Paradoxical Effects of Statins on Aortic Valve Myofibroblasts and Osteoblasts
Implications for End-Stage Valvular Heart Disease

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Objectives—We evaluated the effects of statins on aortic valve myofibroblast (AVMFs) and osteoblast calcification in vitro.

Methods and Results—Cultured porcine AVMFs and M2–10B4 cells were treated with simvastatin and pravastatin. Mevalonate, a 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase metabolite, was added in parallel experiments. Manumycin A, which inhibits protein prenylation, was added to cultures in the absence of statins. Calcification was assessed by counting the number of calcific nodules formed and measuring alkaline phosphatase activity (APA). Statins inhibited calcific nodule formation \( P<0.01 \) and APA \( P<0.01 \) in AVMFs. Mevalonate reversed the statin effect on nodule formation \( P<0.05 \) and APA \( P<0.01 \). Manumycin A had no effect on either parameter. M2–10B4 cells treated with simvastatin formed more calcific nodules compared with controls \( P<0.01 \), although pravastatin had no effect. Both statins, however, resulted in increased APA in M2–10B4 cells \( P<0.01 \). Mevalonate had no impact on nodule numbers or APA in M2–10B4 cells.

Conclusions—Statins inhibit calcification in AVMFs by inhibiting the cholesterol biosynthetic pathway, independent of protein prenylation, but paradoxically stimulate bone cell calcification. Because 15% of patients with end-stage valvular heart disease exhibit mature bone in their aortic valves, statins may differentially regulate calcification within a valve, limiting dystrophic calcification but promoting ossification of formed bone. (Arterioscler Thromb Vasc Biol. 2005; 25:1-6.)

Key Words: calcification ■ aortic valve ■ myofibroblasts ■ protein prenylation ■ HMG-CoA reductase

Aortic valve calcification is the most frequent pathologic process necessitating surgical aortic valve replacement. Advanced disease is associated with high morbidity and mortality, because surgical valve replacement remains the only therapy for this common problem. Although the mechanism underlying valve calcification has not been established, recent data suggest that valve calcification is an active process, much like atherosclerosis, that is preceded by inflammation, lipid deposition, and the accumulation of extracellular matrix proteins.

Additionally, many of the atherosclerotic risk factors, such as total cholesterol, elevated LDL and triglycerides, low HDL, male sex, cigarette smoking, hypertension, and diabetes, are associated with aortic valve calcification. In addition to dystrophic calcification (nonbone mineralization), \( \approx 15\% \) of end-stage aortic valves contain mature heterotopic bone with hematopoietic and stromal elements, clearly indicating that an active pathologic process is occurring.

Evidence indicates that cells residing in arterial and valvular tissue participate in the calcification process. Pericyte-like "calcifying vascular cells" from the medial layer of the aorta and vascular smooth muscle cells of arteries are thought to differentiate into osteoblast-like cells, an event triggered by oxidized LDL cholesterol and inhibited by HDL cholesterol. A clonal population of aortic valve myofibroblasts (AVMFs) that resides in the interstitial layer of the cardiac valves has likewise been found to transdifferentiate into osteoblast-like cells. These cells express alkaline phosphatase as well as the extracellular matrix proteins bone morphogenetic protein 2 (BMP-2), osteopontin, and osteocalcin. Moreover, transforming growth factor-\( \beta \)1, a regulator of osteoblast differentiation, and 25-hydroxycholesterol, a by-product of cholesterol oxidation found in atherosclerotic lesions, stimulate the formation of calcific nodules by aortic medial cells and AVMFs. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have pleiotropic effects. In nonrandomized clinical studies, statins inhibit calcification in atherosclerotic coronary arteries and aortic valves in addition to their established role in lowering serum LDL levels.11–15

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In parallel experiments, mevalonate was added to the culture medium to evaluate the potential mechanisms responsible for statin effects. Mevalonate (Sigma Chemical Co) was stored at −20°C. At the time of use, mevalonate was dissolved in sterile water and added to the medium to achieve a final concentration of 100 μmol/L. Manumycin A (Sigma), a protein prenylation inhibitor, was added to AVMF culture medium in the absence of statin drug to achieve final concentrations of 10 and 20 μmol/L.

**Calcium Assay**

After treatment with statins or Manumycin A, the number of nodules was recorded. Medium was removed and calcium was then assessed with a commercially available kit (Starbio Laboratory).

**Alkaline Phosphatase Assay**

After treatment with increasing doses of statin (0.0, 0.1, 0.2, and 0.4 μmol/L), APA was assayed with a commercially available kit (Sigma Diagnostics, Inc). Cell lysis buffer included 75 mmol/L NaCl, 1 mmol/L MgCl2, 10 mmol/L PEP, pH 7.4, and 0.25% NP40.

**Statistical Analysis**

Student’s t test was used to compare 2 means. ANOVA was used to determine the significance of differences with multiple comparisons. The results are expressed as mean±SE. A probability value of P<0.05 was considered to denote statistical significance.

**Methods**

### Cell Culture

Freshly obtained porcine AVFs were cultured from explants by previously published methods. Aortic valve leaflets were rinsed in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Both sides of the leaflet were then scraped lightly with the blunt end of a scalpel blade. Valve leaflets were gently cut into 1- to 2-mm pieces by rolling the scalpel blade. Valve pieces were then placed in culture plates and covered with the aforementioned medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). On reaching 50% confluence, cells were split into 12-well plates by first soaking them in trypsin-EDTA for 10 minutes at 37°C, then rinsing with the aforementioned medium supplemented with 10% FBS, and finally distributing them among the 12 wells. Cells were then cloned from the nodule-forming 12 wells. Cells were then cloned from the nodule-forming cultures by limiting dilution and single-cell harvesting, according to previously described methods, to select for myofibroblasts. For osteoblast cultures, M2–10B4 murine bone marrow stromal cells (American Type Culture Collection, Manassas, Va) were grown in RPMI-1640 medium (Vitacell) containing 10% FBS, supplemented as indicated earlier for DMEM. Both myofibroblast and osteoblast cultures were maintained at 37°C at 5% CO2, and the medium was changed every 3 to 4 days. For each experiment, entire wells were examined for nodule formation by light microscopy at 10x magnification (Figure 1, available online at http://atvb.ahajournals.org). The number of nodules in each well, which has been shown to be in direct correlation with degree of calcification, was recorded every 3 to 4 days before changing media.

### Drug Preparation and Treatment

Simvastatin, in an inactive lactone form (Merck & Co) was solubilized and activated by adding 0.1 mol/L NaOH (final pH 7.4) to produce the active isomer. The solution was vortexed for 30 minutes, filtered for sterility, and stored at −20°C. Pravastatin (Bristol-Meyers Squibb) is water soluble and was obtained in solution and stored at −20°C. In statin treatment experiments evaluating nodule formation, cultures were incubated with increasing concentrations of simvastatin or pravastatin (0.1, 0.2, 0.4, and 0.6 μmol/L). Both simvastatin and pravastatin significantly inhibited calcific nodule formation compared with untreated control cultures in a dose-dependent manner (data not shown). Figure 1 demonstrates inhibition of calcific nodules formation by 76.5% and 85.7% in the presence of simvastatin (Figure 1A) and pravastatin (Figure 1B), respectively. Statins also reduced the amount of calcium and were correlated directly with the number of nodules (P<0.01, data not shown). Consistent with the clinical effects of statins on aortic valve calcification, these results demonstrate that statins prevent the formation of calcific nodules by AVFs.

### Results

**Statins Inhibit Formation of Calcified Nodules by AVFs**

To determine whether statins inhibit calcific nodule formation, porcine AVFs were incubated with increasing concentrations of simvastatin or pravastatin (0.1, 0.2, 0.4, and 0.6 μmol/L). Both simvastatin and pravastatin significantly inhibited calcific nodule formation compared with untreated control cultures in a dose-dependent manner (data not shown). Figure 1 demonstrates inhibition of calcific nodules formation by 76.5% and 85.7% in the presence of simvastatin (Figure 1A) and pravastatin (Figure 1B), respectively. Statins also reduced the amount of calcium and were correlated directly with the number of nodules (P<0.01, data not shown). Consistent with the clinical effects of statins on aortic valve calcification, these results demonstrate that statins prevent the formation of calcific nodules by AVFs.

**Statin Inhibition of Myofibroblast Calcific Nodule Formation Is Reversed by Mevalonate**

In an effort to determine whether the reduction in nodule formation resulting from statin treatment occurs via statin-mediated inhibition of HMG-CoA reductase, myofibroblast cultures were treated with statins in the presence of mevalonate (100 μmol/L). Mevalonate is a metabolite of HMG-CoA reductase that can be readily absorbed and used by cells in vitro and thereby circumvents the blockade of HMG-CoA reductase produced by statins. Treatment with mevalonate partially reversed the decrease in nodule formation observed with simvastatin (P<0.001) and pravastatin (P<0.05) treatment alone (Figure 1A and 1B). These data show that statins inhibit the processes of calcification in myofibroblasts in vitro and that this inhibition is at least partially mediated by downregulation of HMG-CoA reductase activity.
Statins Inhibit APA of AVMs
In addition to the examination of calcific nodule formation, APA, a marker of osteoblastic differentiation, was measured in myofibroblast cultures treated with increasing concentrations of statin (0.0, 0.1, 0.2, and 0.4 μmol/L). APA was significantly reduced by both simvastatin and pravastatin in a dose-dependent manner (\(P<0.01\) for both statins; Figure 2). Consistent with the effects on nodule formation, the decrease in APA produced by either statin was partially reversed when mevalonate was added to the culture medium (\(P<0.01\); Figure 3), again suggesting that the observed effect is secondary to inhibition of HMG-CoA reductase by statins.

Statins Effects on Myofibroblasts Do Not Occur Through Protein Prenylation
Because HMG-CoA reductase is the first step in a number of biochemical processes, we treated AVMFs with Manumycin A, an inhibitor of farnesyl transferase, in an attempt to further localize the mechanism by which statins inhibit calcific nodule formation.24 Farnesyl transferase is 1 of the key enzymes involved in protein prenylation, a process that occurs downstream of mevalonate in the cholesterol biosynthesis pathway that is thought to be responsible for many of the pleiotropic effects of HMG-CoA reductase inhibitors.24 Statins, like Manumycin A, are thought to inhibit protein prenylation; thus, both would be expected to inhibit calcific nodule formation and APA if they truly share an identical mechanism. No significant changes in the number of calcific nodules or in the levels of APA were observed after treatment with Manumycin A (Figure 4), suggesting that statin effects on myofibroblasts do not occur via the protein prenylation branch of the cholesterol biosynthesis pathway.

Statins Stimulate the Formation of Calcific Nodules by Marrow Stromal Cells Through a Nonmevalonate-Dependent Pathway
Recent data suggest that statins have a significant impact on bone metabolism.16,17 To determine how statins affect bone formation, parallel experiments were performed to directly assess the effects of statins on osteoblast activity. Increasing concentrations of simvastatin and pravastatin were added to M2–10B4 cells, a marrow stromal cell line that undergoes osteoblastic differentiation. Treatment with simvastatin significantly increased the number of calcific nodules (Figure 5A; \(P<0.01\)) and amount of calcium (\(P<0.05\); data not shown) compared with control cultures. Treatment with pravastatin similarly increased the number of calcific nodules formed in M2–10B4 cell cultures; however, the effect was not statistically significant (Figure 5B; \(P>0.05\)). Additionally, treatment with mevalonate (100 μmol/L) did not significantly alter the number of calcific nodules formed after simvastatin treatment (data not shown).

Statins Stimulate Osteoblast Differentiation in Marrow Stromal Cells
The effects of simvastatin and pravastatin on APA in M2–10B4 cells were also measured. Treatment with either statin resulted in increased APA in M2–10B4 cells (\(P<0.01\); Figure 6A and B) compared with control cultures (Figure 6C). This effect was significantly reversed when mevalonate was added to the culture medium (\(P<0.01\)). Error bars depict standard error. Other abbreviations are as defined in text.
6), suggesting that statins stimulate osteoblast differentiation. Similar to the lack of effect on calcific nodule formation, mevalonate did not reverse the stimulatory effect of the statins on APA in M2–10B4 cells (data not shown). Together, this work shows that statin drugs differentially modulate AVMFs and osteoblasts in vitro and that these processes occur via different mechanisms.

**Discussion**

Using a well-characterized in vitro model, we demonstrate for the first time that statin drugs, both water- and lipid-soluble types, inhibit calcification of AVMFs. In myofibroblast cultures, both simvastatin and pravastatin reduced the number of calcific nodules formed and the level of APA. Mevalonate, a downstream metabolite of HMG-CoA reductase, partially reversed the inhibitory effect of simvastatin and pravastatin on nodule formation and on APA, indicating that this effect is at least in part due to inhibition of HMG-CoA reductase. Because only partial reversal was observed with the addition of mevalonate, a higher concentration of mevalonate may be needed to completely inhibit the amount of statin used in the experiments or alternatively, some of the observed effects may be due to an HMG-CoA reductase–independent pathway.

To further elucidate the mechanism responsible for the observed statin-induced inhibition of myofibroblast calcification, we cultured AVMFs in the presence of Manumycin A, an inhibitor of farnesyl transferase, 1 of the key enzymes needed for protein prenylation. Protein prenylation is a process that branches off the cholesterol biosynthesis pathway downstream of mevalonate that mediates a number of cellular processes, including cell growth and protein trafficking.24 Statins, like Manumycin A, are thought to inhibit protein prenylation, thereby explaining a number of their observed pleiotropic effects. Here, we found that Manumycin A had no effect on myofibroblast APA, suggesting for the first time that protein prenylation is not involved in the inhibition of valvular calcification observed with statins. However, Manumycin A does not inhibit geranylgeranyl transferase and thus, does not completely eliminate the
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cultures, others have reported that statins inhibit extracellular matrix protein prenylation signaling pathway. Given that mevalonate reverses the statin inhibition of alkaline phosphatase in AVMF, taken together, these results suggest that statin inhibition of nodule formation is due primarily to events in the cholesterol biosynthetic pathway that are independent of protein prenylation, such as cholesterol synthesis.

In contrast to its effects on myofibroblasts, simvastatin increased the number of calcific nodules observed in marrow stromal cell cultures, and both simvastatin and pravastatin stimulated APA in osteoblasts in vitro. Mevalonate did not reverse APA or nodule number in marrow stromal cells, indicating that the statin effect on these cells is not due to inhibition of HMG-CoA reductase. Although the addition of pravastatin to marrow stromal cells stimulated calcific nodule formation, the effect was not statistically significant. Given that pravastatin increased APA, it is possible that the lack of significant effect is due to lack of power in our experiments. However, our results are consistent with the findings of Izumo et al.25 who found that the lipophilic statins mevastatin and fluvastatin but not the water-soluble pravastatin promoted mineralization of a bone cell line (MC3T3-E1 cells). Clinical study data are needed to evaluate whether statins inhibit extracellular matrix protein production and osteoblastic gene markers in aortic valves26 and clinical progression of aortic valve mineralization.13–15 In contrast, subcutaneous administration of simvastatin and lovastatin has been found to increase the rate of bone formation in rats.16 Furthermore, a number of retrospective clinical studies indicate that statins have a clinically significant effect on bone metabolism, thereby reducing the risk of hip fractures in elderly patients18–20; however, these data are not consistent.22 Nonetheless, together with our results, these studies show that statins have different effects on cardiovascular tissue and bone, supporting a “statin paradox” between cardiovascular mineralization and bone mineralization.

Interestingly, the opposing effects of statins on aortic valve cells and bone cells observed here is congruous with the paradoxical relationship reported between osteoporosis and cardiovascular calcification, the “calcium paradox,” wherein patients with osteoporosis have a higher frequency of cardiovascular calcification.27 Parhami et al25 showed that oxidized lipid may be a common factor underlying both disease processes. Oxidized lipid was found to increase APA, a marker of osteoblast differentiation in calcifying vascular cells, whereas it inhibited the differentiation of bone-derived preosteoblasts. Consequently, statin drugs may decrease arterial and valvular calcification and skeletal fractures in osteoporotic patients through their known effects on lipid levels and subsequent osteoblast differentiation. However, the effect of statins on bone present in end-stage calcified aortic valves remains unclear. It is unknown, for example, whether statins inhibit dystrophic calcification but stimulate growth of bone already present in end-stage ossified aortic valves.4 Also, assessment of variables such as the potency of LDL reduction by statins and the effect on calcification requires clinical data not available from our study.

Several mechanisms have been proposed to explain how statins modulate bone formation and remodeling. HMG-CoA reductase inhibitors, especially simvastatin, stimulate the promoter of BMP-2, an activator of osteoblastic differentiation,16 thus explaining how simvastatin induces osteoblast differentiation.28,29 Here we similarly show that statins stimulate osteoblast differentiation. Furthermore, our results indicate that the stimulation of osteoblast differentiation caused by statins is independent of HMG-CoA reductase. Taken together, these results suggest that the ability of statins to stimulate BMP-2 may be the primary mechanism responsible for the observed statin effects on bone. However, data regarding the effect of statins on osteoblastic differentiation in cell culture are not consistent; Parhami et al25 showed that mevastatin inhibited activity and expression of alkaline phosphatase and mineralization of mouse marrow stromal cells.

The results presented here have significant implications for the use of statin drugs in the management of patients with advanced aortic valve lesions. However, it is important to note that our study was limited by the different culture techniques used for growing myofibroblasts and marrow stromal cells. The AVMFs were derived from primary cultures grown in DMEM, whereas the bone marrow stromal cells used were cultured from the murine M2–10B4 cell line in RPMI-1640 medium. Although our results are congruous with the findings of Parhami et al25 who found that the lipophilic statins mevastatin and fluvastatin but not the water-soluble pravastatin promoted mineralization of a bone cell line (MC3T3-E1 cells).
with a large body of emerging data on the effects of statins, it is possible that the paradoxical effects of statins on our myofibroblasts and marrow stromal cells could be explained by these differences in cell culture techniques. Additionally, our study was not designed to determine whether the statin effect was due to a difference in maturity of the valve versus bone cells. To date, there are no published reports of cultured osteoblasts from human end-stage ossified aortic valves. Cells removed from patients at the time of valve replacement are extremely difficult to culture because they are slow growing and they frequently die. Thus, the effects of statins on human valve osteoblasts remain to be determined. Consequently, a noninvasive method capable of identifying aortic valve ossification is needed that would allow clinical studies to examine the effects of statins on this process.

In conclusion, statins inhibit valve calcification in tissue culture via inhibition of the cholesterol biosynthetic pathway, independent of protein prenylation, but paradoxically stimulate bone cell calcification in a population of cells similar to that present in ossified cardiac valves. Because \( \approx 15\% \) of patients with end-stage aortic valve disease have mature bone and bone cells in their valves, the in vivo effects will be critical to determine. Prospective clinical studies are needed to determine whether statins inhibit aortic valve calcification and progression of aortic valve stenosis. Additionally, patients with end-stage disease who are at the highest risk of having bone in the valve leaflet should be carefully monitored for rapid progression of disease due to stimulation of ossification in the valve tissue.

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