High-dose atorvastatin is associated with impaired myocardial angiogenesis in response to vascular endothelial growth factor in hypercholesterolemic swine

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Objectives: The disappointing results of myocardial angiogenic therapy have been attributed, in part, to endothelial dysfunction present in patients with coronary disease. Statins have established proendothelial properties but seem to have dose-dependent effects on angiogenesis. We investigated the functional and molecular effects of high-dose atorvastatin on vascular endothelial growth factor–induced myocardial angiogenesis in hypercholesterolemic swine.

Methods: Yucatan miniswine (20-30 kg) were fed either a normal (ND group, n = 8) or high-cholesterol diet, with (HC-ATOR group, n = 8) or without (HC group, n = 8) atorvastatin (3 mg · kg⁻¹ · d⁻¹), for 13 weeks. Chronic ischemia was induced by ameroid constrictor placement around the circumflex artery, followed 3 weeks later by perivascular vascular endothelial growth factor administration (2 µg over 4 weeks) with a sustained release osmotic pump. Microvessel relaxation responses, myocardial perfusion, and myocardial expression of angiogenic mediators were assessed 4 weeks later.

Results: Hypercholesterolemic swine demonstrated impaired microvessel relaxation to vascular endothelial growth factor (P < .01 vs ND group) and adenosine diphosphate (P < .001 vs ND group), which was normalized in the HC-ATOR group. After perivascular vascular endothelial growth factor administration, collateral-dependent myocardial perfusion was significantly increased in the ND group but decreased in both the HC and HC-ATOR groups (both P < .01 vs the ND group). The animals in the HC-ATOR group demonstrated increased myocardial expression of the antiangiogenic protein endostatin and increased Akt phosphorylation without significant changes in Akt and endothelial nitric oxide synthase expression.

Conclusions: Atorvastatin treatment reverses hypercholesterolemia-induced endothelial dysfunction without appreciable improvements in collateral-dependent myocardial perfusion in response to vascular endothelial growth factor treatment. Increased myocardial endostatin expression and chronic Akt activation, associated with atorvastatin therapy, might account for the lack of improvement in the angiogenic response to vascular endothelial growth factor therapy.

Although growth factor therapy has been demonstrated to result in myocardial angiogenesis in animal models, clinical trials to date have shown limited benefit in patients. It is becoming increasingly clear that angiogenesis is a complex process that involves interactions between proangiogenic and
antiangiogenic mediators, a functioning endothelium, and the extracellular matrix. Growth factors implicated in the angiogenic response, like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2), act in large part through the activation of tyrosine kinase receptors, leading eventually to the release of nitric oxide (NO). The presence of endothelial dysfunction caused by reduced NO bioavailability is a common finding in patients with coronary artery disease and might contribute to the limited benefit seen in clinical trials of growth factor administration for therapeutic angiogenesis. Indeed, the critical role played by a functioning endothelium has been highlighted in numerous studies by our group, as well as others, in which we demonstrated that hypercholesterolemia-induced endothelial dysfunction is associated with a diminished endogenous and growth factor–induced angiogenic response to chronic ischemia, and reversal of endothelial dysfunction by L-arginine supplementation (an NO substrate) rescues the impaired angiogenic response.

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are commonly used in patients with coronary disease and can improve peripheral and coronary endothelial dysfunction in these patients. Statins can increase NO bioavailability by activating Akt, which subsequently leads to endothelial nitric oxide synthase (eNOS) activation, as well as through its antioxidant effects. However, in vitro and murine studies have suggested a biphasic and dose-dependent effect of statins on endothelial cell proliferation, migration, and apoptosis, which are key events in angiogenesis. We have recently reported an impaired endogenous angiogenic response to statins in a swine model of chronic myocardial ischemia. Furthermore, there is an increased emphasis on high-dose statin treatment in patients with coronary artery disease. The aims of this study were to evaluate the functional and molecular effects of high-dose atorvastatin on coronary microvascular function and the angiogenic response to perivascular, intramyocardial VEGF therapy in a clinically relevant porcine model of hypercholesterolemia-induced endothelial dysfunction.

Materials and Methods

General Experimental Sequence

Twenty-four Yucatan miniswine of either sex (Sinclair Research Inc, Colombia, Mo) were used for the studies. After weaning at 7 weeks of age, animals were divided into 3 groups. The first group was fed a normal pig diet (ND group, n = 8); the second was given a hypercholesterolemic diet (Purina Modified Mini-Pig Grower Diet 5081, Richmond, Ind) consisting of 20% lard, 4% cholesterol, and 1.5% sodium cholate (HC group, n = 8); and the third received a hypercholesterolemic diet with oral atorvastatin (3 mg · kg$^{-1}$ · d$^{-1}$; Pfizer Inc, New York, NY; HC-ATOR group, n = 8) 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, 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 (National Institutes of Health publication no. 5377-3 1996). Briefly, for all surgical procedures, anesthesia was induced with ketamine (10 mg/kg administered intramuscularly), thiopental (5-10 mg/kg administered intravenously), and thiopental 2.5% and maintained with a gas mixture of oxygen at 1.5 to 2 L/min and isoflurane at 0.75% to 3.0%. The animals were intubated and mechanically ventilated at 12 to 20 breaths/min.

The first procedure, performed through a small left anterolateral thoracotomy at 20 weeks of age, consisted of the placement of a 1.75-mm ameroid constrictor 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 subsequently allow for identification, by means of 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 in the left atrium to allow for determination of baseline perfusion after ameroid closure. Left coronary angiography was performed through an 8F sheath (Cordis Corp, Miami, Fla) surgically inserted in the femoral artery by using a catheter with the appropriate distal angulation and high atomic weight contrast (Mallinckrodt Inc, St Louis, Mo) to document ameroid closure. After microsphere injection, an osmotic pump (Model 2ML4; Alzet Inc, Cupertino, Calif) containing human recombinant VEGF (VEGF$^{165}$) was used to provide sustained intramyocardial delivery of the growth factor by using a microcatheter implanted in the ischemic territory. VEGF (2 µg) mixed with 50 units of heparin was delivered as a 2-mL solution over 4 weeks at a rate of 3 µL/h in the ischemic territory, as previously described.

The third procedure was carried out at 27 weeks of age (4 weeks after the second procedure and 7 weeks after ameroid placement). Sternotomy was performed, and 1.5 × 10$^5$ samarium microspheres were injected into the left atrium. 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 refrigerated for myocardial microsphere analyses; and put in 4°C Kreb’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 to confirm occlusion.

In Vitro Assessment of Coronary Microvessel Reactivity
After cardiac harvest, epicardial coronary arterioles (80 to 150 μm in diameter and 1 to 2 mm in length) originating from branches of the left anterior descending and circumflex arteries were dissected from the surrounding tissue with a dissecting microscope (40× magnification) and examined in isolated organ chambers, as described previously. The responses to sodium nitroprusside (SNP; 1 nmol/L to 100 μmol/L), an endothelium-independent cyclic guanosine monophosphate–mediated vasodilator, and adenosine 5′-diphosphate (ADP; 1 nmol/L to 10 μmol/L), VEGF (1 fmol/L to 1 nmol/L), and FGF-2 (1 fmol/L to 1 nmol/L), all endothelium-dependent receptor–mediated vasodilators that act through bioavailable NO, were studied after precontraction by 30% to 50% by using previously reported methods. Assays showed that 8 ILMs, 15 μm in diameter, of different isotopic masses were used at each experimental stage. Gold-labeled microspheres were injected during temporary occlusion of the preconstricted diameter and were analyzed by using multiple tests.

Assessment of Myocardial Perfusion
Myocardial perfusion was assessed during each procedure with isotope-labeled microspheres (ILMs; BioPAL, Worcester, Mass) by using previously reported methods. ILMs, 15 μm in diameter, of different isotopic masses 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-labeled ILMs were used during the second procedure to determine baseline blood flow, and samarium-labeled ILMs were injected during the third procedure. Reference blood samples were obtained from the femoral artery during the second and third procedures. 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, reflecting changes in lateral myocardial perfusion, was determined from the 2 myocardial samples that showed the lowest count of gold-labeled microspheres by using the following equations:

Crude blood flow (tissue sample) = (Withdrawal rate [mL/min]/Weight [tissue sample]) × (Isotope counts [tissue sample]/Isotope counts 

Adjusted blood flow = Crude blood flow (3rd operation) – Crude blood flow at baseline (2nd operation)

Immunohistochemistry
Myocardial sections from the circumflex territory of animals from groups ND, HC, and HC-ATOR were stained with anti-platelet/endothelial cell adhesion molecule-1 (CD-31) antibody diluted to 1:600 (BD Biosciences, San Diego, Calif), as previously described. The sections were counterstained with methyl green and examined for capillary endothelial cell density in a triplicate blinded fashion from 600 × 440 μm (0.264 mm²) cross-sectional fields randomly selected from the center of circumflex territories.

Western Blotting
Whole-cell lysates were isolated from the homogenized myocardial samples with RIPA buffer (Boston Bioproducts, Worcester, Mass) and centrifuged at 12,000 g for 10 minutes at 4°C to separate soluble from insoluble fractions. Protein concentration was measured spectrophotometrically at 595-nm wavelength with a DC protein assay kit (Bio-Rad, Hercules, Calif). Forty micrograms of total protein was fractionated by 4% to 20% gradient 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-eNOS antibody (1:2500; BD Biosciences, San Jose, Calif), anti-phospho-Akt antibody (1:1000), anti-Akt antibody (1:1000; Cell Signaling, Beverly, Mass), anti-endostatin antibody (1:1000; Upstate, Chicago, Ill), and anti-angiostatin antibody (1:1000; EMD Biosciences, San Diego, Calif). Then the membranes were incubated for 1 hour in dilute 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 means of densitometry of radioautograph films.

Data Analysis
Data are reported as means ± standard error of the mean. Microvessel responses are expressed as the percentage relaxation of the preconstricted diameter and were analyzed by using repeated-measures analysis of variance (ANOVA), examining the relationship between vessel relaxation, log concentration of the vasoactive agent, and the experimental group (Graphpad Prizm 4.0; GraphPad, San Diego, Calif). Subgroup testing was done only if the ANOVA P value was less than .05. 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 1-way ANOVAs. Bonferroni corrections were applied to multiple tests.

Results
Animal Model
Three animals died 1 day after ameroid placement, 1 in each group, likely because of arrhythmias. In the HC group 1 additional animal died at the time of the second operation because of intractable ventricular arrhythmias, and 1 animal was killed early in the HC-ATOR group because of iliac artery thrombosis resulting in severe ischemic limb pain.

Serum cholesterol levels were significantly increased in the HC group compared with those in the ND group (20.1 ±
1.7 vs 2.3 (P < .001). Atorvastatin treatment resulted in a 30% reduction in serum cholesterol levels (14.2 ± 1.8 mmol/L, P < .05 vs the HC group). Table 1 depicts the lipid profile of study animals.

### Coronary Microvessel Reactivity

Microvessel responses are depicted in Figure 1. Coronary microvessel relaxation in the hypercholesterolemic animals (HC group) was impaired in response to ADP and VEGF, indicating a marked reduction in bioavailable NO. Atorvastatin supplementation resulted in a normalization of this endothelial dysfunction. Microvessel relaxation in response to FGF-2 was impaired in hypercholesterolemic animals (23% ± 3% vs 47% ± 4%, P < .001) and recovered only partially with atorvastatin treatment (34% ± 3%, P = 0.06 vs the HC group). Endothelium-independent responses to SNP were preserved in all groups (P = .67, ANOVA).

### Myocardial Perfusion

Baseline myocardial perfusion of the circumflex territory, determined 3 weeks after ameroid placement, was similar between groups (0.39 ± 0.06 vs 0.55 ± 0.07 vs 0.51 ± 0.05 mL · min⁻¹ · g⁻¹, ND group vs HC group vs HC-ATOR group; P = .29, ANOVA). As seen in Figure 2, after 4 weeks of VEGF treatment, the normocholesterolemic animals had a significant increase in circumflex territory perfusion, whereas the hypercholesterolemic animals demonstrated reduced perfusion. A similar reduction in myocardial perfusion was observed in the circumflex territory of the atorvastatin-treated animals.

### Capillary Endothelial Cell Density

Figure 3 depicts the results of endothelial cell (CD31) staining. Consistent with the myocardial perfusion results, the hypercholesterolemic animals demonstrated a significant reduction

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**TABLE 1. Lipid profiles of study animals**

<table>
<thead>
<tr>
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<th>ND group</th>
<th>HC group</th>
<th>HC-ATOR group</th>
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<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>2.3 ± 0.4</td>
<td>20.1 ± 1.7</td>
<td>14.2 ± 1.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.10 ± 0.01</td>
<td>0.57 ± 0.09</td>
<td>0.61 ± 0.20</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.47 ± 0.10</td>
<td>2.15 ± 0.18</td>
<td>2.24 ± 0.32</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>1.58 ± 0.25</td>
<td>17.7 ± 1.7</td>
<td>11.85 ± 1.56</td>
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ND, Normal diet; HC, high-cholesterol diet; HC-ATOR, high-cholesterol diet supplemented with atorvastatin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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Figure 1. Coronary microvascular reactivity. Relaxation responses to increasing concentrations of vascular endothelial growth factor (VEGF; A), adenosine 5’-diphosphate (ADP; B), basic fibroblast growth factor (FGF-2; C), and the endothelium-independent vasodilator sodium nitroprusside (SNP; D) in the ischemic territory of animals in the ND, HC, and HC-ATOR groups after preconstriction with U46619 are shown. *P < .01 versus the ND group, †P = .06 versus the HC group. ND, Normal diet; HC, high-cholesterol diet; HC-ATOR, high-cholesterol diet supplemented with atorvastatin.
in endothelial cell density in the ischemic territory, which was further reduced in atorvastatin-treated animals.

Western Blotting
Atorvastatin-treated animals demonstrated significantly increased Akt phosphorylation, whereas total Akt expression in the myocardium was similar between groups. Endothelial NOS expression was similar between groups ($P = .40$, ANOVA; Figure 4, B). Interestingly, myocardial expression of the antiangiogenic protein endostatin was significantly increased in the ischemic territory of atorvastatin-treated animals. Expression of angiostatin was similar among groups.

Discussion
In this study swine treated for 20 weeks with a hypercholesterolemic diet demonstrated impaired coronary microvessel relaxation to ADP and VEGF, whereas microvessel relaxation to SNP was preserved, indicating reduced NO bioavailability and endothelial dysfunction in these animals. Supplementation with atorvastatin reversed the endothelial dysfunction. Despite improved endothelial dysfunction, however, VEGF treatment did not result in improvements in perfusion of the collateral-dependent circumflex territory in atorvastatin-treated animals. This was accompanied by reduced endothelial cell density, which was decreased in the HC group and further reduced in the HC-ATOR group. Furthermore, atorvastatin treatment was associated with a more than 4-fold increase in Akt phosphorylation and a 2-fold increase in the expression of the antiangiogenic mediator endostatin. In summary, this study provides evidence for impaired VEGF-induced myocardial angiogenesis in response to high-dose atorvastatin treatment, despite improved endothelial function in a clinically relevant model of chronic myocardial ischemia.

The process of new blood vessel formation requires a complex interaction between proangiogenic growth factors, antiangiogenic mediators, a functioning endothelium, and the extracellular matrix. Angiogenic growth factors act by binding to tyrosine kinase receptors and initiating intracellular signaling that eventually leads to the release of NO. Specifically, VEGF binds to and activates its receptor, VEGFR2 (KDR), which leads to the activation of phosphatidylinositol 3 kinase and downstream phosphorylation of Akt (protein kinase B). Akt activation has numerous downstream effects, including phosphorylation of the homodimeric eNOS, leading to the release of NO and vasodilation. In vitro studies have demonstrated that exposure to statins leads to a time- and dose-dependent Akt phosphorylation (ser473). In this study we show that statin treatment leads to an approximately 4-fold increase in Akt phosphorylation demonstrated in vivo in myocardial tissue. This chronic Akt activation might interfere with endogenous VEGF signaling, as well as impair the response to exogenous VEGF administration. Furthermore, recent studies have suggested that chronic Akt activation is potentially
Another important molecular finding was the increased expression of endostatin, a 20-kd fragment of collagen XVIII, which has been shown to inhibit endothelial cell proliferation and migration, as well as vascular tube formation. Endostatin is also responsible for endothelial cell apoptosis in vitro and can reduce the expression of hypoxia-inducing factor and interfere with VEGF signaling through down-regulation of VEGF receptor expression. Therefore increased myocardial endostatin expression might also help to explain the lack of improvement in perfusion observed in atorvastatin-treated animals.

Statins are increasingly being used at higher doses in patients with coronary artery disease, not only for cholesterol reduction but also for their many pleiotropic effects. Among these pleiotropic effects is their ability to improve endothelial function by increasing bioavailable NO. A variety of statins have been shown to improve endothelial function in the peripheral circulation, evaluated primarily through flow-mediated or acetylcholine-induced dilation of the brachial artery. Of the 3 studies that evaluated epicardial coronary vasodilation in response to acetylcholine, 2 showed a benefit of statins, and 1 did not. The effects of statins on the coronary microcirculation, which plays a critical role in angiogenesis and is physiologically distinct from large coronary arteries, have not been well studied. In this study we observed that hypercholesterolemia-induced impairments in coronary microvessel relaxation to the endothelium-dependent vasorelaxants ADP and VEGF were normalized in the atorvastatin-treated animals. However, FGF-2–mediated relaxation only improved partially with atorvastatin treatment, and therefore alterations in the downstream FGF-2 signaling might be related to the lack of improvement in myocardial perfusion. Lastly, microvessel relaxation in response to SNP was preserved in all groups, indicating intact vascular smooth muscle cell function.

Although the effects of statins on endothelial function have been extensively studied, their effects on new vessel formation are less clear. Proangiogenic effects of statins have been linked to the phosphorylation and activation of Akt, an important mediator of endothelial cell survival.

Figure 4. Expression of angiogenic mediators. A, Myocardial Akt expression was similar between groups. However, Akt phosphorylation was significantly increased in the HC-ATOR group. *P < .05 versus the ND group. B, Myocardial expression of endothelial nitric oxide synthase (eNOS) was similar between groups. C, Expression of the antiangiogenic protein endostatin was significantly increased in the HC-ATOR group. *P < .05 versus the ND group. ND, Normal diet; HC, high-cholesterol diet; HC-ATOR, high-cholesterol diet supplemented with atorvastatin.
and an activator of eNOS. However, statins have also been shown to have dose-dependent and cell-specific effects on VEGF expression, VEGF receptor function, and Akt activation. In addition, endothelial cell migration, a critical event in angiogenesis, has been demonstrated in vitro to be affected by statins in a dose-dependent manner, with lower doses enhancing migration and higher doses inhibiting it. Furthermore, apoptosis of vascular smooth muscle cells in response to statins has been demonstrated in models of vascular injury and neointimal formation. In a clinically relevant model of chronic myocardial ischemia, we have demonstrated that high-dose atorvastatin does not lead to improvement in perfusion of the collateral-dependent territory, despite improvements in microvascular function. These changes in myocardial perfusion were accompanied by reduced endothelial cell density in atorvastatin-treated animals. These seemingly discordant findings of improved microvessel relaxation and impaired angiogenesis underscore the complexity of the angiogenic process, which is a function of various proangiogenic and antiangiogenic influences. In our model we found that FGF-2 signaling remained impaired in the atorvastatin-treated animals. Furthermore, chronic Akt activation, which might impair VEGF signaling, and increased expression of endostatin might help to explain the antiangiogenic effects of atorvastatin.

Model Strengths and Limitations

The swine model of chronic myocardial ischemia has been used extensively in preclinical studies of growth factor- and cell-based angiogenic therapy and provides a reliable and physiologically relevant measure of myocardial perfusion. The dose and delivery method for VEGF was chosen based on previous preclinical studies conducted in our laboratory. However, because the molecular studies were only performed at a single time point, 7 weeks after ameroind placement, it is likely that many of the acute changes in angiogenic mediators in response to chronic ischemia and VEGF treatment are not captured in this model. Furthermore, we used only one high dose of atorvastatin and therefore were not able to evaluate dose-dependent effects.

Conclusions

Despite significant improvements in hypercholesterolemia-induced endothelial dysfunction, high-dose atorvastatin did not result in improved collateral-dependent myocardial perfusion in response to VEGF in a clinically relevant swine model of chronic myocardial ischemia. Atorvastatin supplementation was associated with increased Akt activation and increased endostatin expression, which might account for the attenuated angiogenic response. These antiangiogenic effects of statins warrant further study in patients with end-stage coronary artery disease, who stand to benefit from angiogenic therapy.

References