Atorvastatin impairs the myocardial angiogenic response to chronic ischemia in normocholesterolemic swine

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Objective: Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, used routinely in patients with coronary disease, can improve endothelial function but can have biphasic and dose-dependent effects on angiogenesis. In vitro evidence suggests that the proangiogenic effects of statins are linked to activation of Akt, a mediator of endothelial cell survival and an activator of endothelial nitric oxide synthase. We investigated the functional and molecular effects of atorvastatin supplementation on microvascular function and the endogenous angiogenic response to chronic myocardial ischemia in normocholesterolemic swine.

Methods: Yucatan miniswine were fed a normal diet with (ATOR, $n = 7$) or without (control, $n = 8$) atorvastatin (1.5 mg/kg/d) for 20 weeks. Chronic ischemia was induced by ameroid constrictor placement around the circumflex artery. Myocardial perfusion was assessed at 3 and 7 weeks using isotope-labeled microspheres. In vitro microvessel relaxation responses and myocardial protein expression were evaluated.

Results: Endothelium-dependent relaxation to adenosine diphosphate and endothelium-independent relaxation to sodium nitroprusside were intact in both groups. The ATOR group demonstrated impaired microvessel relaxation to vascular endothelial growth factor ($53\% \pm 3\%$ vs $70\% \pm 7\%$, ATOR vs NORM at 10^{-10} mol/L, $P = .05$) and fibroblast growth factor-2 ($35\% \pm 3\%$ vs $57\% \pm 5\%$, ATOR vs NORM at 10^{-10} mol/L, $P = .04$). Baseline-adjusted myocardial perfusion in the ischemic circumflex territory was significantly reduced in the ATOR group (-0.29 ± 0.10 mL/min/g vs NORM, $P = .009$). Phosphorylation of Akt was significantly increased in the ATOR group ($+235\% \pm 72\%$, $P = .009$ vs NORM), as was the myocardial expression of endostatin, an antiangiogenic protein ($+51\% \pm 9\%$, $P < .001$ vs NORM). Expression of vascular endothelial growth factor, Tie-2, fibroblast growth factor receptor-1, and endothelial nitric oxide synthase was similar in both groups.

Conclusions: Atorvastatin supplementation is associated with impaired growth factor-mediated microvessel relaxation and a significant reduction in collateral-dependent perfusion. Chronic Akt activation, increased myocardial expression of endostatin, and impaired growth factor signaling may account for the diminished endogenous angiogenic response observed with atorvastatin treatment.

Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are commonly used in patients with coronary artery disease with or without hypercholesterolemia and can improve peripheral and coronary endothelial dysfunction in these patients.¹ Numerous clinical studies have demonstrated the

Abbreviations and Acronyms

eNOS	= endothelial nitric oxide synthase
FGF	= fibroblast growth factor
ILM	= isotope-labeled microsphere
VEGF	= vascular endothelial growth factor

benefits of statins in preventing cardiovascular events in patients with coronary disease and after coronary bypass surgery.^{2,3} However, in vitro and murine evidence has suggested a biphasic and dose-dependent effect of statins on angiogenesis.^{4,5} Proangiogenic effects of statins have been linked to phosphorylation and activation of Akt (Ser 473),⁶ which has several downstream effects, including the promotion of endothelial cell survival and activation of endothelial nitric oxide synthase (eNOS). On the other hand, high doses of statins have an inhibitory effect on endothelial cell migration, and the antioxidant effects of statins can potentially interfere with reactive oxygen species-mediated angiogenic signaling. In the setting of hypercholesterolemia-induced endothelial dysfunction, we have demonstrated that high-dose atorvastatin supplementation (3 mg/kg) reduces cholesterol levels and improves endothelial function but fails to improve the endogenous⁷ or growth factor-induced⁸ myocardial angiogenic response. To further examine the effects of atorvastatin under normocholesterolemic conditions, we evaluated the functional and molecular effects of atorvastatin at a lower dose (1.5 mg/kg) on the microvascular function and the endogenous angiogenic response in a clinically relevant porcine model of chronic myocardial ischemia.

Materials and Methods**General Experimental Sequence**

Fifteen Yucatan miniswine of either sex (Sinclair Research Inc, Colombia, Mo) were used for the studies. After weaning at 7 weeks of age, the animals were divided into 2 groups. The first group was fed a regular pig chow (NORM group; N = 8), and the second group was fed a regular pig chow supplemented with atorvastatin at an oral dose of 1.5 mg/kg/d (ATOR group; n = 7) for the duration of the study (total 20 weeks).

All animals underwent an identical experimental protocol involving 3 separate procedures on each animal. Anesthesia was performed as reported previously,⁹ and all animals received humane care in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and the National Research Council's *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animals and published by the National Institutes of Health (Publication No. 5377-3 1996).

The first procedure, performed through a small left anterolateral thoracotomy at 20 weeks of age, consisted of ameroid constrictor (1.75-mm) placement around the proximal circumflex artery and the injection of 1.5×10^7 gold-labeled microspheres into the left atrium during temporary circumflex coronary occlusion to subse-

quently allow for identification, by shadow labeling, of the myocardial territory at risk.

The second procedure, also performed through a left anterolateral thoracotomy, 3 weeks after ameroid placement, consisted of 1.5×10^7 lutetium microspheres injected into the left atrium during rest conditions and 1.5×10^7 europium microspheres injected during rapid atrial pacing (150 beats/min) to allow for determination of baseline perfusion after ameroid closure. To document ameroid closure, left coronary angiography was performed through an 8F sheath surgically inserted in the femoral artery, using a catheter with the appropriate distal angulation and high atomic weight contrast.

The third procedure was carried out at 27 weeks of age (4 weeks after the second procedure and 7 weeks after ameroid placement). A sternotomy was performed, 1.5×10^7 samarium microspheres were injected into the left atrium during rest conditions, and 1.5×10^7 lanthanum microspheres were injected during pacing (150 beats/min). Euthanasia was then performed with 10 mL/kg of a saturated KCl solution administered intravenously. Cardiac samples were harvested and snap-frozen for molecular studies, sectioned, weighed, and dried for myocardial microsphere analyses, and put in 4°C Krebs's solution for in vitro assessment of coronary microvascular reactivity. Ameroid constrictors were resected along with a segment of circumflex artery and examined under low-power magnification.

In Vitro Assessment of Coronary Microvessel Reactivity

After cardiac harvest, epicardial coronary arterioles (80–150 μ m in diameter and 1–2 mm in length) originating from branches of the left anterior descending and circumflex arteries were dissected from the surrounding tissue with a 40 $\times$ dissecting microscope and examined in isolated organ chambers, as described previously.⁹ The responses to sodium nitroprusside (1 nM to 100 μ M), an endothelium-independent cyclic guanosine monophosphate-mediated vasodilator, adenosine 5'-diphosphate (1 nM to 10 μ M), an endothelium-dependent receptor-mediated vasodilator, and growth factors, vascular endothelial growth factor (VEGF) (1 fM to 1 nM) and fibroblast growth factor (FGF)-2 (1 fM to 1 nM), which act through bioavailable nitric oxide, were studied after precontraction by 30% to 50% of the baseline diameter with the thromboxane A2 analog U46619 (0.1–1 μ mol/L). Relaxation responses were defined as the percentage of relaxation of the precontracted diameter, and 6 to 8 vessels were examined in each group from the left anterior descending and circumflex territories.

Assessment of Myocardial Perfusion

Myocardial perfusion was assessed during each procedure with isotope-labeled microspheres (ILMs) (BioPAL, Worcester, Mass) using methods previously reported.⁹ Isotope-labeled microspheres, 15 μ m in diameter, of different isotopic mass were used at each experimental stage. Gold-labeled microspheres were injected during temporary circumflex occlusion at the time of ameroid placement to identify myocardial samples that originated from the circumflex coronary distribution (those with the lowest count of gold-labeled microspheres). Lutetium and europium-labeled ILMs were used during the second procedure to determine baseline blood flow at rest and with pacing. Samarium and lanthanum-

labeled ILMs were injected at rest and during atrial epicardial pacing at 150 beats/min during the third procedure. After euthanasia, 10 circumferential transmural left ventricular sections were collected for ILM assays in each animal, weighed, and dried. Each sample was exposed to neutron beams, and microsphere densities were measured in a gamma counter. Adjusted myocardial blood flow (at rest and with pacing), reflecting changes in lateral myocardial perfusion, was determined from the 2 myocardial samples that showed the lowest count of red microspheres by using the following equation:

$$\text{Adjusted blood flow} = \text{crude blood flow (third surgery)} \\ - \text{crude blood flow at baseline (second surgery)}$$

Immunohistochemistry

Myocardial sections from the circumflex territory of NORM and ATOR animals were stained with antiplatelet endothelial cell adhesion molecule-1 (CD-31) antibody diluted to 1:600 (BD Biosciences Pharmingen, San Diego, Calif), as previously described.⁹ The sections were counterstained with methyl green and examined for capillary endothelial cell density in a triplicate, blinded fashion from $700 \times 550 \mu\text{m}$ (0.385 mm^2) cross-sectional fields randomly selected from the center of the ischemic circumflex territory.

Western Blotting

Whole-cell lysates were isolated from the homogenized myocardial samples with a radioimmunoprecipitation assay buffer (Boston Bioproducts, Worcester, Mass) and centrifuged at $12,000g$ for 10 minutes at 4°C to separate soluble from insoluble fractions. Protein concentration was measured spectrophotometrically at a 595-nm wavelength with a DC protein assay kit (Bio-Rad, Hercules, Calif). Forty micrograms of total protein were fractionated by 4% to 20% gradient and sodium dodecylsulfate polyacrylamide gel electrophoresis (Invitrogen, San Diego, Calif) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass). Each membrane was incubated with specific antibodies as follows: anti-VEGF antibody diluted to 1:250 (Calbiochem, San Diego, Calif), anti-eNOS antibody diluted to 1:2500 (BD Biosciences, San Jose, Calif), anti-endostatin antibody diluted to 1:1000 (Upstate, Chicago, Ill), anti-phospho-Akt (Cell Signaling, Danvers, Mass), FGF receptor-1 (Zymed, San Francisco, Calif). Then the membranes were incubated for 1 hour in diluted appropriate secondary antibody (Jackson Immunolab, West Grove, Pa). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Bands were quantified by densitometric of radioautograph films.

Data Analysis

Data are reported as means $\pm$ standard error of the mean. Microvessel responses are expressed as percent relaxation of the precontracted diameter and were analyzed using 2-way, repeated-measures analysis of variance examining the relationship among vessel relaxation, log concentration of the vasoactive agent of interest, and the experimental group. Immunoblots are expressed as a ratio of protein to loading band density and were analyzed after digitization and quantification of x-ray films with ImageJ 1.33 (National Institutes of Health, Bethesda, Md). Blots and ILM data

were analyzed with 2-tailed t tests. Bonferroni corrections were applied to multiple tests. Statistical analyses were conducted using SAS Version 9.1 (SAS Inc, Cary, NC), and figures were generated using GraphPad Prism (GraphPad, San Diego, Calif).

Results

Coronary Microvessel Reactivity

Figure 1 shows the results of microvessel relaxation studies. Baseline diameter was similar between the NORM and ATOR groups ($125 \pm 12 \mu\text{m}$ vs $133 \pm 8 \mu\text{m}$, NORM vs ATOR, $P = .59$), as was the degree of precontraction with the thromboxane A2 analog, U46619 ($41\% \pm 6\%$ vs $34\% \pm 3\%$, $P = .32$). There were no significant differences in microvessel relaxation to any vasodilators in the nonischemic left anterior descending territory. In the ischemic circumflex territory, atorvastatin-treated animals demonstrated impaired microvessel relaxation to VEGF ($P = .05$) and basic FGF-2 ($P = .04$). Relaxation to adenosine 5'-diphosphate and sodium nitroprusside was similar between groups.

Myocardial Perfusion

Three weeks after ameroid placement, baseline myocardial perfusion of the ischemic circumflex territory at rest was similar between the NORM and ATOR groups ($0.49 \pm 0.07 \text{ mL/min/g}$ vs $0.58 \pm 0.04 \text{ mL/min/g}$; $P = .23$). During the subsequent 4 weeks, there was an increase in baseline-adjusted circumflex territory perfusion ($+0.11 \pm 0.08 \text{ mL/min/g}$) in the NORM group, reflecting the endogenous response to chronic myocardial ischemia. The atorvastatin-treated animals, however, demonstrated a reduction in baseline-adjusted circumflex territory perfusion compared with the NORM group ($-0.19 \pm 0.06 \text{ mL/min/g}$, $P = .009$; Figure 2). Similar trends were seen under pacing conditions ($+0.20 \pm 0.06$ vs -0.26 ± 0.09 , $P < .001$).

Endothelial Cell Density

Endothelial cell density (Figure 3) was significantly reduced in the ischemic territory of atorvastatin-treated animals compared with controls (111 ± 11 vs 152 ± 12 counts/high-power field; $P = .02$).

Molecular Studies

Myocardial expression of proangiogenic and antiangiogenic mediators is depicted in Figure 4. Atorvastatin-treated animals demonstrated a marked increase in Akt phosphorylation (>3 -fold, $P = .009$) without significant changes in total Akt expression. Expression of VEGF, eNOS, Tie-2, and FGF receptor-1 was similar between groups. Expression of endostatin, an antiangiogenic protein, was significantly and consistently increased in the atorvastatin-treated animals (1.5-fold, $P < .001$).

Figure 1. Microvessel response to endothelium-dependent vasodilator adenosine diphosphate and endothelium-independent vasodilator sodium nitroprusside were similar between groups. Both VEGF and FGF-2-mediated microvessel relaxation were impaired in the ischemic territory of atorvastatin-treated animals. * $P = .05$. ADP, Adenosine diphosphate; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

Discussion

In this study, we found that normocholesterolemic swine treated with high-dose atorvastatin exhibited a significant impairment in the angiogenic response to chronic myocardial ischemia. This conclusion was supported by decreased perfusion of the collateral-dependent circumflex territory and the histologic finding of reduced endothelial cell density in the ischemic territory of atorvastatin-treated animals. We

also found that atorvastatin-treated animals demonstrated reduced coronary microvessel relaxation in response to VEGF and FGF-2, suggesting impairments in growth factor-mediated signaling. Exploration of molecular pathways revealed increased expression of the potent antiangiogenic protein en-

Circumflex Territory Perfusion (adjusted for baseline)

Figure 2. Atorvastatin-treated animals demonstrated decreased baseline-adjusted perfusion of the ischemic circumflex territory compared with controls, both at rest and under pacing conditions (* $P = .009$, ** $P < .001$).

CD31+ Endothelial Cell Density

Figure 3. Endothelial cell density was determined by counting the number of CD31+ cells per high power field (0.263 mm²) in myocardial sections from the ischemic circumflex territory. Atorvastatin-treated swine demonstrated significantly reduced endothelial density compared with controls ($P = .02$).

Figure 4. Myocardial expression of endostatin was significantly increased in atorvastatin-treated animals (* $P < .001$). Phosphorylation of Akt was also increased with atorvastatin treatment (** $P = .009$). Expression of Akt, eNOS, VEGF, Tie-2, and FGF receptor-1 was similar between groups. eNOS, endothelial nitric oxide synthase; FGFR, fibroblast growth factor receptor.

dostatin and increased Akt activation in atorvastatin-treated animals. In summary, high-dose atorvastatin supplementation resulted in a reduced angiogenic response to chronic myocardial ischemia in normocholesterolemic swine.

There are a number of possible explanations for antiangiogenic effects of statins. Weis and colleagues⁵ demonstrated that endothelial cell proliferation, migration, and differentiation are impaired in the presence of high-dose cerivastatin. Furthermore, endothelial cell release of VEGF is diminished and endothelial cell apoptosis is increased under these conditions. Urbich and colleagues⁴ corroborated these findings using atorvastatin and established that statins exert these effects on endothelial cells through the downstream activation of Akt. Park and colleagues¹⁰ also observed the antiangiogenic effects of simvastatin in response to VEGF and FGF-2 in chick chorioallantoic membranes; they observed that statins interfered with VEGF signaling in vitro. Last, statin treatment has also been shown to reduce serum levels of VEGF in hypercholesterolemic patients.¹¹

We previously demonstrated, in a swine model of hypercholesterolemia-induced endothelial dysfunction, that atorvastatin supplementation (3 mg/kg/d) improves endothelial function but does not improve the endogenous⁷ or growth factor-induced⁸ angiogenic response to chronic myocardial ischemia. This dose is approximately equivalent, in humans, to 160 mg per day and from a clinical perspective may represent a supratherapeutic dose. To validate our findings, in this study, we evaluated the effects of a clinically relevant but a high dose of oral atorvastatin (1.5 mg/kg/d, ie, approximately equivalent to 80 mg/d) on myocardial angiogenesis. In contrast with control animals that demonstrated an endogenous angiogenic response to chronic ischemia, atorvastatin-treated animals exhibited reductions in perfusion of the ischemic, collateral-dependent circumflex territory.

Three important findings from this study provide mechanistic insight into the observed impairment in angiogenic

response. First, in ex vivo microvessel relaxation studies we found that both vascular smooth muscle function and endothelium-dependent nitric oxide release were preserved in atorvastatin-treated animals. However, the microvessel relaxation responses to VEGF and FGF-2 were attenuated, suggesting an impairment in VEGF and FGF-2 signaling (independent of nitric oxide bioavailability) at the level of the receptor or downstream mediators. Second, we found that phosphorylation and activation of the downstream mediator, Akt, was chronically increased approximately 3-fold in the myocardium of atorvastatin-treated animals. Because VEGF exerts its downstream effects, in part, through Akt activation,¹² this persistent Akt activation resulting from statin exposure may interfere with native VEGF signaling. Furthermore, chronic Akt activation has been demonstrated to have detrimental effects on the myocardium in the setting of ischemia-reperfusion.¹³ Last, increased expression of the potent antiangiogenic protein endostatin has consistently been observed by our group in the setting of atorvastatin treatment. Endostatin, a 20-kD cleavage product of collagen XVIII, has been shown to inhibit endothelial cell proliferation, migration, and vascular tube formation, and to promote endothelial cell apoptosis.^{14,15} Increased endostatin in the myocardium of atorvastatin-treated animals may contribute to reduced new vessel formation in response to chronic ischemia.

In light of the increasing evidence suggesting antiangiogenic properties of statins, it is critical to define the relevance of these findings to patients with coronary disease who stand to benefit from long-term statin treatment. One of the many proposed pleiotropic effects of statins is the stabilization of atherosclerotic plaque, which can reduce the chance of plaque rupture, thereby reducing the incidence of acute coronary thrombosis and myocardial injury.¹⁶ Plaque rupture has been clearly linked to plaque angiogenesis, that is, through the disruption of the fragile blood vessels found in the vaso vasorum of atherosclerotic arteries. Antiangiogenic

genic properties of high-dose statins may, in fact, be responsible for the plaque stabilization effects that have been observed. There is some suggestion that high-dose statins may also have antineoplastic effects.¹⁷ Given the critical role of angiogenesis in the pathophysiology of neoplastic processes, it is possible that the observed antineoplastic properties of statins are due to their antiangiogenic effects.

Statins, particularly high-dose statins, have well-documented benefits in patients with coronary disease; therefore, any application of these preclinical data to the clinical setting would first require well-controlled, randomized studies in patients with well-defined end points to evaluate the long-term effects of high-dose statins on collateral vessel formation. Unfortunately, these studies will be limited, in part, by the currently available imaging modalities that lack the sensitivity and resolution to clearly detect small changes in collateral vessel formation. If proven true in clinical studies, however, these antiangiogenic properties of statins would be an important consideration in weighing the risks and benefits of long-term statin therapy in patients with end-stage coronary disease in whom collateral vessel development can be a lifesaving process.

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