High-Dose Atorvastatin Improves Hypercholesterolemic Coronary Endothelial Dysfunction Without Improving the Angiogenic Response

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Background—Although 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) can restore endothelial function in coronary disease, in vitro and murine studies have shown their effects on myocardial angiogenesis to be biphasic and dose dependent. We investigated the functional and molecular effects of high-dose atorvastatin on the endogenous angiogenic response to chronic myocardial ischemia in hypercholesterolemic swine.

Methods and Results—Yucatan pigs were fed either a normal (NORM group; n=7) or high-cholesterol diet, with (CHOL-ATR group; n=7) or without (CHOL group; n=6) atorvastatin (3 mg/kg per day) for 13 weeks. Chronic ischemia was induced by ameroid constrictor placement around the circumflex artery. Seven weeks later, microvessel relaxation responses, myocardial perfusion, and myocardial protein expression were assessed. The CHOL group demonstrated impaired microvessel relaxation to adenosine diphosphate (29±3% versus 61±6%, CHOL versus NORM; P<0.05), which was normalized in the CHOL-ATR group (67±2%; P=NS versus NORM). Collateral-dependent myocardial perfusion, adjusted for baseline, was significantly reduced in the CHOL group (−0.27±0.07 mL/min per gram versus NORM; P<0.001) as well as the CHOL-ATR group (−0.35±0.07 mL/min per gram versus NORM; P<0.001). Atorvastatin treatment was associated with increased phosphorylation of Akt (5.7-fold increase versus NORM; P=0.001), decreased vascular endothelial growth factor expression (−68±8%; P<0.001 versus NORM), and increased expression of the antiangiogenic protein endostatin (210±48%; P=0.004 versus NORM).

Conclusions—Atorvastatin improves hypercholesterolemic-induced endothelial dysfunction without appreciable changes in collateral-dependent perfusion. Increased myocardial expression of endostatin, decreased expression of vascular endothelial growth factor, and chronic Akt activation associated with atorvastatin treatment may account for the diminished angiogenic response. (Circulation. 2006;114[suppl I]:I-402–I-408.)

Key Words: angiogenesis ● endothelium ● hypercholesterolemia ● ischemia ● HMG-CoA reductase inhibitors

Numerous studies have demonstrated that angiogenesis is a complex process involving a balance of proangiogenic and antiangiogenic mediators. 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).1–3 Endothelial dysfunction, with diminished NO bioavailability, is a common finding in patients with coronary artery disease and may contribute to the limited benefit seen in clinical trials of growth factor administration for therapeutic angiogenesis.4 In a model of hypercholesterolemia-induced endothelial dysfunction, we have demonstrated that both the endogenous and growth factor–induced angiogenic responses to chronic ischemia are attenuated.5–7 Modulation of endothelial dysfunction by increasing NO bioavailability may therefore enhance the angiogenic response.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are commonly used in patients with coronary disease and can improve peripheral and coronary endothelial dysfunction in these patients.8 Statins can increase NO bioavailability by activating Akt, which subsequently leads to endothelial nitric oxide synthase (eNOS) activation,9 as well as through its antioxidant effects.10 However, in vitro and murine studies have suggested a biphasic and dose-dependent effect of statins on angiogenesis.11,12 We evaluated the functional and molecular effects of high-dose atorvastatin on the microvascular function and the endogenous angiogenic response to chronic myocardial ischemia in a clinically relevant,
porcine model of hypercholesterolemia-induced endothelial dysfunction.

Methods

General Experimental Sequence

Twenty 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 regular pig chow (NORM group; n=7), 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 (CHOL group; n=7), and the third received a hypercholesterolemic diet with oral atorvastatin (3 mg/kg per day) (CHOL-ATR group; n=6) 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 (NIH publication No. 5377-3, 1996). Briefly, for all surgical procedures, anesthesia was induced with ketamine (10 mg/kg IM), thiopental (5 to 10 mg/kg IV), and thiopental 2.5%.

The first procedure, performed via small left anterolateral thoracotomy at 20 weeks of age, consisted of the placement of a 1.75-mm incision after ameroid closure. To document ameroid closure, left coronary angiography was performed through an 8F sheath (Cordis Corporation, Miami, Fla) surgically inserted in the femoral artery, with the use of a catheter with the appropriate distal angulation and rapid atrial pacing (150 bpm). Euthanasia was then performed with 10 mL/kg of a saturated potassium chloride solution.

The second procedure, also performed via left anterolateral thoracotomy, 3 weeks after ameroid placement, consisted of 1.5×10⁷ Lutetium microspheres injected into the left atrium during rest conditions and 1.5×10⁶ Europium microspheres injected during rapid atrial pacing (150 bpm) to allow for determination of baseline perfusion after ameroid closure. To document ameroid closure, left coronary angiography was performed through an 8F sheath (Cordis Corporation, Miami, Fla) surgically inserted in the femoral artery, with the use of a catheter with the appropriate distal angulation and high atomic weight contrast (Mallinckrodt Inc, St Louis, Mo).

The third procedure was performed with animals at 27 weeks of age (4 weeks after the second procedure and 7 weeks after ameroid placement). Sternotomy was performed, 1.5×10⁷ Samarium microspheres were injected into the left atrium during rest conditions, and 1.5×10⁶ Lanthanum microspheres were injected during pacing (150 bpm). 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 Krebs’ solution 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 was fractionated by 4% to 20% gradient SDS–polyacrylamide gel electrophoresis (Invitrogen, San Diego, Calif) and transferred to PVDF membranes (Millipore, Bedford, Mass). Each membrane was incubated with specific antibodies as follows: anti-VEGF antibody (dilution 1:250) (Calbiochem, San Diego, Calif), anti-eNOS antibody (1:2500; BD Biosciences, San Jose, Calif), anti–phospho-Akt antibody (1:1000), anti–Akt antibody (1:1000), anti–Bcl-2 antibody (1:1000), anti–phospho-Bcl-2 antibody (1:1000), anti–caspase-3 antibody (1:1000), anti–apoptosis-inducing factor (AIF) antibody (1:1000; Cell Signaling, Beverly, Mass), anti–FGF-2 antibody (1:1000; US Biological, Swampscott, Mass), anti–Tie-2 antibody (1:200; Santa Cruz, Santa Cruz, Calif), anti–endostatin antibody (1:1000; Upstate, Chicago, Ill), anti–angiostatin antibody, and anti–matrix metalloproteinase-9 (MMP-9) antibody. 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

Assessment of Myocardial Perfusion

Myocardial perfusion was assessed during each procedure with isotope-label microspheres (ILM; BioPAL, Worcester, Mass) by methods previously reported. ILM, 15 μm 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 circulation (those with the lowest count of gold-labeled microspheres). Lutetium- and Europium-labeled ILM were used during the second procedure to determine baseline blood flow at rest and with atrial pacing at 150 bpm. Samarium- and Lanthanum-labeled ILM were injected at rest and during pacing 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 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 with the use of the following equations:

\[
\text{Crude Blood Flow (Tissue Sample)} = \frac{[\text{Withdrawal Rate (mL/min)} \times \text{Weight (Tissue Sample)}]}{[\text{Isotope Counts (Tissue Sample)} \times \text{Isotope Counts (Reference Blood Sample)}]}
\]

Adjusted Blood Flow = Crude Blood Flow (Third Surgery) – Crude Blood Flow at Baseline (Second Surgery)

Immunohistochemistry

Myocardial sections from the circumflex territory of NORM, CHOL, and CHOL-ATR animals were stained with anti–platelet–endothelial cell adhesion molecule-1 (PECAM-1; CD31) 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-μm² (0.264 mm²) cross-sectional fields selected randomly from the center of circumflex territories.

Western Blotting

Whole-cell lysates were isolated from the homogenized myocardial samples with a RIPA 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 was fractionated by 4% to 20% gradient SDS–polyacrylamide gel electrophoresis (Invitrogen, San Diego, Calif) and transferred to PVDF membranes (Millipore, Bedford, Mass). Each membrane was incubated with specific antibodies as follows: anti-VEGF antibody (dilution 1:250) (Calbiochem, San Diego, Calif), anti-eNOS antibody (1:2500; BD Biosciences, San Jose, Calif), anti–phospho-Akt antibody (1:1000), anti–Akt antibody (1:1000), anti–Bcl-2 antibody (1:1000), anti–phospho-Bcl-2 antibody (1:1000), anti–caspase-3 antibody (1:1000), anti–apoptosis-inducing factor (AIF) antibody (1:1000; Cell Signaling, Beverly, Mass), anti–FGF-2 antibody (1:1000; US Biological, Swampscott, Mass), anti–Tie-2 antibody (1:200; Santa Cruz, Santa Cruz, Calif), anti–endostatin antibody (1:1000; Upstate, Chicago, Ill), anti–angiostatin antibody, and anti–matrix metalloproteinase-9 (MMP-9) antibody. 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

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 40 dissecting microscope and examined in isolated organ chambers, as described previously. The responses to sodium nitroprusside (SNP; 1 nmol/L to 10 mmol/L), two endothelium-dependent receptor-mediated vasodilators that act via bioavailable NO, were studied after preconstriction by 20% to 50% of the baseline diameter with the thromboxane A₂ analogue U46619 (0.1 to 1 μmol/L). Relaxation responses were defined as the percent relaxation of the preconstricted diameter, and 6 to 8 vessels were examined in each group from the left anterior descending artery and the circumflex territories.

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detection system (Amersham, Piscataway, NJ). Bands were quantified by densitometry of radioautograph films.

Data Analysis
Data are reported as mean±SEM. Microvessel responses are expressed as percent relaxation of the preconstricted diameter and were analyzed with the use of 2-way repeated-measures ANOVA examining the relationship between vessel relaxation, log concentration of the vasoactive agent of interest, and the experimental group (SAS version 9.1, Cary, NC). 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 ANOVAs. Bonferroni corrections were applied to multiple tests, and probability values of <0.05 were considered statistically significant.

Statement of Responsibility
The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Serum Cholesterol Levels
Total serum cholesterol levels were significantly higher in the hypercholesterolemic diet animals (P<0.001) than in the NORM group (86±7 mg/dL) but were not significantly different between the CHOL and CHOL-ATR groups (386±70 versus 490±46 mg/dL; P=0.26).

Coronary Microvessel Reactivity
Results of microvessel relaxation studies are summarized in Figure 1. Compared with the NORM group, animals in the CHOL group demonstrated impaired microvessel relaxation to endothelium-dependent vasorelaxants ADP (P=0.005) and VEGF (P=0.001), as well as the endothelium-independent vasorelaxant SNP (P=0.004). The CHOL-ATR group demonstrated significant improvement and near normalization of endothelium-dependent relaxation to ADP and VEGF, as well as endothelium-independent relaxation to SNP (all P<0.001 versus CHOL).

Myocardial Perfusion
At 3 weeks after ameroid placement, circumflex territory blood flow at rest was similar in all 3 groups (NORM, 0.46±0.07 mL/min per gram; CHOL, 0.43±0.04 mL/min per gram; CHOL-ATR, 0.36±0.03 mL/min per gram). At 7 weeks, whereas the NORM group had an increase in myocardial flow at rest (0.23±0.09 mL/min per gram), the CHOL (−0.003±0.06 mL/min per gram; P=0.04 versus NORM) and CHOL-ATR (−0.03±0.06 mL/min per gram; P=0.02 versus NORM) groups demonstrated mild reductions in circumflex territory flow (ANOVA F value=3.85; Figure 2). These differences were also evident during rapid atrial pacing (ANOVA F value=4.24) with the baseline-adjusted circumflex flow higher in the NORM (0.16±0.08 mL/min per gram) group compared with CHOL (−0.04±0.08 mL/min per gram; P=0.09 versus NORM) and CHOL-ATR groups (−0.11±0.03 mL/min per gram; P=0.005 versus NORM).

Capillary Endothelial Cell Density
Figure 3 shows the density of PECAM-1 (CD31)–positive capillary endothelial cells (cells per high-power field) in the ischemic territory of pigs from both diet groups 7 weeks after ameroid placement. The CHOL group demonstrated a signifi-
significant decrease in endothelial cell density compared with NORM (232±11 versus 186±18; *P=0.05). Endothelial cell density in the CHOL-ATR group was improved compared with the CHOL (*P=0.003 versus CHOL) and the NORM groups (279±22; *P=0.09 versus NORM).

Western Blotting
To explore possible molecular mechanisms for the impaired angiogenic response in CHOL and CHOL-ATR animals, we examined the expression of angiogenic and antiangiogenic mediators as well as markers of apoptosis in the myocardial tissue.

Expression of Proangiogenic Mediators: VEGF, eNOS, Phospho-Akt, Akt, FGF-2, and Tie-2
The results of the Western blots and the densitometric analyses of proangiogenic mediators are summarized in Figure 4. In the ischemic territory, the expression of VEGF was mildly reduced in the CHOL group compared with the NORM group (−19±15%; *P=0.32 versus NORM) but significantly reduced in CHOL-ATR group (−68±8%; *P<0.001 versus NORM). There were no differences in the expression of eNOS, FGF-2, or Tie-2 between the groups. Akt phosphorylation was similar between the NORM and CHOL groups (*P=0.66). However, atorvastatin treatment caused a significant increase in Akt phosphorylation (5.7-fold increase in CHOL-ATR versus NORM; *P=0.001). Expression of Akt was similar in all groups.

Expression of Antiangiogenic Mediators: Endostatin, MMP-9, and Angiostatin
Figure 5 displays the results of Western blots of antiangiogenic mediators endostatin and angiostatin as well as of the active form of MMP-9, the major enzyme responsible for
cleavage of collagen XVIII to form endostatin. Increased expression of the antiangiogenic protein endostatin was evident in the CHOL group (94.38% \(P=0.07\)) and significantly more so in the CHOL-ATR group (210.48% \(P=0.004\)). MMP-9 expression was similar between NORM and CHOL groups but was significantly increased in atorvastatin-treated animals (\(P=0.03\) versus NORM). Myocardial expression of angiostatin varied between animals but was not significantly different between groups.

Markers of Myocardial Apoptosis: Caspase-3, Bcl-2, Phospho-Bcl-2, and AIF

Western blot results of apoptotic markers are displayed in Figure 6. There were no significant differences in the expression of total caspase-3, Bcl-2, and AIF between the groups. However, there was a trend toward increased levels of Bcl-2 phosphorylation (expressed as a ratio of phospho-Bcl-2/total Bcl-2) in the CHOL (\(P=0.09\) versus NORM) and CHOL-ATR (\(P=0.10\) versus NORM) groups.

Discussion

In this study swine treated for 20 weeks with a hypercholesterolemic diet demonstrated endothelial dysfunction, which was significantly improved with the oral administration of high-dose atorvastatin (3 mg/kg per day). Despite increased NO bioavailability and improved endothelial dysfunction, treatment with high-dose atorvastatin did not result in improvements in perfusion of the collateral-dependent circumflex territory. These functional changes were associated with a >5-fold increase in Akt phosphorylation, decreased expression of VEGF, and a 2-fold increase in the expression of the antiangiogenic mediator, endostatin, in the atorvastatin-treated animals. Surprisingly, atorvastatin-treated animals demonstrated a trend toward increased endothelial cell density. Although antiangiogenic effects of high-dose statins have previously been suggested in vitro models, this study provides in vivo evidence for impaired myocardial angiogenesis despite improved endothelial function in a clinically relevant model of chronic myocardial ischemia. In addition, the molecular findings of reduced VEGF, Akt activation, and increased endostatin expression in the myocardium suggest potential mechanistic explanations for the functional observations.

Endothelial Function, NO, and the Angiogenic Response

The process of new vessel formation requires a complex interaction between proangiogenic growth factors, antiangiogenic mediators, a functioning endothelium, and the extracellular matrix. Numerous disease states, eg, hypercholesterolemia and diabetes, that often coexist with coronary disease can lead to endothelial dysfunction,\(^5\) as well as alterations in the angiogenic response.\(^5,13\) We have previously demonstrated that hypercholesterolemia-induced endothelial dysfunction is associated with decreased NO bioavailability and an attenuated endogenous and growth factor–induced angiogenic response and that treatment with the NOS substrate l-arginine reverses the endothelial dysfunction as well as
normalizes collateral-dependent myocardial perfusion.\textsuperscript{15} These and other studies have highlighted the critical role of the endothelium-derived NO in the angiogenic response.

**Statins and Endothelial Function**

Among the pleiotropic effects of statins is their ability to improve endothelial function by increasing bioavailable NO, which has been demonstrated in numerous studies in animal models as well as in humans.\textsuperscript{8} In this study we observed improvement of hypercholesterolemia-induced impairment in coronary microvessel relaxation in response to the endothelium-dependent vasorelaxants ADP and VEGF. In addition, we also observed improvements in endothelium-independent microvessel relaxation with atorvastatin, which may be due to the effects of statins on vascular smooth muscle relaxation. Recent studies in obese Zucker rats\textsuperscript{16} and in hyperlipoproteinemic rabbits\textsuperscript{17} have suggested that statins improve vascular smooth muscle relaxation, possibly through the inhibition of the small GTP-binding protein Rho, which can induce myosin light chain phosphorylation, thus limiting vasorelaxation.

**Statins and Angiogenesis**

Although the effects of statins on endothelial function have been studied extensively, their effects on the angiogenic response are less clear. Proangiogenic effects of statins are linked to the phosphorylation and activation of Akt,\textsuperscript{18} an important mediator of endothelial cell survival and an activator of eNOS.\textsuperscript{9} However, statins have also been shown to have dose-dependent and cell-specific effects on VEGF expression.\textsuperscript{12,19} 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.\textsuperscript{11} Furthermore, apoptosis of vascular smooth muscle cells in response to statins has been demonstrated in models of vascular injury and neointimal formation.\textsuperscript{20,21} 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. This lack of improvement was observed despite the reversal of endothelial dysfunction and increased phosphorylation and activation of Akt. In addition, we observed a trend toward increased endothelial cell density in atorvastatin-treated animals. These results imply that although endothelial cell density may be enhanced, the formation of vessels capable of improving myocardial perfusion was not improved by statin administration.

These functional effects may be explained by the reduced myocardial VEGF expression and increased endostatin expression in atorvastatin-treated animals. The expression of endostatin, a 20-kD fragment of collagen XVIII, has been shown to inhibit endothelial cell proliferation and migration, as well as vascular tube formation.\textsuperscript{22,23} Endostatin has also been demonstrated to cause endothelial cell apoptosis in vitro.\textsuperscript{24} In addition, endostatin can reduce the expression of hypoxia-inducing factor-1a\textsuperscript{25} and interfere with VEGF signaling through downregulation of VEGF receptor expression.\textsuperscript{26} We have previously reported that diet-induced hypercholesterolemia is associated with an increased myocardial expression of endostatin, which is associated with reduced perfusion of the collateral-dependent myocardial territory.\textsuperscript{5} Therefore, the atorvastatin-induced increase in myocardial endostatin expression may explain the lack of improvement in perfusion observed despite improved endothelial function and suggests a possible new mechanism for the antiangiogenic effects of statins.

Recent studies have also suggested that while many growth factors exert their effects acutely through the phosphorylation and activation of Akt,\textsuperscript{27} chronic activation of Akt is potentially detrimental to the myocardium, particularly in the setting of ischemia/reperfusion injury.\textsuperscript{28} Numerous in vitro studies have demonstrated the ability of statins to induce Akt activation, particularly in endothelial cells.\textsuperscript{12,18} In this study we found that long-term treatment (20 weeks) with high-dose atorvastatin (3 mg/kg per day) led to a 5-fold increase in phosphorylated Akt in the myocardium. Chronic Akt activation may, therefore, have a role to play in the impaired angiogenic response after chronic myocardial ischemia in the presence of high-dose statins.

**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.\textsuperscript{7,29,30} However, because the molecular studies were performed at a single time point, 7 weeks after ameroid placement, it is likely that many of the acute changes in angiogenic mediators in response to chronic ischemia are not captured in this model. Additionally, morphometric assessment of new vessel formation by quantification of CD31\textsuperscript{+} endothelial cells leads inevitably to the identification of all vessels, which may include newly formed, leaky, and unstable capillaries that may not be capable of improving myocardial perfusion.

**Conclusions**

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

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**Disclosure**

Dr Sellke is on the speakers’ bureau for Bayer Corporation.
References

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