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 myofibroblasts (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; 25:592-597.)

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 bone matrix proteins. Additionally, many of the atherosclerotic risk factors, such as total cholesterol, elevated low-density lipoprotein (LDL) and triglycerides, low high-density lipoprotein (HDL), male sex, cigarette smoking, hypertension, and diabetes, are associated with aortic valve calcification. In addition to dystrophic calcification (non-bone mineralization), ≈15% of end-stage aortic valves contain mature heterotopic bone with hematopoietic and stromal elements, clearly indicating that an active pathologic process is occurring.
3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have pleiotropic effects. In non-randomized clinical studies, statins inhibit calcification in atherosclerotic coronary arteries and aortic valves in addition to their established role in lowering serum LDL levels. Paradoxically, in rodent models, statins stimulate osteoblast differentiation by acting on the BMP-2 promoter, thereby inducing bone formation, but whether statins result in fewer fractures in a susceptible osteoporotic population is controversial. The observed effects of statin drugs on valve calcification may be the product of numerous noncongruent effects on the various components of a calcific valve lesion: osteoblasts, lipids, dystrophic calcium deposits, and inflammatory mediators of calcification and ossification.

In the current study, we attempted to define the effects of statins on aortic valves and bone in vitro and to delineate the mechanism by which their effects occur, in hopes that this knowledge may aid in establishing medical management of valvular calcification. Increasing doses of lipid-soluble simvastatin and water-soluble pravastatin were added to AVMs and osteoblast cell cultures. The statin effect on calcification and osteoblast differentiation was measured by counting the number of calcific nodules formed and by measuring alkaline phosphatase activity (APA). Mevalonate, a metabolite of HMG-CoA reductase, and Manumycin A, an inhibitor of protein prenylation, were each added to cell cultures to aid in determining the mechanism responsible for statin effects on calcification.

**Methods**

**Cell Culture**

Freshly obtained porcine AVMFs 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-mm² 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 2 mmol/L NaCl, 1 mmol/L MgCl₂, 10 mmol/L PEP, pH 7.4, and 0.25% NP40.

**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, statins were diluted to 0.1, 0.2, 0.4, or 0.6 μmol/L in sterile water and added to culture medium when cells reached 80% to 90% confluence. Medium with statins was refreshed every 3 to 4 days. All experiments were performed in triplicate.

**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.

**Results**

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

To determine whether statins inhibit calcific nodule formation, porcine AVMFs 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 nodule 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 AVMFs.

**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.

**Statin 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. 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. 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. 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 ($P<0.01$) 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 ($P>0.05$). 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 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. 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
Figure 6. Statins stimulate APA in M2–10B4 cells. APA was measured in cell culture plates after the addition of increasing concentrations of statin. Simvastatin (A) and pravastatin (B) both stimulated APA in M2–10B4 cells in a dose-dependent fashion (*P<0.05, **P<0.01). Error bars depict standard error. Abbreviations are as defined in text.

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., 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 statin solubility or anti-inflammatory action affects mineralization of valve and bone tissue.

Our findings that statins decrease valve calcification while increasing bone cell calcification highlights an interesting “statin paradox.” Consistent with our work in myofibroblast cultures, others have reported that statins inhibit extracellular bone matrix protein production and osteoblastic gene markers in aortic valves and clinical progression of aortic valve mineralization. In contrast, subcutaneous administration of simvastatin and lovastatin has been found to increase the rate of bone formation in rats. 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 patients; however, these data are not consistent. 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. Parhami et al 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. 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, thus explaining how simvastatin increases osteoblast differentiation. 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 al 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 a large body of emerging data on the effects of statins, it is possible that the paradoxical effects of statins on our myocardial cells 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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References

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