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Calcification and C-reactive protein in atherosclerosis : effects of calcium blocking and cholesterol lowering therapy

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Chapter 4

Modulation of Calcification of Vascular Smooth Muscle Cells in Culture by Calcium Antagonists, Statins, and their Combination

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Abstract

Background Atherosclerosis is the principal cause of coronary artery disease, stroke and peripheral artery disease and is the major cause of mortality in the Western hemisphere. Vascular calcification is a prominent feature of atherosclerosis. Calcium antagonists (CAs), originally developed as anti-hypertensive drugs, have been implicated to have anti-atherosclerotic effects as well, but to date their anti-atherosclerotic effects are disputed. Statins have proven anti-atherosclerotic effects, one being a plaque-stabilizing effect. The purpose of the present study was to assess the effects of CAs and statins on the process of vascular calcification in atherosclerosis.

Methods In an *in vitro* model of vascular calcification neonatal rat vascular smooth muscle cells (VSMCs) underwent calcification in the absence and presence of the CA amlodipine, the statin atorvastatin, and their combination. Calcification was measured by the o-cresolphthalein method. Osteopontin expression was assessed by Western blotting. VSMC proliferation was quantified by a commercial colorimetric assay (XTT-based). Apoptotic nuclei were scored by the presence of chromatine condensation as observed from Hoechst 33342-stained nuclei.

Results Incubation of neonatal rat VSMCs with various concentrations of amlodipine (0.01-1 µmol/L) had no effect on calcification. However, incubation of VSMCs with atorvastatin (2-50 µmol/L) resulted in a dose-dependent increase of calcium deposition. At a concentration of 10 µmol/L atorvastatin, calcification was increased 2-fold compared to atorvastatin-free incubated cells. Combining the treatments (0.1 µmol/L amlodipine + 10 µmol/L atorvastatin) resulted in a 2-fold increase in calcification which is similar to the increase observed with atorvastatin (10 µmol/L) alone.

Atorvastatin (50 µmol/L) depressed VSMC proliferation by 50% ($p < 0.01$), whereas amlodipine (1 µmol/L) inhibited VSMC proliferation by only 26% ($p < 0.05$).

Atorvastatin (50 µmol/L), but not amlodipine (1 µmol/L), nor the combination of atorvastatin and amlodipine (10 µmol/L + 0.1 µmol/L, resp.), was associated with considerable apoptosis.

Conclusion *In vitro* calcification of VSMCs is not inhibited by amlodipine, and is stimulated by 10 µmol/L atorvastatin. The latter finding may explain the plaque-stabilizing effect reported for statins.

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

Introduction

Atherosclerosis is the principal cause of coronary artery disease, stroke and peripheral artery disease and is the major cause of mortality in the Western hemisphere [1]. Vascular calcification is a prominent feature of atherosclerosis and it is associated with an increased risk of myocardial infarction [2]. Vascular calcification refers to the deposition of calcium phosphate mineral, most often in the form of hydroxyapatite, in the vessel wall. Calcification of the vessel wall and heart valves is associated with ageing, diabetes and uraemia [3-5].

Vascular calcification is now considered to be an organized, regulated process comparable to bone mineralization. The presence of various components associated with bone mineralization, such as bone morphogenetic proteins, osteocalcin, osteopontin, osteoblast-like cells, and matrix vesicles, in atherosclerotic lesions supports this concept. Vascular cells such as vascular smooth muscle cells (VSMCs) and pericyte-like cells play an important role in vascular calcification [6].

Calcium ions play critical roles in physiological and pathophysiological signal transduction in VSMCs. Therefore it is reasonable to assume that calcium antagonists (CAs) influence the process of atherosclerosis and calcification. The lipophilic CA amlodipine has been shown to restore cholesterol-induced membrane bilayer abnormalities in vascular smooth muscle cells (VSMCs) derived from the atherosclerotic rabbit aorta [7,8], thereby restoring normal calcium homeostasis. Other proposed mechanisms through which CAs may affect atherosclerosis development include inhibition of proliferation and migration of VSMCs [9-12], and inhibition of lipoprotein oxidation [13,14]. In addition, CAs modify binding of monocytes to the endothelium and activate synthesis of matrix components [15]. The effects of CAs on atherosclerotic calcification have not been widely studied however.

Lipids may contribute to atherosclerotic calcification [16,17]. Besides their potent lipid-lowering effects, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) exert pleiotropic effects on vascular wall cells, which include improvement of endothelial function, stabilization of the atherosclerotic plaque, and suppression of inflammation (for review [18-20]). Recently statins have been shown to decrease the progression of coronary artery calcification and aortic valve calcification [21,22], and the mechanisms by which statins influence vascular calcification are now under investigation.

Several *in vitro* and *in vivo* studies have demonstrated that a combination of CA and statin therapy might be more atheroprotective than either treatment alone [23-26]. These studies have shown that combination therapy with CA and statin improves endothelial function and arterial compliance, diminishes LDL atherogenicity, and slows progression of atherosclerosis. The

effects of the combination therapy with CA and statin on vascular calcification have not been widely studied.

We have developed an *in vitro* model of vascular calcification using neonatal rat vascular smooth muscle cells (VSMC). This model was used to study the effect of the CA amlodipine and the statin atorvastatin, alone and in combination, on *in vitro* calcification of neonatal rat VSMCs.

Materials and methods

Cell culture

Vascular smooth muscle cells (VSMCs) were isolated by outgrowth from aortic explants. 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 hours. Thereafter the tissues were immersed in growth medium. 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 (remaining) 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), 12-well plates or on cover slips.

In vitro calcification of VSMCs

Calcification of VSMC cultures was induced by the method of Shioi et al. [27] with minor modifications. Briefly, VSMCs were cultured in DMEM supplemented with 10% FBS and antibiotics as described in the previous paragraph. Cells were detached using trypsin and subsequently cultured in 12-well plates. When the cells reached confluence, the growth medium was switched to calcification medium supplemented with amlodipine, atorvastatin or a combination of these drugs. Cells treated with calcification medium without added drugs were used as positive control. 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 $CaCl_2$, 10 mmol/L sodium pyruvate, 10 μmol/L insulin, 50 μg/mL ascorbic acid, 10 mmol/L β-glycerophosphate and 100 nmol/L dexamethazon. The medium was replaced with fresh

medium every 3 days. After 3 weeks of incubation calcification was quantified as described in the next paragraph.

Quantification of calcium deposition

Neonatal VSMCs were grown in 12-well plates as described in the previous paragraph and treated with calcification medium for 3 weeks. VSMCs were decalcified by incubation with 0.6 N HCl for 24 hours. The calcium contents of the supernatants were determined spectrophotometrically using the o-cresolphthalein method (Roche Diagnostics). After decalcification the cells were washed with ice-cold 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.

Sample drug preparation

The CA amlodipine (Pfizer Inc.) was dissolved in absolute ethanol at a concentration of 1 mmol/L. This stock solution was to be diluted at least 1:1,000 in culture medium.

Atorvastatin (Pfizer Inc.), an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, was dissolved in absolute ethanol at a concentration of 10 mmol/L. This stock solution was to be diluted at least 1:200 in culture medium.

Amlodipine and/or atorvastatin were added to the calcification medium at start of induction of calcification. Treatment with the drugs was continued until calcium deposition was quantified, typically 2-3 weeks after start of calcification induction.

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 μg/ml leupeptin, 10 μg/ml aprotinin) and sonicated. The cell debris was pelleted and the total protein concentration of the supernatant was determined using the Pierce assay. Cell lysates were mixed with sample buffer and heated for 10 min at 70°C. Protein extracts (10 μg) 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), dilution 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).

Proliferation assay

To detect changes in VSMC proliferation resulting from treatment with the various drugs, VSMCs were seeded at a density of $5 \times 10^3 - 2 \times 10^4$ cells/well and allowed to attach overnight. Subsequently the standard culture medium (DMEM + 10% FBS + antibiotics) was replaced by standard culture medium supplemented with amlodipine, atorvastatin or a combination of both. Cell proliferation was assessed at day 4 and day 9 using the Cell Proliferation Kit II (XTT)(Roche Diagnostics) which is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability.

Detection of apoptosis

To detect changes in cell viability resulting from treatment with the various drugs, we determined the percentage of apoptotic cells in culture. Apoptosis was assessed by ultrastructural features. Apoptotic cells were identified by having a condensed chromatin structure. To identify (apoptotic) cell nuclei, VSMC cultures were stained with Hoechst 33342 (10 μg/mL; Molecular Probes) for 10 min in the dark. Immunofluorescent images were obtained using a fluorescence microscope (Nikon Eclipse) equipped with 20x, 40x and 100x objectives and a digital camera (Nikon DXM1200).

Statistical analysis

Results are expressed as mean ± 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 Kruskall-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

Neonatal rat VSMCs exhibit a calcifying capacity by incubation with a specific calcification medium containing 10 mmol/L β-glycerophosphate, 8 mmol/L $CaCl_2$, 10 mmol/L sodium pyruvate, 1 μmol/L insulin, 50 μg/mL ascorbic acid and 100 nmol/L dexamethazone. Within 21 days cultures treated with this calcification medium demonstrated extensive calcium deposition (15-20 μmol calcium/mg protein).

Effects of amlodipine on in vitro *VSMC calcification*

VSMCs were incubated for 2-3 weeks in calcification medium supplemented with varying concentrations of amlodipine (0.01 – 1 μmol/L). Incubation of neonatal rat VSMCs with amlodipine had no effect on VSMC calcification, at none of the concentrations tested [fig. 1A].

Effects of atorvastatin on in vitro *VSMC calcification*

VSMCs were incubated for 2-3 weeks in calcification medium supplemented with varying concentrations of atorvastatin (2-50 μmol/L). Atorvastatin dose-dependently increased VSMC calcification [fig. 1B]. At a concentration of 2 μmol/L atorvastatin calcium deposition was increased by 30% (p = 0.042) when compared to VSMCs incubated with atorvastatin-free calcification medium. At concentrations of 10 and 50 μmol/L atorvastatin calcium deposition was increased 2.3-fold and 6.1-fold, respectively (p < 0.001) when compared to VSMCs incubated with atorvastatin-free calcification medium.
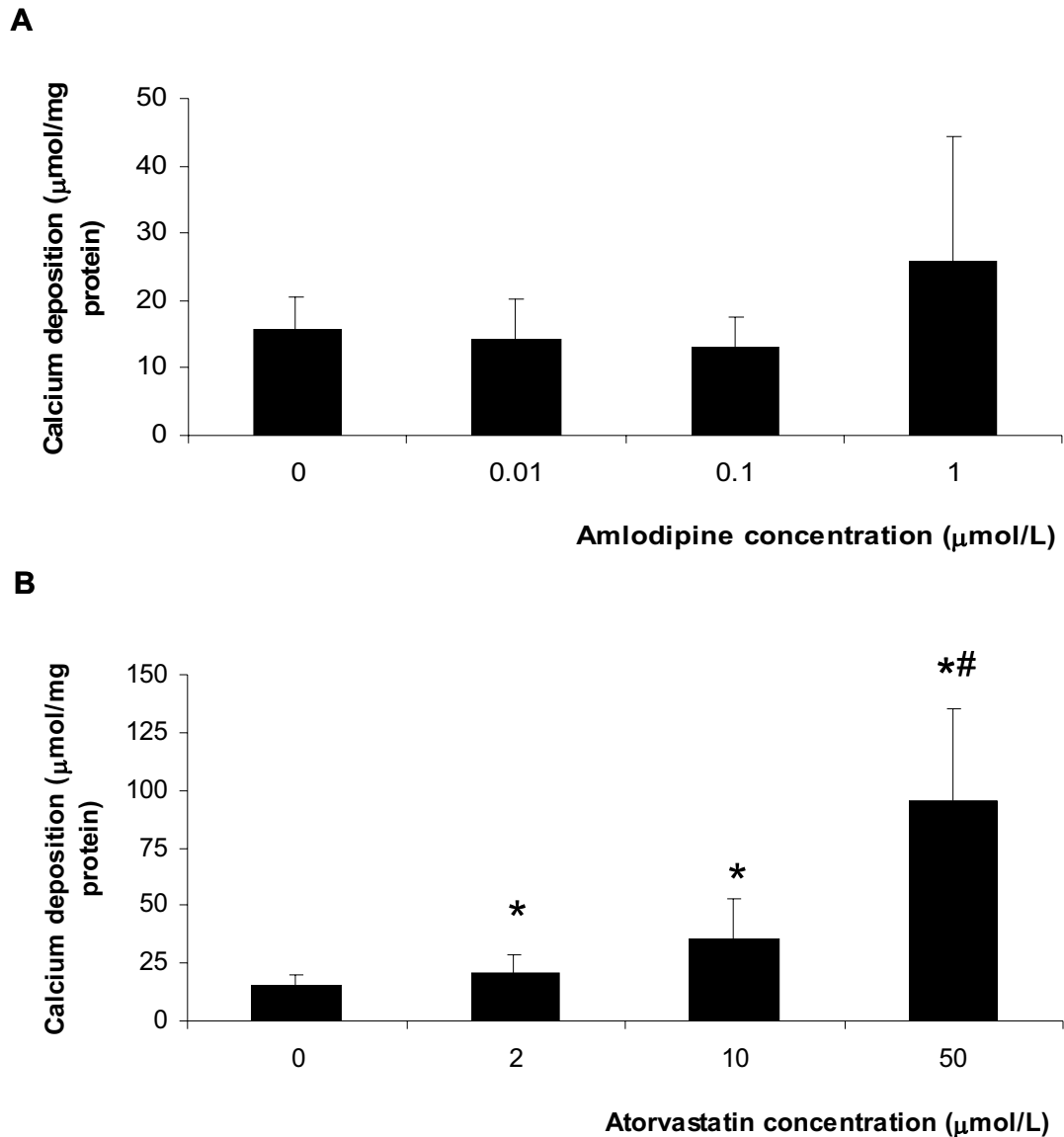
A

B

Figure 1. A. Dose-dependent effects of amlodipine on calcification of neonatal rat VSMCs. **B.** Dose-dependent effects of atorvastatin on calcification of neonatal rat VSMCs.

VSMCs were treated for 21 days with calcification medium containing varying concentrations of atorvastatin or amlodipine. Calcium deposition was quantified by o-cresolphthalein method. The data are presented as mean ± SEM (n = 15). * p < 0.05 when compared to untreated control cultures. # p < 0.05 when compared to all other treatments.

Effect of a combination of CA and statin treatment on in vitro *VSMC calcification*

VSMCs were incubated for 2-3 weeks in calcification medium supplemented with 0.1 μmol/L amlodipine, 10 μmol/L atorvastatin, or a combination of these drugs in the same concentrations. Incubating VSMCs with 0.1 μmol/L amlodipine had no effect on VSMC calcification. Incubation of VSMCs with 10 μmol/L atorvastatin resulted in a 2.2-fold

increased calcium deposition when compared to control cultures treated with calcification medium only [fig. 2] ($p < 0.001$). Combining the amlodipine and atorvastatin treatments resulted in a 2.2-fold increased calcium deposition when compared to control cultures ($p = 0.026$). The combination of treatments resulted in significantly more calcium deposition than treatment with amlodipine alone ($p = 0.003$), and as much calcium deposition compared with treatment with atorvastatin alone (n.s.).
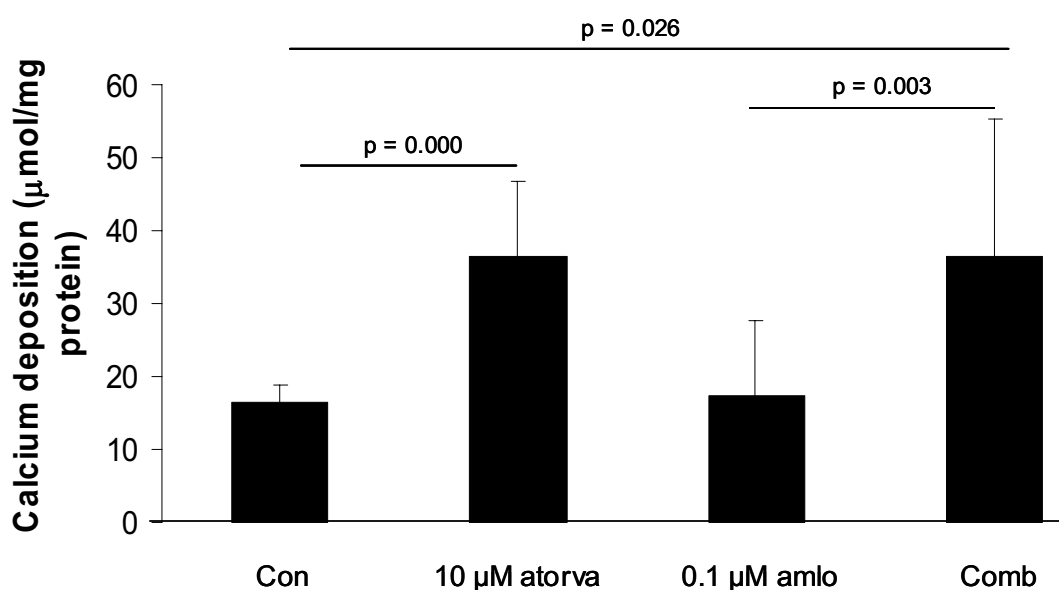

Figure 2. Effects of amlodipine (0.1 μM), atorvastatin (10 μM) and a combination of both treatments (0.1 μM amlo + 10 μM atorva) on neonatal rat VSMC calcification. VSMCs were treated for 21 days with calcification medium containing either atorvastatin or amlodipine, or a combination of both. Calcium deposition was quantified by o-cresolphthalein method. The data are presented as mean $\pm$ SEM (n = 15).

Effects of amlodipine, atorvastatin, and their combination on osteopontin expression in calcifying VSMCs

After VSMCs had been incubated in calcification medium for 2-3 weeks, osteopontin (OPN) expression had been induced considerably as compared to the OPN expression in VSMCs cultured in DMEM + 10% FBS + antibiotics, which was undetectable (chapter 3, fig. 5). Incubation with calcification medium containing amlodipine (0.1 μmol/L) inhibited OPN

expression by 75% as compared to VSMCs cultured in amlodipine-free calcification medium. Incubation of VSMCs in calcification medium supplemented with atorvastatin (10 μmol/L) inhibited the OPN roughly to the same extent as observed for VSMCs treated with amlodipine (0.1 μmol/L). Also, combination therapy with 0.1 μmol/L amlodipine and 10 μmol/L atorvastatin inhibited OPN expression as much as either treatment alone [fig. 3].
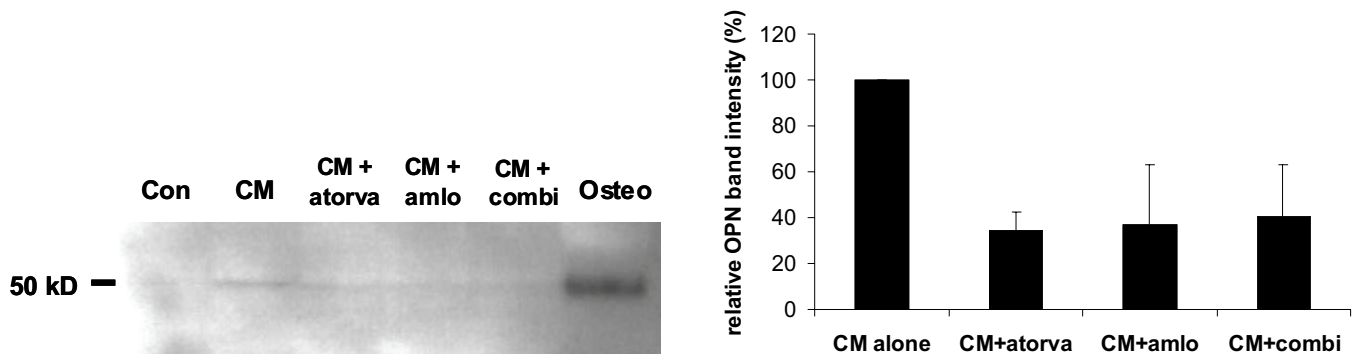

Figure 3. Immunoblot analysis of VSMCs incubated with amlodipine (0.1 μmol/L), atorvastatin (10 μmol/L), and the combination of amlodipine and atorvastatin (0.1 μmol/L + 10 μmol/L, resp.) in the calcification medium. Equal amounts of protein were analyzed by Western blotting with anti-osteopontin antibody. Values represent mean ± SEM (n = 3)

Effects of amlodipine, atorvastatin, and their combination on proliferation of calcifying VSMCs

To detect changes in VSMC proliferation due to treatment with the various drugs, VSMC proliferation was assessed at day 4 and day 9 using the Cell Proliferation Kit II (XTT)(Roche Diagnostics). After 4 days of incubation in calcification medium, amlodipine (1 μmol/L) had decreased VSMC proliferation by 21% ($p < 0.001$) as compared to control cultures (fig. 4A). After 9 days of incubation VSMC proliferation had increased by 14% ($p < 0.05$) when treated with 0.01 μmol/L amlodipine, but at a concentration of 1 μmol/L VSMC proliferation had decreased by 26% ($p < 0.01$) as compared to control cultures [fig. 4A]. Atorvastatin, at a concentration of 50 μmol/L, decreased VSMC proliferation with 50% ($p < 0.001$)[fig. 4B]. Treatment of VSMCs with both 0.1 μmol/L amlodipine and 10 μmol/L atorvastatin did not result in significant changes in cell proliferation.
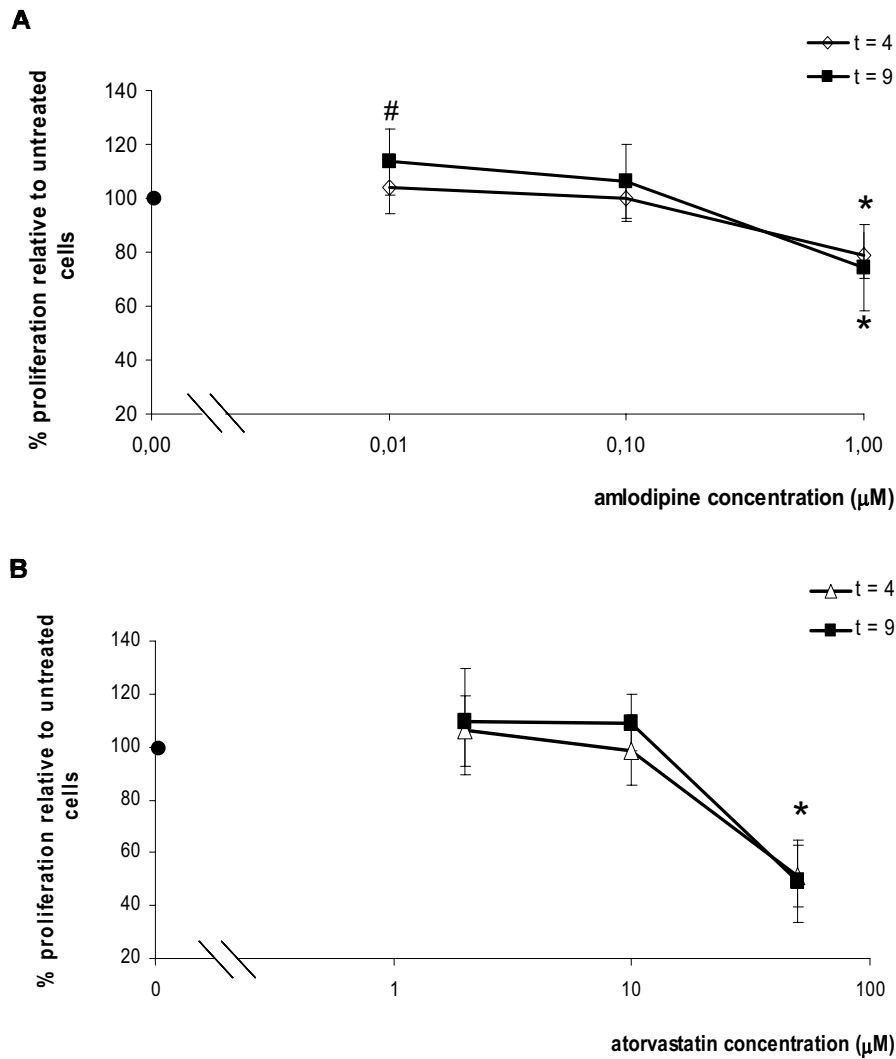
Figure 4. Dose-dependent effects of amlodipine (**A**) and atorvastatin (**B**) on proliferation of VSMCs incubated in calcification medium. VSMC proliferation was assessed at day 4 and day 9 using the Cell Proliferation Kit II (XTT)(Roche Diagnostics). VSMC proliferation is depicted as percentage relative to cells incubated in calcification medium only (=100%).Indicated are mean values ± SEM, n = 6.
# $p < 0.05$ vs. control cultures. * $p < 0.05$ vs. control cultures (t-test).

Effects of amlodipine, atorvastatin, and their combination on apoptosis of calcifying VSMCs

Viability of VSMCs that were incubated with various concentrations of amlodipine and atorvastatin was tested. Untreated cells, or VSMCs incubated with amlodipine (0.01 – 1 µmol/L) or atorvastatin (2 and 10 µmol/L) for 72 h hardly contained any apoptotic nuclei. At the highest concentration (1 µmol/L), amlodipine caused apoptosis of on average 0.05% of the cells (n.s. compared to control). At the highest concentration of atorvastatin (50 µmol/L),

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however, 5.6% of the nuclei were apoptotic (p < 0.05 vs. control, 2 and 10 μmol/L atorvastatin)[fig. 5].

If treated with 0.1 μmol/L amlodipine and 10 μmol/L atorvastatin for 72 h, the number of apoptotic nuclei did not significantly differ from those observed in 10 μmol/L atorvastatin only, 0.1 μmol/L amlodipine only, or control VSMCs.
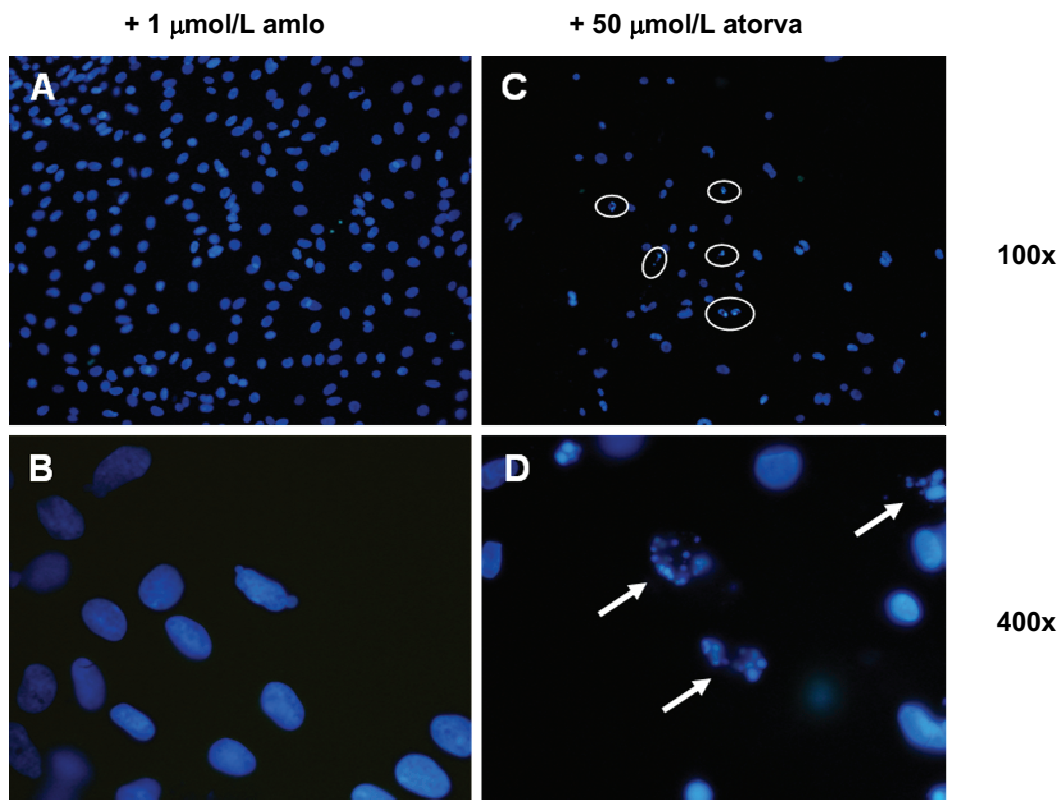

Figure 5. VSMCs were treated with 1 μmol/L amlodipine (**A-B**) or 50 μmol/L atorvastatin (**C-D**) for 72 h. Nuclei were visualized with Hoechst 33342. Apoptotic nuclei are circled in panel C and indicated with arrows in panel D. Microscopy: magnification x 100 in panels A and C, x 400 in panels B and D.

Discussion

Vascular wall calcification is a prominent feature of advanced atherosclerosis. Calcification of the vessel wall is associated with increased wall stiffness. Coronary calcification is associated with an increased risk of death from coronary heart disease and increased risk of myocardial infarction in high-risk asymptomatic adults [2]. To study the pathogenesis of vascular calcification the use of *in vitro* models is indispensable. We therefore developed an

in vitro model of vascular calcification using neonatal rat VSMCs. We used this model to study the effect of the CA amlodipine and the statin atorvastatin, alone and in combination, on *in vitro* calcification of neonatal rat VSMCs. We found that at none of the concentrations tested (0.01-1 µmol/L) did amlodipine have any effect on VSMC calcification in this model, nor on development of apoptosis. However, a slight antiproliferative effect was noted at the highest amlodipine concentration used (1 µmol/L).

CAs have been shown to influence several processes associated with atherosclerosis development such as restoring cholesterol-induced membrane bilayer abnormalities thereby restoring normal calcium homeostasis [7,8], inhibition of proliferation and migration of VSMCs [9-12], inhibition of lipoprotein oxidation [13,14], modifying the binding of monocytes to the endothelium and activating synthesis of matrix components [15]. Fleckenstein-Grün *et al* [28] have demonstrated that calcium overload is a potent inducer of atherosclerosis in rats. In conventional human coronary artery plaques a positive correlation between the degree of calcium accumulation and the severity of atherosclerosis has been found, and it was concluded that the development of conventional human coronary artery plaques is governed by a progressive calcium overload [28]. The vitamin D_3-treated rat is an animal model with calcium overload-induced atherosclerosis. In this model nilvadipine was demonstrated to have the most potent protective action against aortic calcium deposition [29] when compared to nifedipine, nicardipine and verapamil. Diltiazem did not have any effect on aortic calcium deposition. Scanning and transmission electron microscopy demonstrated that vitamin D_3 treatment induced degenerative changes in endothelial cells. Nilvadipine exerted a protective effect against these degenerative changes. Fleckenstein-Grün *et al* [30] have also examined long-term progression of calcific degeneration (degeneration as a result of calcium overload) in coronary arteries of rats after intoxication with one dose of vitamin D_3. Verapamil prevented progression of calcium incorporation and induced a regression of pre-established mural calcium overload. Therefore, in this model of atherosclerosis, inhibition of transmembrane Ca^{2+} influx into VSMC by CA has a protective effect on vascular calcification.

In the present study amlodipine did not affect the calcium deposition by neonatal VSMCs. We did not observe an effect of amlodipine on proliferation or apoptosis of VSMCs. Amlodipine did however reduce the amount of OPN expression induced by incubation with calcification medium.

Statins possess potent lipid-lowering effects. Besides, statins exert pleiotropic effects on vascular wall cells, which include improvement of endothelial function, stabilization of the atherosclerotic plaque, and suppression of inflammation (for review [18-20]). Since calcification is an important aspect of atherosclerosis development, we were interested to see whether statins have an effect on calcification of the vascular wall. In the present study we observed a dose-dependent stimulatory effect of atorvastatin on calcification of VSMCs incubated in

calcification medium. Several other studies have also demonstrated effects of statins that can be considered as pro-calcification effects. Sugiyama *et al.* [31] demonstrated that lovastatin stimulates expression of bone-morphogenetic protein-2 (BMP-2) in human VSMCs *in vitro*. Lovastatin and simvastatin increased bone formation when injected subcutaneously over the calvaria of mice and increased cancellous bone volume when orally administered to rats [32]. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells [33].

However, recently statins have also been shown to decrease the progression of coronary artery calcification and aortic valve calcification [21,22]. This is in accordance with the findings of Kizu *et al.*, who have demonstrated that statins inhibit calcification in an *in vitro* model of inflammatory vascular calcification. [34]. Using interferon-γ, 1α,25-dihydroxyvitamin D$_3$, tumor necrosis factor-α and oncostatin M to induce calcification in human VSMCs, it was demonstrated that cerivastatin and atorvastatin dose-dependently inhibited calcification. However, one should realize that this model differs from the model we have used in the present study. Whereas Kizu *et al.* used inflammatory mediators to induce calcification of the VSMCs, we used increased levels of CaCl$_2$ and organic phosphate in the culture medium to promote calcification. Statins are known to have anti-inflammatory effects, and therefore their inhibitory effect on calcification induced by inflammatory mediators is to be expected.

Reynolds *et al.* [35] have demonstrated that human VSMCs undergo vesicle-mediated calcification in response to increased calcium and phosphate concentrations in the culture medium. Increased calcium and phosphate concentrations resulted in increased release of vesicles and stimulation of apoptosis. Calcification was initiated by release of membrane-bound matrix vesicles from living cells and also by apoptotic bodies from dying cells. Vesicles released by VSMCs after prolonged exposure to calcium and phosphate contained preformed basic calcium phosphate and calcified extensively. The present study confirms that statins stimulate apoptosis in VSMCs [36,37]. Apoptotic bodies can calcify extensively. So, a likely mechanism of induction of calcification by atorvastatin is through induction of apoptosis. Indeed, atorvastatin stimulates both apoptosis and calcification in our model.

Prolonged intensive lipid-lowering has demonstrated to increase calcium content of plaques as assessed by MRI *in vivo* [38], and lowered plaque lipid content at about the same extent, thus having no pronounced effect on plaque size. Since atherosclerotic plaque composition plays an important role in plaque stability, with lipids destabilizing the plaque and calcification possibly stabilizing the plaque, the effect of statins on plaque composition may be very favorable. In addition to lipid-lowering and calcification-inducing effects, we have observed that statins have an anti-proliferative effect on VSMCs. Less proliferation of VSMCs could destabilize the plaque. However, this only occurs at high doses of atorvastatin, whereas calcification induction occurs at lower doses.

Since several *in vitro* and *in vivo* studies have demonstrated that combining CA and statin therapy might be more atheroprotective than either treatment alone [23-26], we also tested the effect of combining amlodipine and atorvastatin therapy on VSMC calcification. Combining both treatments resulted in a similar increase in VSMC calcification as observed for atorvastatin alone. Therefore, the atorvastatin effect on calcium deposition appears to be dominant, without a significant contribution of amlodipine.

Van de Poll *et al.* have performed several studies on the effect of amlodipine, atorvastatin and their combination on atherosclerotic plaque development and atherosclerotic calcification in ApoE*3-Leiden mice [39,40]. These studies have demonstrated a strong atheroprotective effect of atorvastatin. In addition, amlodipine significantly reduced mineralization of the plaques, but did not affect the lipid pool. Co-treatment showed no synergistic effects [39]. Effects of amlodipine, atorvastatin and their combination on pre-existing, advanced atherosclerosis were also investigated in ApoE*3-Leiden mice [40]. When compared to the late-control group, amlodipine, atorvastatin, and their combination reduced lesion area by 25%, 39% and 46% respectively. Lesion severity and relative plaque contents of collagen, cholesterol and calcification were equal in all treatment groups. Neither treatment resulted in regression of atherosclerotic plaque size. So, both amlodipine and atorvastatin significantly retarded the progression of existing plaques. No additive effect of the combination of amlodipine and atorvastatin was observed [38].

Amlodipine reduced mineralization of the plaque in animals when treatment was started at the same time atherosclerosis was induced [39]. If atherosclerosis was already present amlodipine treatment did retard the progression of atherosclerosis but had no effect on the calcium content of the atherosclerotic plaque [40]. In the present study, we did not observe any effect of amlodipine on calcification of neonatal rat VSMCs. The major difference between the studies described by van de Poll *et al.* and the present study is the model system. Van de Poll *et al.* used an hypercholesterolemic *in vivo* model whereas we used an *in vitro* model with calcification medium.

We also determined whether the protein osteopontin (OPN), a noncollagenous matrix protein which has been associated particularly with calcified atherosclerotic plaques [41], was induced in situations that favour calcification. OPN is expressed by macrophages, endothelial cells and VSMCs within the atherosclerotic plaque [42]. Using a OPN$^{-/-}$ and apolipoprotein (apo) E$^{-/-}$ double knockout mouse model, Matsui et al. [43] 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 [44]. The release of soluble OPN near sites of vascular calcification may represent an

adaptive mechanism aimed at preventing vascular calcification. Amlodipine, atorvastatin and their combination inhibited OPN expression at the protein level.

In conclusion, *in vitro* calcification of neonatal rat VSMCs is not affected by amlodipine treatment, but is stimulated by atorvastatin treatment. The latter finding may explain the plaque-stabilizing effect reported for statins.

Acknowledgement

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