Atorvastatin Protects Vascular Smooth Muscle Cells From TGF-β1-Stimulated Calcification by Inducing Autophagy via Suppression of the β-Catenin Pathway

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Abstract

Background: Arterial calcification is a major event in the progression of atherosclerosis. It is reported that statins exhibit various protective effects against vascular smooth muscle cell (VSMC) inflammation and proliferation in cardiovascular remodeling. Although statins counteract atherosclerosis, the molecular mechanisms of statins on the calcium release from VSMCs have not been clearly elucidated. Methods: Calcium content of VSMCs was measured using enzyme-linked immunosorbent assay (ELISA). The expression of proteins involved in cellular transdifferentiation was analyzed by western blot. Cell autophagy was measured by fluorescence microscopic analysis for acridine orange staining and transmission electron microscopy analysis. The autophagic inhibitors (3-MA, chloroquine, NH₄Cl and bafilomycin A1) and β-catenin inhibitor JW74 were used to assess the effects of atorvastatin on autophagy and the involvement of β-catenin on cell calcification respectively. Furthermore, cell transfection was performed to overexpress β-catenin. Results: In VSMCs, atorvastatin significantly suppressed transforming growth factor-β1 (TGF-β1)-stimulated calcification, accompanied by the induction of autophagy. Downregulation of autophagy with autophagic inhibitors significantly suppressed the inhibitory effect of atorvastatin on cell calcification. Moreover, the beneficial effect of atorvastatin on calcification and autophagy was reversed by β-catenin overexpression. Conversely, JW74 supplement enhanced this effect. Conclusion: These data demonstrated that atorvastatin protect VSMC from TGF-β1-stimulated calcification by inducing autophagy through suppression of the β-catenin pathway, identifying autophagy induction might be a therapeutic strategy for use in vascular calcification.
Introduction

Vascular calcification, such as aortic and coronary calcification, is prevalent in cardiovascular diseases [1]. Calcium deposition is found frequently and in greater amounts in older adults and more advanced lesions, and it reduces vascular wall elasticity and leads to heart attacks and stroke. The main feature of vascular calcification is cell osteogenic phenotype transition. Vascular smooth muscle cells (VSMCs) are involved in the development of atherosclerosis by migration, proliferation, and secretion of several growth cytokines [2], and they play a significant role in vascular calcification [3]. Many osteogenic factors are synthesized by VSMCs, including alkaline phosphatase (ALP), type I collagen, bone morphogenetic proteins (BMPs) and osteocalcin, and they are usually associated with the induction of bone formation [2, 4, 5]. In addition, a variety of factors, such as transforming growth factor-β1 (TGF-β1), angiotensin-II, reactive oxygen species, β-glycerophosphate, insulin and vitamin C also could induce VSMC osteogenic differentiation and calcification [6-8].

Considering the high risk of mortality and morbidity related to vascular calcification, therapeutic strategies for prevention and therapy of this process are necessary. Statins, including atorvastatin, simvastatin, rosuvastatin and others, are a group of drugs used to lower the level of cholesterol in the blood. It has been reported that statins exhibit various protective effects against VSMC inflammation and proliferation in cardiovascular remodeling [9]. Results from clinical trials suggest an association of statins usage with slowed progression of calcific aortic stenosis and coronary artery calcification [10-12]. It is showed that statins could prevent calcium deposition and ALP activity, inhibit apoptosis and restore the vitamin K–dependent protein family [7, 13]. Moreover, statins exhibit stabilizing effects on vulnerable atherosclerotic plaques, and inhibit calcification of atherosclerotic plaques in the apoE-deficient mice [13]. However, there are limited studies about the benefit of statins on the release of calcium from VSMCs, and it is necessary to identify the molecular mechanism of statins in the early stage of vascular calcification processes.

TGF-β1 binds to TGF-β receptor I and II (TGF-βRI and II) and plays a crucial role in vascular calcification [6]. It has been shown to be capable of promoting VSMC differentiation and matrix formation in the artery wall [6], and inducing rapid activation of β-catenin signaling to modulate cell osteogenic differentiation [14]. β-catenin is a central component of the canonical Wnt signaling pathway and it is involved in the mitogenic effect of oxLDL in human VSMCs [15] and vascular calcific vasculopathy [16]. Activation of β-catenin signaling is evidenced by the translocation of β-catenin into the nuclei, and it enhances osteogenic factor expression in phosphate-, calcitriol-, and warfarin-induced calcification [17, 18].

Autophagy is an important survival mechanism in the cellular functions. It is necessary in the cells for maintaining organelle quality control, acting in parallel with the ubiquitin proteasomal degradation pathway to suppress the accumulation of polyubiquitinated and aggregated cellular proteins [19]. Autophagy plays a protective role in the progression of certain human disorders, including cancer, neurodegeneration and heart diseases [20]. Cell autophagy can influence the cellular oxidative stress through the degradation of damaged intracellular material [21]. The protective role of autophagy in advanced atherosclerotic plaques is illustrated by in vitro findings showing that VSMC death induced by excess free cholesterol is a kind of cellular defense mechanism to promote cell survival [22]. In the kidney, autophagy also has the cytoprotective effects by downregulating and preventing excess collagen accumulation [23]. Moreover, autophagy is an endogenous protective mechanism counteracting phosphate-induced vascular calcification by reducing matrix vesicle release [24]. The pharmacological autophagy inducer valproic acid could significantly ameliorated phosphate-induced calcification in rat aortic ring explants and bovine aortic smooth muscle cells, indicating that activation of autophagic response could be developed to treat aging or disease-related vascular calcification and osteoporosis [24].

In the present study, we showed that the protective effect of atorvastatin on VSMC calcification was attributed to the induction of autophagy, which was dependent on the
β-catenin pathway. Our results revealed the role of autophagy as a cytoprotective mechanism to negatively regulate osteoblastic differentiation of VSMCs, and suggested that autophagy induction might be a therapeutic strategy for use in vascular calcification.

**Materials and Methods**

**Materials**

TGF-β1 was supplied by Peprotech Inc (Rocky Hill, NJ, USA). Atorvastatin, 3-Methyladenine (3-MA), chloroquine, ammonium chloride (NH₄Cl) and bafilomycin A1 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Antibodies for α-actin, ALP, type I collagen, BMP-2, microtubule-associated protein 1 light chain 3 (LC3), GAPDH, Histone H2B and β-catenin were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for osteocalcin, Beclin-1 and Atg5 were from Epitomics Company (Burlingame, CA, USA).

**Cell culture and treatment**

Male Sprague-Dawley rats were sacrificed, the aorta was removed, and the VSMCs were isolated as previously described [25]. VSMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained under 5% CO₂ at 37°C in a humidified atmosphere. When VSMCs were cultured in DMEM, the cells were characterized by the expression of known marker protein α-actin using immunofluorescence assay (Fig. 1A). Before stimulation with TGF-β1 [26], VSMCs were washed with phosphate buffer saline (PBS) and re-cultured in serum-free medium for 24 h. Then the cells were treated with various agents for the corresponding experiments. The effects of the agents in our studies were compared with the same concentration of dimethyl sulfoxide (DMSO) as vehicle.

**Transfection of VSMCs**

To overexpressed the expression of β-catenin in VSMCs, cells were transfected either with empty vector or the same vector containing a cDNA encoding wild-type β-catenin (WT β-catenin). Briefly, cells were seeded in plates and grown for 24 h until they reached 50–60% confluence, then VSMCs were transfected with WT β-catenin or empty vector using transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The overexpression of β-catenin by the transfection of WT β-catenin was confirmed by western blot analysis.

**Cell viability assays**

Cell viability was assessed by the MTT assays. Cells were seeded at 5 × 10³ per well in 96-well plates overnight. After the treatment of agents for the corresponding experiments, cells were incubated with 5 mg/ml MTT for 3 h, and subsequently solubilized in 200 μl DMSO. Cell viability was determined by measuring the absorbance at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Experimental conditions were tested in quintuplicate, and the data were expressed as the means ± SEM.

**Analysis of calcification**

To determine the cell calcification, calcium content was measured using QuantiChrom™ Calcium Assay Kit (Bioassay Systems, Hayward, CA) according to manufacturer’s instruction. The absorbance was measured using an ELISA reader at 612 nm. Experiments were tested in quintuplicate, and the data were expressed as the means ± SEM.

**Nuclear and cytosolic fractionation**

After cultured, VSMCs were rinsed with ice cold PBS. The extraction of nuclear and cytosolic protein was obtained by a modified protocol from previous report [27]. Briefly, the harvested VSMCs were collected in hypotonic lysis buffer (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.2 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 1 mmol/l dithiothreitol) with protease inhibitor cocktail and incubated on ice for 5 min. The cell lysate was chilled on ice for 10 min and then vigorously shaken for 10 min in the presence of 0.6% Nonidet P-40. The nuclear fraction was precipitated by centrifugation. The supernatants containing the cytosolic proteins were collected. Nuclear fractionation was extracted by addition of high-salt buffer (20 mmol/l HEPES, pH 7.9, 400 mmol/l KCl, 0.2 mmol/l EDTA, 0.2 mmol/l phenylmethylsulfonyl
fluoride, and 1 mmol/l dithiothreitol with protease inhibitor cocktail) with continuous shaking, then centrifuged and the supernatants were collected.

**Western blot analysis**

Lysates from VSMCs were prepared and western blot analysis was performed as described previously [28]. The proteins were separated by 10% SDS-PAGE, then transferred onto PVDF membranes and blotted with specific antibodies against ALP (1:200), type I collagen (1:200), BMP-2 (1:200), LC3 (1:200), GAPDH (1:200), Histone H2B (1:200), β-catenin (1:200), osteocalcin (1:1000), Beclin-1 (1:1000) or Atg5 (1:1000) at 4°C overnight, and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10000) for 2 h. The blot was detected using the Chemiluminescence plus Western blot analysis kit (Millipore). All bands were evaluated by densitometry with Quantity One V4.6.2 software (Bio-Rad, USA). Bands of interest were normalized against GAPDH or Histone H2B and data were presented as relative density ratios.

**Transmission electron microscopy (TEM) analysis**

To determine the formation of autophagosomes, cells were fixed in 0.1 mol/l sodium cacodylate buffer and postfixed in 0.1 mol/l sodium cacodylate buffer 1% OsO₄ solution. After dehydration in an ethanol gradient, samples were incubated with propyleneimine, impregnated with a mixture of propyleneimine/LX-112 (Ladd Research Industries, 1:1), and embedded in LX-112. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Jeol-100 CX II TEM.

**Detection of acidic vesicular organelles**

Cells were plated on coverslips and allowed to attach. Following treatment with DMSO (vehicle), TGF-β1 or atorvastatin, cells were stained with 1 μg/ml acridine orange in PBS for 15 min, washed with PBS and examined under fluorescence microscope (Olympus, Japan).

**Immunofluorescence assay**

Cells were fixed in 4% paraformaldehyde solution on slides and washed with PBS, followed by incubation in 10% normal goat serum blocking solution and β-catenin antibody (1:50). Cells were washed with PBS and incubated in TRITC conjugated secondary antibody (1:100) for 60 min at room temperature. The cells were washed with PBS, and then stained with 1 mg/ml DAPI for 15 min. Excess dye was washed off with PBS, and visualized using fluorescence microscope. Moreover, for a quantitative analysis, Mander's coefficient of colocalization for β-catenin (red) with DAPI (blue) was determined on six cells for each condition per experiment.

**Statistical analysis**

Data analysis was performed by using SPSS statistical software (SPSS, Inc., Chicago, IL) [29]. All data were presented as means ± SEM. The variance of the data were analysed by ANOVA, followed by Tukey's post hoc test. A value of P<0.05 was considered to be significant.

**Results**

**Atorvastatin suppresses TGF-β1-stimulated VSMC calcification**

It is demonstrated that TGF-β1 could regulate vascular calcification and VSMC differentiation at the concentration of 2 ng/ml [26]. Therefore, VSMCs were incubated with TGF-β1 (2 ng/ml) for 6, 12, 24, 48 and 72 h. As shown in Fig 1B, TGF-β1 stimulation increased calcium content in a time-dependent manner. TGF-β1 also increased the expression of ALP, BMP-2 and osteocalcin in VSMC (Fig. 1C). However, the expression of type I collagen was not affected by TGF-β1.

To determine the effect of atorvastatin on VSMC calcification, cells were cultured with atorvastatin for 24 h and then incubated with TGF-β1 for additional 24 h. As shown in Fig 1D, atorvastatin significantly suppressed calcium content of cells stimulated by TGF-β1 (48.2±11.3% of TGF-β1 treatment group), and it did not compromise the cell viability (Fig.
Fig. 1. Effect of atorvastatin on TGF-β1-stimulated VSMC calcification. (A) Immunohistochemistry for α-actin in VSMCs (magnification, ×400). (B) VSMCs were incubated with TGF-β1 (2 ng/ml) for 6, 12, 24, 48 and 72 h, and calcium content of cells was measured using QuantiChrom™ Calcium Assay Kit. Calcium content was reported as microgramme of calcium per milligram protein (μg/mg protein). (C) Expression of ALP, type I collagen, BMP-2 and osteocalcin was analyzed by western blot. *, p<0.05 versus vehicle. (D) VSMCs were cultured with atorvastatin (10 μmol/l) for 24 h and then incubated with 2 ng/ml TGF-β1 for additional 24 h. Calcium content was measured. The results were calculated as the ratio of the absorbance of the atorvastatin-treated cells/absorbance of TGF-β1-stimulated cells. (E) Cell viability was determined by the MTT assays (n = 6). All values were presented as means±SEM from three independent experiments (n=6). (F) ALP, type I collagen, BMP-2 and osteocalcin expression were analyzed by western blot. Bands of interest were normalized against GAPDH and data were provided as relative density ratios. *, p<0.05 versus TGF-β1-stimulated group.
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Inhibitory effect of atorvastatin on calcification is caused by inducing autophagy

Previous study showed that autophagy controlled osteogenic differentiation of mesenchymal stem cells and autophagy inhibitors suppressed these processes [30], so we determined the effects of autophagy in VSMC calcification. As shown in Fig 2A, autophagic vacuolization was rarely detected in normal, atorvastatin-treated alone, and TGF-β1-stimulated VSMCs by TEM. However, after TGF-β1-stimulation, atorvastatin induced a large number of autophagic vacuolization distributed throughout the whole cytoplasm at concentration exerting inhibition of calcification. These results were further confirmed by fluorescence microscopy following staining with the lysosomotropic agent acridine orange (Fig. 2B). Beclin-1 was a tumor suppressor protein in the lysosomal degradation pathway of autophagy, which was the most important up-regulator of autophagy. Moreover, Atg5 was involved in autophagic vacuole formation, and it contributed to autophagic cell death through interacting with Fas-associated protein with death domain [31]. Western blot analysis showed that the expression of Beclin-1 and Atg5 was also increased after atorvastatin treatment (Fig. 3A). LC3 was cleaved by a cysteine protease to produce LC3 I. The conversion of LC3 I to LC3 II was indicative of increased autophagic activity and it was considered a reliable marker of autophagosome formation. In our experiments, the effect of atorvastatin on autophagy was confirmed by the enhanced conversion of the cleaved LC3 I into LC3 II in cells (Fig. 3A).
Fig. 3. Effect of atorvastatin on autophagy in TGF-β1-stimulated VSMC calcification. VSMCs were cultured with atorvastatin (10 μmol/l) for 24 h and then incubated with 2 ng/ml TGF-β1 for additional 24 h. (A) Total protein lysates were analyzed by western blot for Beclin-1, Atg5, LC3 and GAPDH. *, p<0.05 versus TGF-β1 plus atorvastatin group. (B) 3-MA (5 mmol/l) pretreatment for 2h abolished the inhibitory effect of atorvastatin on calcium content in VSMCs. The results were calculated as the ratio of the absorbance of the agent-treated cells/absorbance of TGF-β1-stimulated cells. *, p<0.05. (C) Expression of ALP, type I collagen, BMP-2 and osteocalcin was analyzed by western blot after atorvastatin treatments. (D) Chloroquine (2.5 μmol/l), NH₄Cl (5 mmol/l), and bafilomycin A1 (2.5 nmol/l) pretreatment abolished atorvastatin-induced autophagy. Bands of interest were normalized against GAPDH and data were provided as relative density ratios. *, p<0.05 versus TGF-β1 plus atorvastatin group. (E) Calcium content was measured after autophagy inhibitor treatment. The results were calculated as the ratio of the absorbance of the agent-treated cells/absorbance of TGF-β1-stimulated cells. All values were presented as means±SEM (n=6). *, p<0.05.
To explore the relationship between calcification and autophagy, we used a specific autophagic inhibitor 3-MA [24], and we found that 3-MA (5 mmol/l) pretreatment for 2h

![Fig. 4. Role of β-catenin signaling in TGF-β1-stimulated calcification. (A) VSMCs were cultured in the presence of 2 ng/ml TGF-β1 for the indicated times (6, 12, 24 and 48 h). Total protein lysates were collected and analyzed by western blot for TGF-βRI and β-catenin. (B) VSMCs were incubated with 2 ng/ml TGF-β1 for 24 h, and then β-catenin translocation into the nucles was determined by fluorescence microscopy. Cells were stained with the antibody specific for β-catenin (red). Nuclei were stained with DAPI (blue) (magnification, ×400). Mander's coefficient of colocalization for β-catenin (red) with DAPI (blue) was provided. (C) Cell nuclear and cytoplasm extracts were prepared and the extract was analyzed for β-catenin by western blot. Bands of interest were normalized against GAPDH or Histone H2B and data were provided as relative density ratios. *, p<0.05 versus vehicle. (D) After pretreatment with TGF-β1 (2 ng/ml) for 24 h with or without JW74 (10 μg/ml) for 12 h, β-catenin expression was analyzed by western blot. (E) Calcium content of VSMCs was measured after treatment. The results were calculated as the ratio of the absorbance of the agent-treated cells/absorbance of TGF-β1-stimulated cells. *, p<0.05 versus TGF-β1-stimulated group. (F) Cell viability was determined by the MTT assays (n = 6). All values were presented as means±SEM from three independent experiments (n=6).]
significantly suppressed the effect of atorvastatin on VSMC autophagy (Fig. 2A, 2B and 3A) as well as calcification (Fig. 3B and 3C). Moreover, the pretreatment of VSMCs with another three autophagic inhibitors namely chloroquine (2.5 μmol/l) [32], NH₄Cl (5 mmol/l) [33], and bafilomycin A1 (2.5 nmol/l) [34] also showed the similar results (Fig. 3D and 3E).

**Downregulation of β-catenin is associated with the inhibitory effect of atorvastatin on cell calcification**

TGF-β could bind to TGF-βRI, then it induced rapid nuclear translocation of β-catenin to modulate cell osteogenic differentiation [14]. As shown in Fig. 4A, TGF-βRI and total β-catenin expression were markedly increased after TGF-β1 stimulation, accompanied by
the β-catenin translocation into the nucleus (Fig. 4B and 4C). To further explore the role of β-catenin signaling in VSMC calcification, cells were treated with the β-catenin inhibitor JW74. The addition of JW74 (10 μg/ml) suppressed TGF-β1-stimulated total β-catenin expression (Fig. 4D), and it clearly abrogated TGF-β1-stimulated calcium content in cells (Fig. 4E). In addition, JW74 did not compromise the VSMC viability (Fig. 4F). These results indicated that TGF-β1-stimulated calcification was associated with upregulation of β-catenin.

To determine whether the inhibitory effect of atorvastatin on cell calcification was dependent on the downregulation of the β-catenin, we evaluated the expression of TGF-βRI and nuclear β-catenin after atorvastatin treatment. As shown in Fig. 5A, atorvastatin treatment decreased TGF-β1-stimulated both TGF-βRI and nuclear β-catenin expression. Furthermore, we overexpressed β-catenin by the transfection of WT β-catenin (Fig. 5B) and examined the cell calcification. As shown in Fig. 4C, the beneficial effect of atorvastatin on calcification was abolished by β-catenin overexpression, and it was confirmed by the detection of osteogenic factor expression (Fig. 5D). Similarly, the enhanced effect of atorvastatin on cell autophagy was also reversed by β-catenin overexpression (Fig. 5E). In addition, the β-catenin inhibitor JW74 supplement enhanced the effect of atorvastatin on VSMC calcification and autophagy (Fig. 5C, 5D and 5E).

Discussion

Vascular calcification is a major risk factor for cardiovascular morbidity and mortality, and it is prevalent in the patients with diabetes and atherosclerosis [35]. Considering that vascular calcification is correlated with the risk of cardiovascular disease, many studies have attempted to interrupt the procession of this disease [36]. Recently, there is some evidence that atorvastatin could reduce arterial calcification and plasma calcium concentration [7], but the mechanism is not clear yet. The present study demonstrated that atorvastatin significantly suppressed early calcium content and osteogenic differentiation marker protein expression in TGF-β1-stimulated VSMCs via the induction of autophagy (Fig. 6). These findings provided additional insight into the protective effects of statins against arterial calcification and supported the previous idea that statins had a potential role in vascular calcification.

During TGF-β1-stimulated calcification, we found that TGF-β1 upregulated the β-catenin expression, resulting in promoting calcification. Previous studies showed that the β-catenin signaling pathway could regulate the cell apoptosis and autophagy [37-39]. Inhibition of β-catenin significantly increased LC3II expression and induced autophagic cell death [38, 39]. Conversely, the increase of β-catenin signalling reduced Beclin-1 expression and rescued endothelial cells from endostatin-induced autophagy [37]. Here, the similar results were showed and we found that cell autophagy was reversed by β-catenin overexpression, indicating the critical role of β-catenin in the atorvastatin-induced autophagy. The interaction between β-catenin signaling and Smad2/3 pathway had been reported to be significant
in regulating cell function [40]. Cellular Smad2/3 became rapidly phosphorylated by the activated TGF-βRI kinases after TGF-β stimulation [41]. Therefore, more detailed studies are warranted to define the effects of atorvastatin on the TGF-β-induced Smad2/3 pathway during cell autophagy.

In addition, we investigated the association of atorvastatin-induced autophagy and calcification in VSMCs. In TGF-β1-stimulated calcification, atorvastatin increased cell autophagy which was characterized morphologically by the accumulation of numerous cytoplasmic autophagic vacuoles of lysosomal origin, followed by mitochondrial dilation and enlargement of the endoplasmic reticulum. A relationship between calcification and autophagy was evident from the results that both of them were suppressed by the autophagy inhibitors, indicating that autophagy was one target of atorvastatin in inhibiting VSMC calcification. However, the present study was different with the previous report which showed that autophagy was favoring the osteogenic differentiation of mesenchymal stem cells [30]. So we speculate that autophagy played a unique role in the different cells to serve as a cytoprotective method to maintain cellular homeostasis. Thus, further investigations are needed to identify more evidence.

In summary, our studies revealed that atorvastatin suppressed TGF-β1-stimulated VSMC calcification by inducing autophagy through downregulation of β-catenin signaling, suggesting a potential role of autophagy in vascular pathophysiology. The present reports provided evidence of a protective role of atorvastatin in VSMC calcification and further indicated the signaling mechanism, which could potentially contribute to the treatment of the related cardiovascular diseases.

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