the cell layer was normalized to protein content.

Immunocytochemistry

Subconfluent cultures at early passage were examined for the presence of α -smooth muscle actin, SM myosin heavy chain and calponin using specific antibodies (anti-SM actin; clone 1A4, Sigma; anti-SM myosin heavy chain, SanverTech; anti-calponin, Sigma). For immunocytochemistry the cells were cultured on cover slips for several days. After fixation in 1% formalin in PBS on ice for 30 min, the cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min on ice. Subsequently the cells were incubated with the primary antibody (anti-SM actin, 1:400; anti-SM myosin heavy chain, 1:100; anti-calponin, 1:10,000, all diluted in PBS) for several hours at 4°C. Secondary antibodies were conjugated with fluorescein isothiocyanate (FITC) or Cy3 (Sigma). To identify cell nuclei, the cell cultures were also stained with Hoechst 33342 (10 μ g/mL; Molecular Probes) for 10 min. Immunofluorescent images were obtained using a fluorescence microscope (Nikon Eclipse) equipped with 20x, 40x and 100x objectives and a digital camera (Nikon DXM1200).

Western blot analysis

Neonatal rat VSMCs were grown in 12-well culture plates and incubated with various reagents for approximately 15 days. Cells were then scraped, and washed with ice-cold phosphate-buffered saline (PBS). The cell pellet was lysed in lysis buffer containing protease inhibitors (20 mmol/L TrisHCl, pH 7.4, 100 mM NaCl, 5 mmol/L EDTA, 1% Triton X-100, 10% glycerol, 1 mmol/L AEBSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin) and sonicated. The cell debris was pelleted and the total protein concentration of the supernatant was measured using the Pierce assay. Cell lysates were mixed with sample buffer and heated for 10 min at 70°C. Protein extracts (10 μ g) were size-fractionated on NuPage Novex 12% Bis-Tris gels (Invitrogen) under reducing conditions and subsequently electro-transferred to Hybond polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) overnight at 4°C. Non-specific binding sites were blocked by incubating the membranes in a blocking solution (20 g/L ECL Advance Blocking Agent (Amersham Biosciences)) in TBS-Tween (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.05% Tween) for 1 h on an orbital shaker. The primary antibody, rabbit anti-human osteopontin (LF-124, gift of Dr. L.W. Fisher, National Institutes of Health, Bethesda, Maryland, USA), was diluted in antibody diluent (1:50,000) and incubated

with the membranes for 1 hour at room temperature. The membrane was washed four times with TBS-Tween for 10 min and incubated with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology), 1:10,000 for 1 h at room temperature. The membranes were washed again, four times in TBS-Tween for 10 min, and incubated in ECL Advance Detection reagent (Amersham Biosciences) for 5 min. Light emission was detected by exposure to Hyperfilm ECL (Amersham Biosciences). The intensity of protein bands was determined using image analysis software (Scion, available at www.scioncorp.com).

Statistical analysis

Results are expressed as mean \pm SEM. For statistical analysis SPSS 10.0 for Windows was used. Because the data were not normally distributed, non-parametric tests were used for comparisons between groups. Overall comparisons between groups were performed with the Kruskal-Wallis test. If only two groups were compared, Mann-Whitney rank sum tests were used. P values less than 0.05 were regarded as significant.

Results

Characterization of neonatal rat VSMCs

Cells that have been cultured from aortic explants [fig. 1A] for approx. 7 days were transferred to cover slips and subsequently characterized by immunofluorescence. All cells stained positive for α -smooth muscle actin [fig. 1B] and most cells stained positive for smooth muscle myosin heavy chain and calponin [fig. 1C and 1D], confirming the smooth muscle cell nature of these cells. VSMCs of passage 3 to 6 were subsequently used in calcification experiments.

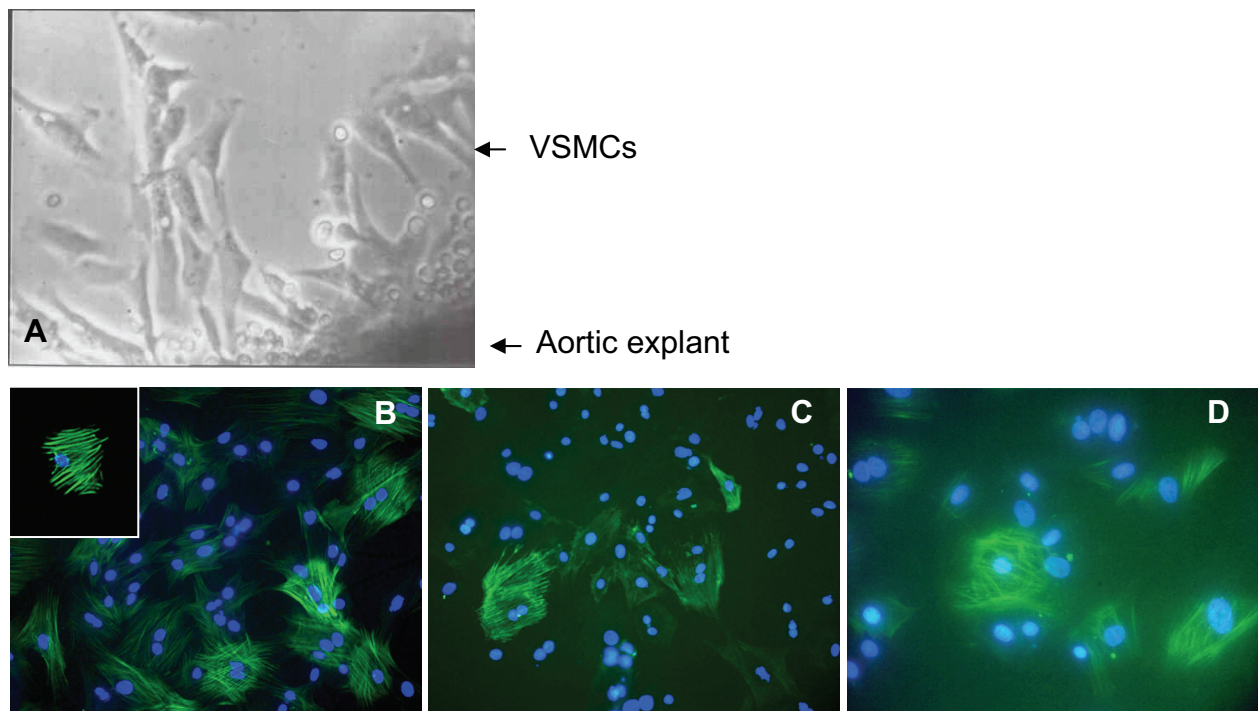


Figure 1. **A.** Neonatal rat VSMC isolated by outgrowth, phase contrast. Immunofluorescent staining of neonatal rat VSMC incubated with **B.** (and insert) Anti-smooth muscle actin Ab, **C.** Anti-smooth muscle myosin Ab and **D.** Anti-calponin Ab. Original magnification B and C x 100, D and insert x 200. VSMC = vascular smooth muscle cell.

Induction of calcification

VSMCs exposed to calcification medium for 3 to 4 weeks stained positive with Von Kossa staining [fig. 2]. To exclude the possibility that the calcium depositions were caused by the high concentration of calcium in the medium alone, VSMCs were also incubated with control

medium (DMEM, 10% FBS and antibiotics) supplemented with 8 mmol/L CaCl_2 (final concentration). These cells did not stain positive with Von Kossa ($n = 4$) (data not shown), indicating that the calcium deposition observed in VSMCs treated with calcification medium is a result of active deposition by the cells.

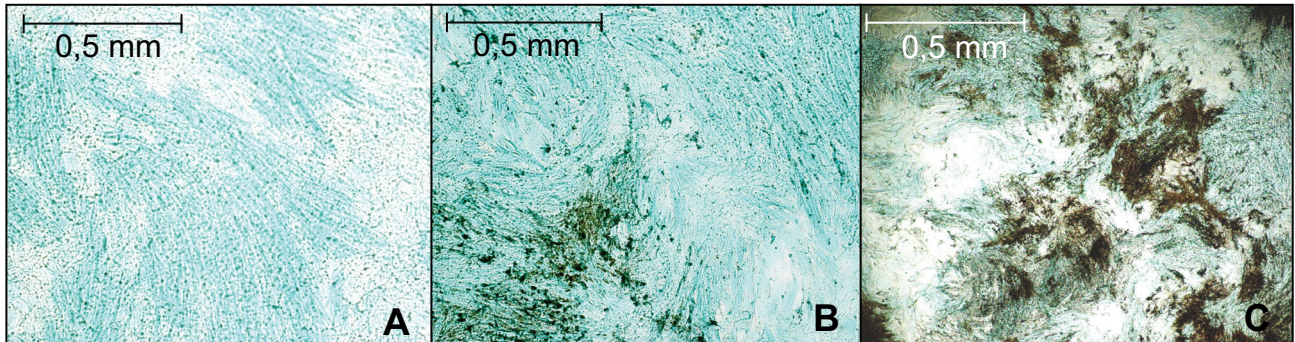


Figure 2. Von Kossa staining (black = positive staining) of neonatal VSMCs cultured in calcification medium for **B.** 3 weeks to **C.** 4 weeks. Panel **A** depicts cells that were cultured in control medium which consists of DMEM (high glucose), 15% FBS and antibiotics. DMEM = Dulbecco's Modified Eagle's medium, FBS = fetal bovine serum, VSMC = vascular smooth muscle cell.

Calcium deposition in VSMCs and dependence of extracellular Ca^{2+} concentration

Cells were incubated with calcification medium supplemented with varying Ca^{2+} concentrations for 21 days. At Ca^{2+} concentrations of 3 mmol/L or lower hardly any calcium deposition was observed, but at Ca^{2+} concentrations > 3 mmol/L a dose-dependent increase in calcium deposition was observed [fig. 3A]. The amount of calcium deposition was positively correlated to the calcium concentration in the culture medium ($p = 0.01$). We chose to continue with 8 mmol/L of Ca^{2+} -ions added to the calcification medium.

Calcium deposition in VSMCs and dependence of extracellular phosphate concentration

Shioi *et al.* have demonstrated that β -glycerophosphate accelerates *in vitro* calcification of VSMCs and induces extensive calcium deposition in a manner analogous to *in vitro* mineralization by osteoblasts¹³. In our model of vascular calcification, the amount of calcium deposition by neonatal rat VSMCs was positively correlated to the β -glycerophosphate concentration in the calcification medium ($p = 0.01$)[fig. 3B].

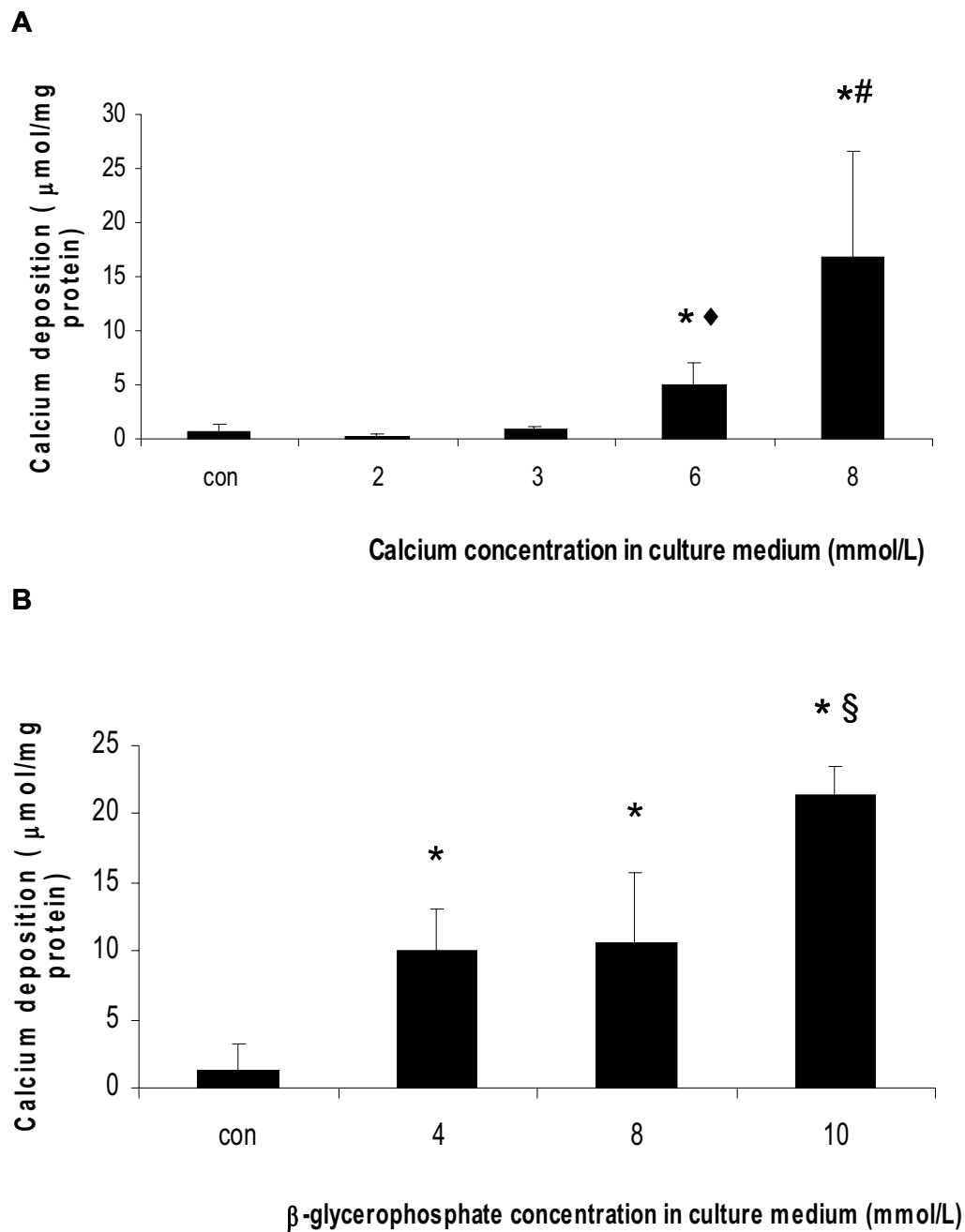


Figure 3. A. Dose-dependent effects of CaCl_2 on calcification of neonatal rat VSMCs. **B.** Dose-dependent effects of β -glycerophosphate on calcification of neonatal rat VSMCs.

VSMCs were treated for 21 days with calcification medium containing varying concentrations of Ca^{2+} ions or β -glycerophosphate. Control cultures (= con) were incubated with DMEM, 10% FBS and antibiotics. Calcium deposition was quantified by o-cresolphthalein method. The data are presented as mean \pm SEM (n = 3 to n = 9). * p < 0.05 vs. control cultures. ♦ p < 0.05 vs. 2 and 3 mM calcium in culture medium. # p < 0.05 vs. all other calcium concentrations used. § p < 0.05 vs. all other β -glycerophosphate concentrations used.

Calcium deposition of VSMCs and dependence of extracellular dexamethazone concentration

To determine the contribution of added dexamethazone, we incubated the VSMCs with varying amounts of dexamethazone (10 – 1000 nmol/L) in the calcification medium. No significant differences in calcium deposition were observed between the cells incubated with 10, 100 and 1000 nmol/L dexamethazone [fig. 4A].

Calcium deposition of VSMCs and dependence of extracellular ascorbic acid concentration

To determine the contribution of ascorbic acid to calcium deposition, we incubated VSMCs with various concentrations of ascorbic acid (0, 5, 50 and 500 µg/mL) in the calcification medium. At the highest concentration (500 µg/mL) ascorbic acid was associated with significantly more calcium deposition than at the lower concentrations used [fig. 4B]. However, 500 µg/mL ascorbic acid caused a considerable decrease of the pH of the culture medium. Since calcium deposition was positively correlated with ascorbic acid concentration ($p = 0.01$), we decided to use 50 µg/mL ascorbic acid in future calcification studies, the highest concentration of ascorbic acid that did not cause acidification of the medium.

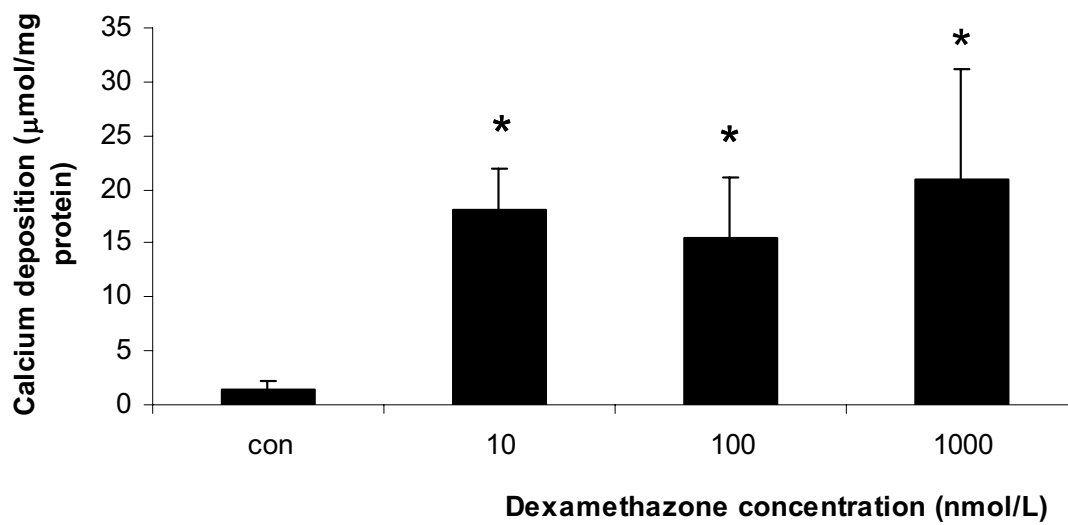
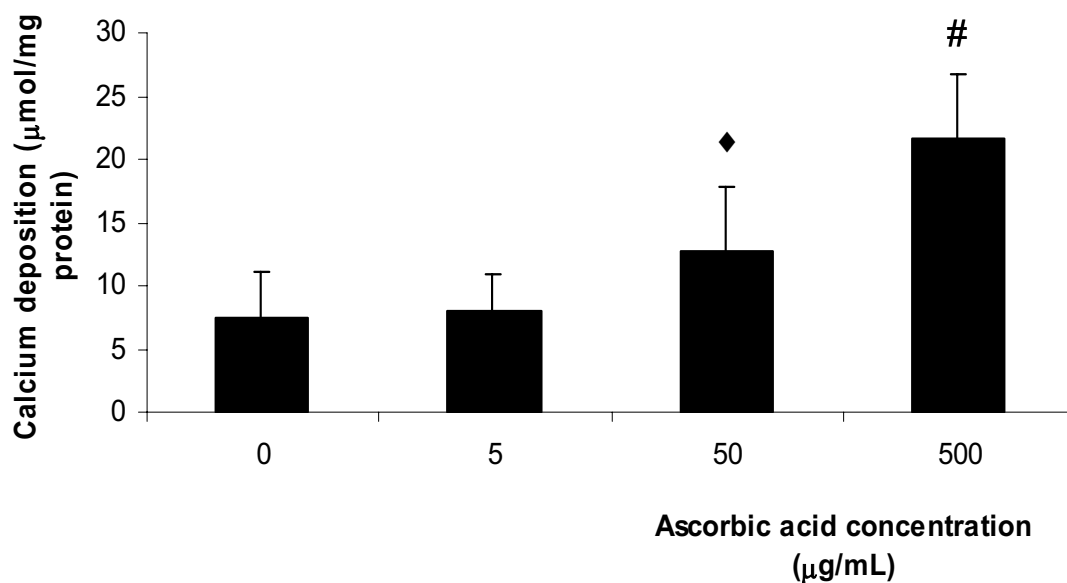
A**B**

Figure 4. A. Dose-dependent effects of dexamethazone on calcification of neonatal rat VSMCs. **B.** Dose-dependent effects of ascorbic acid on calcification of neonatal rat VSMCs.

VSMCs were treated for 21 days with calcification medium containing varying amounts of dexamethazone or ascorbic acid. Control cultures (= con) were incubated with DMEM, 10% FBS and antibiotics. Calcium deposition was quantified by o-cresolphthalein method. The data are presented as mean \pm SEM (n = 6 to n = 12). * p < 0.05 vs. control cultures. # p < 0.05 vs. all lower ascorbic acid concentrations used. ♦ p < 0.05 vs. 0 and 5 μ g/mL ascorbic acid.

Osteopontin expression

We examined the effect of increasing extracellular Ca^{2+} concentration on osteopontin (OPN) expression by neonatal rat VSMCs. Extracellular Ca^{2+} concentration positively affected OPN expression [fig. 5A]. While treatment of VSMCs with 1.8 - 4 mmol/L CaCl_2 had little effect on OPN expression, 6 and 8 mmol/L CaCl_2 induced considerable OPN expression. OPN band intensity was positively correlated to both the calcium concentration in the culture medium and the amount of calcium deposition ($p < 0.01$).

Increasing the amount of β -glycerophosphate in the culture medium, in the presence of 8 mmol/L Ca^{2+} , had effects on OPN expression that were quite similar to those induced by increasing concentrations of Ca^{2+} in the culture medium. At the highest concentration of β -glycerophosphate OPN expression was clearly induced [fig. 5B]. OPN band intensity and the β -glycerophosphate concentration in the culture medium were positively correlated ($p < 0.01$).

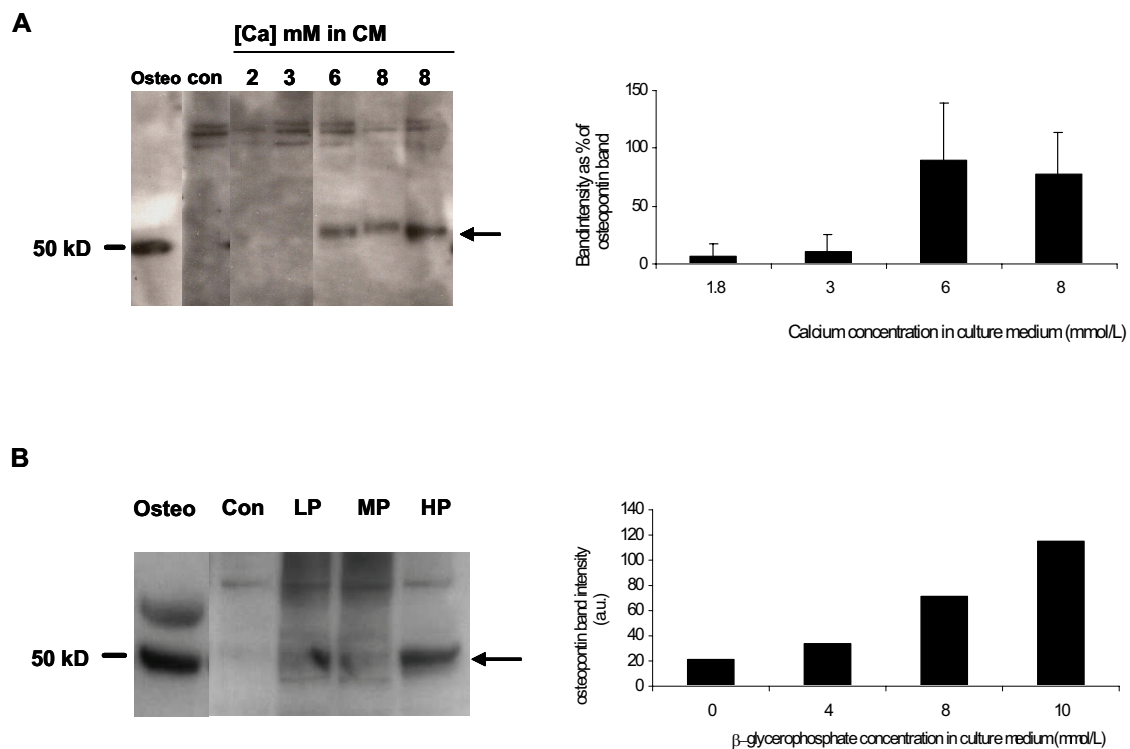


Figure 5. A. Immunoblot analysis of VSMCs incubated with varying concentrations of CaCl_2 or **B.** incubated with varying concentrations of β -glycerophosphate in the calcification medium. Equal amounts of protein were analyzed by Western blotting with anti-osteopontin antibody. Con = control cultures (untreated), LP = 4 mmol/L β -glycerophosphate, MP = 8 mmol/L β -glycerophosphate, HP = 10 mmol/L β -glycerophosphate.

Discussion

Vessel wall calcification is a prominent feature of atherosclerosis. Calcification of the vessel wall is associated with increased wall stiffness resulting in a diminished ability of the vessel wall to adapt its diameter upon changes in blood pressure. Coronary calcification has been associated with an increased risk of coronary heart disease ¹⁵, probably due to colocalization of calcification and atherosclerotic plaques in extensive atherosclerosis. To study the processes involved in vascular calcification the use of *in vitro* models is indispensable. Recent data have indicated that vascular calcification is an organized process in which vascular smooth muscle cells (VSMCs) are the principal component ⁷.

Normally, VSMCs are located in the media of the arterial wall and are embedded in a matrix consisting of elastin, collagen and proteoglycans synthesized by the VSMCs. The fully differentiated, mature VSMCs proliferate at an extremely low rate and have a contractile phenotype. The VSMC has evolved a repertoire of appropriate contractile proteins, receptors, ion channels and signal transduction pathways to carry out this specialized function. The VSMC retains remarkable plasticity, even in adult animals. It can undergo rapid and reversible changes in its phenotype in response to changes in local environmental conditions ¹⁶. During vasculogenesis the VSMC's principal function is proliferation and production of matrix components of the blood vessel wall. Probably due to production of a variety of cytokines by subendothelial macrophages, proliferation and migration of VSMCs to the intimal layer can occur, thereby contributing to the process of atherosclerosis.

Neonatal VSMCs resemble the VSMCs in atherosclerotic plaque since they retain the proliferative phenotype. Neonatal aortic explants exhibit rapid outgrowth of VSMCs and we chose to use these cells to develop a model of vascular calcification. VSMCs were shown to migrate from the aortic explant within several days, and were identified as VSMCs by immunohistochemistry.

Dialysis patients have accelerated atherosclerosis, with extensive calcification of both the intima and the media ¹⁷. Hyperphosphatemia has been implicated in this process ¹⁸. Inorganic phosphate (Pi) levels have previously been shown to regulate human aortic smooth muscle cell (HSMC) culture mineralization *in vitro* ^{19,20}. When cultured in media containing normal physiological levels of Pi (1.4 mmol/L), HSMCs grew in monolayers and did not mineralize. In contrast, if HSMCs were cultured in media containing Pi levels comparable to those seen in hyperphosphatemic individuals (>1.4 mmol/L), dose-dependent increases in cellular calcium deposition occurred. Similar effects were observed in bovine VSMCs ²¹ in which β -glycerophosphate and uremic sera induced calcification. In the present study the β -glycerophosphate concentration and the amount of calcium deposition in neonatal rat VSMCs were correlated positively. Osteoblast cell viability decreases in a dose-

and time-dependent manner when treated with increasing amounts of Pi ²². Apoptosis appears to be an important factor in atherosclerotic calcification ²³, and high phosphate levels induce apoptosis. So, apoptosis may be the link that connects high phosphate levels to atherosclerotic calcification.

In neonatal rat VSMCs the calcium concentration of the culture medium is also an important determinant of calcium deposition. In the present study the calcium concentration of the calcification medium was correlated dose-dependently to the amount of calcium deposition by neonatal rat VSMCs. This observation was not a result of passive calcium deposition since incubating the cells with control medium (DMEM, 10% FBS and antibiotics) supplemented with 8 mmol/L Ca²⁺ did not result in a positive Von Kossa staining, indicating that the calcium deposition observed in VSMCs treated with calcification medium is a result of active deposition by the cells.

Shioi *et al.* ¹³ have previously demonstrated that culturing bovine vascular smooth muscle cells (BVSMC) in the presence of β -glycerophosphate, ascorbic acid, and insulin can induce diffuse calcification in a manner analogous to *in vitro* mineralization of osteoblasts. β -Glycerophosphate appeared to be the most important for inducing calcium deposition. This is in accordance with the results of the present study, showing that both β -glycerophosphate and calcium levels are crucial for the induction of calcification. Ascorbic acid augments this process. Dexamethazone was demonstrated to be less vital in this model of neonatal rat VSMC calcification.

The present study has shown that increased calcium concentrations in the calcification medium were positively correlated to osteopontin (OPN) expression. OPN is a noncollagenous matrix protein which has been associated particularly with calcified atherosclerotic plaques ²⁴. OPN is expressed by macrophages, endothelial cells and VSMCs within the atherosclerotic plaque ²⁵. Using a OPN^{-/-} and apolipoprotein (apo) E^{-/-} double knockout mouse model Matsui *et al.* ²⁶ have demonstrated that OPN promotes atherosclerosis development but inhibits vascular calcification. *In vitro* studies have also demonstrated that addition of OPN to bovine aortic SMC cultures dose-dependently inhibited calcification, an effect that was opposed by addition of calcium to the culture medium ²⁷. The release of OPN near sites of vascular calcification may represent an adaptive mechanism aimed at preventing vascular calcification.

A high concentration of β -glycerophosphate also induced OPN expression in our model, which is in accordance with the results described by Chen *et al.* ²¹ who observed that OPN expression increased when bovine VSMCs were incubated with β -glycerophosphate and uremic sera.

In conclusion, extracellular β -glycerophosphate and Ca^{2+} concentrations are important determinants of *in vitro* calcification of neonatal rat VSMCs. Ascorbic acid augments this process. Dexamethazone was demonstrated to be less vital in this model of neonatal rat VSMC calcification. OPN expression is induced by incubating the VSMCs with both calcium and β -glycerophosphate. This *in vitro* model of VSMC calcification allows the study of arterial wall calcification and the factors, including pharmacotherapeutics, influencing it.

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