Pravastatin Improves Function in Hibernating Myocardium by Mobilizing CD133\(^+\) and cKit\(^+\) Bone Marrow Progenitor Cells and Promoting Myocytes to Reenter the Growth Phase of the Cardiac Cell Cycle

Gen Suzuki, Vijay Iyer, Thomas Cimato, John M. Canty, Jr

Abstract—3-Hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors have been reported to increase circulating bone marrow progenitor cells and variably improve global function in heart failure. The potential role of improved perfusion versus direct effects of statins on cardiac myocytes has not been established. We chronically instrumented swine with a left anterior descending artery (LAD) stenosis to produce chronic hibernating myocardium with regional contractile dysfunction in the absence of heart failure. Hemodynamics, function, perfusion, and histopathology were assessed in pigs treated for 5 weeks with pravastatin (n=12) versus untreated controls (n=10). Regional LAD wall thickening was depressed under baseline conditions (LAD 3.7±0.3 versus 6.6±0.3 in remote regions, P<0.01). It remained unchanged in untreated animals but increased from 3.8±0.6 to 5.2±0.5 mm after pravastatin (P<0.01). There was no increase in myocardial perfusion at rest or during vasodilation. Pravastatin mobilized circulating CD133\(^+\)/cKit\(^+\) bone marrow progenitor cells and increased myocardial tissue levels (LAD CD133\(^+\) cells from 140±33 to 884±167 cells/10\(^6\) myocyte nuclei and cKit\(^+\) cells from 223±49 to 953±123 cells/10\(^6\) myocyte nuclei). Pravastatin increased myocytes in mitosis (phospho–histone-H3; 9±5 to 43±7 nuclei/10\(^6\) myocyte nuclei, P<0.05) and the growth phase of the cell cycle (Ki67; 410±82 to 1261±235 nuclei/10\(^6\) myocyte nuclei, P<0.05) in diseased but not normal hearts. As a result, pravastatin increased LAD myocyte nuclear density from 830±41 to 1027±55 nuclei/mm\(^2\) (P<0.05). These data indicate that, in the absence of impaired endothelial function and heart failure, dysfunctional hibernating myocardium improves after pravastatin. This effect is independent of myocardial perfusion and related to mobilization of CD133\(^+\)/cKit\(^+\) bone marrow progenitor cells which stimulate myocyte proliferation resulting in quantitative increases in myocyte nuclear density.  (Circ Res. 2009;104:255-264.)

Key Words: statins ■ hibernating myocardium ■ cardiac repair ■ bone marrow progenitor cells

Although 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors were originally developed as lipid-lowering drugs and were demonstrated to retard the progression of atherosclerosis and improve endothelial function, pleiotrophic actions of statins may be of equal importance in reducing death and disability from cardiovascular disease. Statins have been shown to have anti-inflammatory, antihypertrophic, and proangiogenic actions that may favorably impact ventricular function independently of their actions on stabilizing and/or regressing atherosclerotic lesions.\(^1,2\) In addition, statins have been demonstrated to mobilize endothelial progenitor cells (EPCs) into the circulation in humans and animals.\(^3,4\) Preclinical studies have demonstrated that EPCs can arise from bone marrow and incorporate into the vascular wall.\(^5\) Some studies have also demonstrated the ability of bone marrow progenitor cell (BMPC) populations to differentiate into cardiac myocytes.\(^6,7\) Several small randomized clinical studies have demonstrated objective increases in myocardial function after statins\(^8,9\) but this may not be a class effect.\(^10\) Although angiogenesis could play a role in some of these actions, improvements in myocardial function have also been demonstrated in nonischemic cardiomyopathy.

We performed the present study to identify the effects of pravastatin on myocardial perfusion and ischemic myocardial dysfunction that are independent of cholesterol lowering. We used a well-characterized porcine model of collateral-dependent myocardium resulting in hibernating myocardium from a chronic LAD stenosis. Because regional rather than global left ventricular (LV) dysfunction is present, the confounding role of statins on inflammation, cytokines, and neurohormonal activation in heart failure are absent.\(^11\) In addition, because hypercholesterolemia and atherosclerosis are absent, the effects of pravastatin on angiogenesis were

Original received October 1, 2008; revision received November 7, 2008; accepted December 4, 2008.
From the Veterans Affairs Western New York Healthcare System, Departments of Medicine and Physiology and Biophysics, and Center for Research in Cardiovascular Medicine, University at Buffalo, The State University of New York.
Correspondence to John M. Canty, Jr, Division of Cardiology, University at Buffalo, Biomedical Research Building, Room 345, 3435 Main St, Buffalo, NY 14214, E-mail canty@buffalo.edu
© 2009 American Heart Association, Inc.
Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.108.188730
studied independently of inhibiting plaque progression and improving endothelium-dependent vasodilation. Our results demonstrate that pravastatin mobilizes BMPCs in hibernating hearts and promotes myocytes to reenter the growth and mitotic phase of the cell cycle. As a result, myocyte nuclear density is increased and myocardial function improves without a change in myocardial perfusion.

Materials and Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research. Detailed experimental and histological protocols are described in the online data supplement, available at http://circres.ahajournals.org.

Mobilization of BMPCs With Pravastatin

We initially assessed the dose dependency of pravastatin to mobilize BMPCs in normal swine using flow-cytometry (FACS, n=11). Baseline measurements were compared to those 2 weeks after daily treatment with low-dose (20 mg/d) or high-dose (160 mg/d) pravastatin. We assessed changes in peripheral blood (30 mL) as well as bone marrow (30 mL). Data from FACS analysis are expressed as CD133+ or cKit+ cells per million mononuclear cells.

Effects of Pravastatin on Flow and Function in Hibernating Myocardium

Pigs were chronically instrumented with a 1.5-mm Delrin stenosis on the proximal LAD as described previously (online data supplement).12 We performed baseline physiological studies 4 months after instrumentation (n=22) when hibernating myocardium with reductions in LAD wall thickening and resting LAD perfusion were present. Under propofol sedation, a Millar catheter was inserted into the left ventricle for microsphere injection. Regional wall thickening was assessed with transthoracic echocardiography. Microsphere flow was assessed at rest and following adenosine vasodilation. Animals were subsequently treated with pravastatin (160 mg/d, n=12) for 5 weeks and compared to untreated controls (n=10). At the end of the study, physiological studies were repeated and the heart was excised for 5-triphenyltetrazolium chloride (TTC), flow, and histological analysis.

Myocyte Nuclear Density and Morphometry

Samples from hibernating LAD and normal remote regions were fixed and paraffin-embedded. Trichrome-stained sections were used to quantify connective tissue.13 Periodic acid Schiff–stained sections were used to quantify myocyte nuclear diameter, nuclear length, and regional myocyte nuclear density. Histological results in hibernating animals were compared to normal sham animals (pravastatin, n=10; untreated, n=5).

Quantitative Analysis of Myocyte Proliferation and CD133+/cKit+ Cells

To identify myocyte proliferation, anti-Ki67 and anti–phospho–histone-H3 staining were performed.14 CD133+/cKit+ BMPCs in myocardial tissue were quantified using confocal immunofluorescence (online data supplement). Preliminary studies demonstrated that tissue CD133+/cKit+ BMPCs were CD45 negative. To evaluate whether CD133+/cKit+ BMPCs differentiated into a cardiac lineage we costained BMPCs with GATA-4 and cardiac troponin I antibodies.14

Statistics

Data are expressed as means±SE. Differences between groups were assessed by 2-way ANOVA and the post hoc Holm–Sidak test. Temporal physiological changes between initial and final studies were assessed by paired t tests. P<0.05 was considered significant.

Results

Pigs were in good health at the time of study, and TTC staining showed no significant infarction. Initial physiological studies were performed 130±3 days after instrumentation and repeated 5 weeks after pravastatin (166±3 days).

Pravastatin Dose-Dependently Mobilizes CD133+ and cKit+ BMPCs

Figure 1 summarizes flow cytometric data before and after low- and high-dose pravastatin for 2 weeks. There were no differences in circulating mononuclear cells (n=11, 11.6×10^6±1.8×10^6 cells/mL at baseline versus 16.6×10^6±4.7×10^6 cells/mL after pravastatin; P=NS). In contrast, pravastatin dose-dependently increased both CD133+ and cKit+ cells in blood and bone marrow aspirates. Circulating and bone marrow CD133+ and cKit+ cells were much higher after a 160-mg dose of pravastatin. For example, circulating CD133+ cells averaged 18±3 cells/10^6 mononuclear cells at baseline, 32±5 cells/10^6 mononuclear cells after 20 mg pravastatin (P<0.05 versus baseline), and 272±54 cells/10^6 mononuclear cells after 160 mg of pravastatin (P<0.05 versus baseline and 20 mg of pravastatin).

Effects of Pravastatin on Function and Flow in Hibernating Myocardium

Hemodynamics (supplemental Table I) and echocardiographic measurements of function (supplemental Table II) are summarized in the online data supplement. Hemodynamics were similar between treatment groups with the exception of LV end-diastolic pressure, which tended to be lower in animals receiving pravastatin. Pravastatin increased LAD systolic wall thickening (percentage WT, 28±2.8% versus 48±5.1% after pravastatin, P<0.05; supplemental Table II) with systolic excursion (ESWT-EDWT) summarized in Figure 2A. There were no differences in remote regions. Serial analysis of function within the same animal confirmed a similar improvement, with no change in untreated controls. Likewise, global function was normal and did not change after pravastatin (ejection fraction, 64±5% versus 69±2% after pravastatin; P=NS). Although pravastatin improved regional function in hibernating myocardium, there was no effect on resting flow (Figure 2B). When analyzed in terms of relative full-thickness perfusion, relative reductions in resting flow (LAD/remote, 0.79±0.07 in pravastatin versus 0.70±0.05 in untreated) were similar, with no serial changes between studies in the same animal. Likewise, relative adenosine flow (LAD/remote 0.27±0.04 in pravastatin versus 0.23±0.02 in untreated) was not significantly different, nor were there serial changes over time. Thus, although pravastatin increased LAD wall thickening, there was no functionally significant angiogenesis.

Increase in Myocardial CD133+ and cKit+ Cells After Pravastatin

We analyzed myocardial tissue to determine whether the increase in circulating CD133+/cKit+ BMPCs produced corresponding increases in myocardial tissue levels (Figure 3A and 3B). Pravastatin increased CD133+ cells in hibernating
myocardium (LAD, 884±167 versus 140±33 cells/10⁶ myocyte nuclei in untreated animals, \( P<0.01 \)). Although CD133⁺ cells were rare in sham hearts (17±5 cells/10⁶ myocyte nuclei), myocardial levels also increased after pravastatin (673±122 cells/10⁶ myocyte nuclei, \( P<0.001 \)). Increases in CD133⁺ cells were global and occurred in nonischemic remote regions in hibernating and sham hearts (944±183 cells/10⁶ myocyte nuclei in hibernating remote and 607±79 cells/10⁶ myocyte nuclei in sham remote). Like CD133⁺ cells, myocardial cKit⁺ cells were rare in normal hearts (27±3 cells/10⁶ myocyte nuclei) and increased after pravastatin (hibernating LAD, 953±123 cells/10⁶ myocyte nuclei; normal LAD, 1031±244 cells/10⁶ myocyte nuclei), as well as their corresponding remote regions. Dual staining in 6 animals demonstrated that 33±4% of cKit⁺ cells also costained for CD133. Thus, pravastatin produced global increases in myocardial CD133⁺ and cKit⁺ cell levels that were independent of ischemia or LV dysfunction.

**GATA4 Expression in CD133⁺ and cKit⁺ Cells**

To identify whether CD133⁺ and cKit⁺ BMPCs could differentiate into a cardiac lineage, they were costained for the cardiac transcription factor GATA4 (Figure 4A, a). These were a small fraction of CD133⁺/GATA4⁺ cells. They rarely coexpressed troponin I (Figure 4A, b). Whereas CD133⁺/GATA4⁺ cells increased after pravastatin (43±9 versus 8±5 cells/10⁶ myocytes in untreated LAD; Figure 4B), the proportion of CD133⁺ BMPCs expressing GATA4 was unchanged (4.4±2.9% in untreated versus 6.2±1.6% in pravastatin; \( P=NS \)). This indicates that increases in GATA4⁺ BMPCs were secondary to mobilizing CD133⁺ BMPCs to the heart and not from pravastatin enhancing the differentiation.
tion of CD133⁺ BMPCs to myocytes. Similar results were found for cKit⁺ cells.

**Increased Myocytes in the Growth Phase of the Cell Cycle After Pravastatin**

We quantified the frequency of myocyte nuclei expressing Ki67, a marker of the growth phase of the cell cycle and phospho–histone-H3, a marker of mitosis (Figure 5). Untreated hibernating animals had low values of Ki67 positivity (LAD, 410±82 nuclei/10⁶ myocyte nuclei; remote, 285±88 nuclei/10⁶ myocyte nuclei). After pravastatin, Ki67 positivity increased (LAD, 1261±235 nuclei/10⁶ myocyte nuclei; remote, 969±132 nuclei/10⁶ myocyte nuclei; *P*<0.05 versus untreated). Surprisingly, although CD133⁺ and cKit⁺ cells increased in sham hearts, there was no increase in Ki67 positivity after pravastatin (LAD, 274±82 nuclei/10⁶ myocyte nuclei; remote, 284±69 nuclei/10⁶ myocyte nuclei; *P*=NS versus untreated). Likewise, myocyte nuclear phospho–histone-H3 positivity was selectively increased in pravastatin-treated hibernating animals versus sham-treated animals (LAD, 43±7 versus 9±5 nuclei/10⁶ myocyte nuclei; remote, 34±9 versus 6±3 nuclei/10⁶ myocyte nuclei; *P*<0.05, respectively). Thus, pravastatin only increased myocytes in the growth phase of the cell cycle in diseased hearts.

**Effects of Pravastatin on Myocyte Nuclear Density in Hibernating Myocardium**

To determine whether increases in cell cycle markers led to myocyte regeneration, we assessed the effects of pravastatin on myocyte nuclear density (Figure 6). Whereas LAD connective tissue was mildly increased in hibernating animals receiving pravastatin (LAD, 8.3±1.9%; *P*<0.05), it was similar to untreated animals (LAD, 6.9±0.7%; *P*<0.05). In untreated animals, LAD myocyte nuclear density was reduced (LAD, 830±41 nuclei/mm²; remote, 1027±55 nuclei/mm²; *P*<0.05), as we have demonstrated previously. After pravastatin, LAD myocyte nuclear density increased from 830±41 to 1054±19 nuclei/mm² (*P*<0.01), but values remained significantly lower than normal myocardium (*P*<0.05). Measurements
of myocyte diameter (Figure 7) demonstrated that increases in myocyte nuclear density after pravastatin were accompanied by a reduction in myocyte size (mean diameter, 13.2 ± 0.6 μm, versus 15.7 ± 0.4 μm; P < 0.05). The diameter of Ki67-positive myocytes was even smaller (11.9 ± 0.2 μm, P < 0.05). Thus, the increase in myocyte nuclear density coupled with a reduction in myocyte size is consistent with myocyte proliferation after pravastatin in hibernating myocardium.

Figure 3. Pravastatin increases myocardial CD133+ and cKit+ BMPCs in hibernating and sham animals. A, Both cKit+ (a) and CD133+ (b) cells (stained green) localized in interstitial regions and were frequently clustered. Myocytes were stained with troponin I (red) and nuclei with TO-PRO-3 (blue). B, There was a global increase in myocardial CD133+ and cKit+ cells in LAD and remote regions after 5 week of pravastatin. Levels were similar in hearts from hibernating and sham animals.

Figure 4. Coexpression of GATA4 in CD133+ and cKit+ BMPCs. A, Example of a CD133+ cell costaining for GATA4 (a) and troponin I (b). Similar changes were seen in cKit+ cells. B, Pravastatin increased myocardial CD133+ and cKit+ BMPCs coexpressing GATA4 in normal and hibernating hearts. Because the percentage of CD133+/cKit+ BMPCs coexpressing GATA4 remained fairly constant (4.4 ± 2.9% vs 6.2 ± 1.6% after pravastatin), the increase reflected a greater total number of CD133+/cKit+ cells in the tissue (Figure 3B).
Discussion

There are several important new findings from our study. First, pravastatin dose-dependently increases CD133<sup>+</sup> and cKit<sup>+</sup> BMPCs in bone marrow and blood. This leads to corresponding increases in myocyte nuclear density and reductions in myocyte diameter in hibernating myocardium. Although statins mobilized CD133<sup>+</sup> and cKit<sup>+</sup> BMPCs to normal hearts, evidence of cardiac myocyte proliferation was absent. Collectively, the observations are consistent with the notion that chronic mobilization of CD133<sup>+</sup>/cKit<sup>+</sup> BMPCs with pravastatin leads to significant myocyte regeneration in diseased hearts.

Mobilization of BMPCs and Localization in the Heart

There is now substantial support for the notion that BMPCs can facilitate myocyte regeneration under selected conditions. Orlic et al demonstrated the ability of cKit<sup>+</sup> bone marrow cells injected into murine infarcts to regenerate myocardium. In a subsequent study, myocyte regeneration was effected by mobilizing BMPCs with stem cell factor and granulocyte colony-stimulating factor. Although controversial, the ability of BMPCs to repair the heart in humans is supported by myocyte chimerism in transplant recipients. This, as well as other preclinical work, stimulated rapid translation of these findings to early clinical trials using a variety of largely unfractionated intracoronary and intramyocardial adult bone marrow preparations in patients with myocardial infarction. Although the therapy appears safe and functional effects are generally favorable, the magnitude of improvement is modest (reviewed in a recent metaanalysis). Importantly, BMPC mobilization strategies using granulocyte colony-stimulating factor in humans have not demonstrated significant functional improvement. Thus, myocardial homing, as well as mobilization, is required for a physiological effect.

Our results demonstrate that pravastatin affords an alternative approach to effect bone marrow–mediated cardiac repair. Because statins have been demonstrated to mobilize EPCs, previous basic and clinical studies have focused on evaluating their effect on angiogenesis and vasculogenesis rather than myocyte regeneration. A variety of HMG-CoA reductase inhibitors can mobilize bone marrow–derived CD34<sup>+</sup> and CD133<sup>+</sup> EPCs. These can differentiate into endothelial cells and have been demonstrated to improve perfusion in the severely ischemic murine hind limb model. Dimmeler et al demonstrated that simvastatin can also increase bone marrow cKit<sup>+</sup> cells. Previous studies have established that EPC mobilization involves activation of the phosphatidylinositol 3-kinase/Akt pathway by statins. Our results extend these studies in swine by showing a parallel dose-dependent increase in cKit<sup>+</sup> and CD133<sup>+</sup> BMPCs after pravastatin.

Discussion

There are several important new findings from our study. First, pravastatin dose-dependently increases CD133<sup>+</sup> and cKit<sup>+</sup> cells in bone marrow and blood. This leads to corresponding increases in myocardial tissue from normal and diseased hearts. Second, in hibernating myocardium, pravastatin improves regional wall thickening with no demonstrable effect on myocardial perfusion, indicating that functional improvement is independent of angiogenesis. Third, immunohistochemical analysis of myocardial tissue demonstrates that improved function is accompanied by an increase in myocytes in the proliferative phase of the cell cycle. This leads to corresponding increases in myocyte nuclear density and reductions in myocyte diameter in hibernating myocardium. Although statins mobilized CD133<sup>+</sup> and cKit<sup>+</sup> BMPCs to normal hearts, evidence of cardiac myocyte proliferation was absent. Collectively, the observations are consistent with the notion that chronic mobilization of CD133<sup>+</sup>/cKit<sup>+</sup> BMPCs with pravastatin leads to significant myocyte regeneration in diseased hearts.

Mobilization of BMPCs and Localization in the Heart

There is now substantial support for the notion that BMPCs can facilitate myocyte regeneration under selected conditions. Orlic et al demonstrated the ability of cKit<sup>+</sup> bone marrow cells injected into murine infarcts to regenerate myocardium. In a subsequent study, myocyte regeneration was effected by mobilizing BMPCs with stem cell factor and granulocyte colony-stimulating factor. Although controversial, the ability of BMPCs to repair the heart in humans is supported by myocyte chimerism in transplant recipients. This, as well as other preclinical work, stimulated rapid translation of these findings to early clinical trials using a variety of largely unfractionated intracoronary and intramyocardial adult bone marrow preparations in patients with myocardial infarction. Although the therapy appears safe and functional effects are generally favorable, the magnitude of improvement is modest (reviewed in a recent metaanalysis). Importantly, BMPC mobilization strategies using granulocyte colony-stimulating factor in humans have not demonstrated significant functional improvement. Thus, myocardial homing, as well as mobilization, is required for a physiological effect.

Our results demonstrate that pravastatin affords an alternative approach to effect bone marrow–mediated cardiac repair. Because statins have been demonstrated to mobilize EPCs, previous basic and clinical studies have focused on evaluating their effect on angiogenesis and vasculogenesis rather than myocyte regeneration. A variety of HMG-CoA reductase inhibitors can mobilize bone marrow–derived CD34<sup>+</sup> and CD133<sup>+</sup> EPCs. These can differentiate into endothelial cells and have been demonstrated to improve perfusion in the severely ischemic murine hind limb model. Dimmeler et al demonstrated that simvastatin can also increase bone marrow cKit<sup>+</sup> cells. Previous studies have established that EPC mobilization involves activation of the phosphatidylinositol 3-kinase/Akt pathway by statins. Our results extend these studies in swine by showing a parallel dose-dependent increase in cKit<sup>+</sup> and CD133<sup>+</sup> cells in bone marrow and blood. This supports the notion that the primary action of statins is to increase the proliferation of CD133<sup>+</sup> and cKit<sup>+</sup> cells in bone marrow. It is also possible that CD133<sup>+</sup>/cKit<sup>+</sup> BMPCs arise from other tissue reservoirs which will require further study. The corresponding global increase in myocardial CD133<sup>+</sup> and cKit<sup>+</sup> cell levels in normal as well as hibernating hearts is probably a function of their circulating concentration. Although the number of CD133<sup>+</sup>/GATA-4<sup>+</sup> and cKit<sup>+</sup>/GATA-4<sup>+</sup> cells increased, pravastatin did not enhance differentiation into a cardiac

Figure 5. Pravastatin increases Ki67 and phospho–histone-H3–positive myocytes in hibernating hearts. A, Pravastatin increased myocyte Ki67 (green) (a and b) and phospho–histone-H3 (pHH3) (c), indicating an increased number of myocytes in the growth and mitotic phase of the cell cycle. Ki67- and pHH3-positive nuclei were confirmed as myocytes by costaining with troponin I (red). B, Quantitative data demonstrated significant increases in Ki67 and pHH3 in the LAD and remote regions in treated pigs with hibernating myocardium. In contrast, these were not altered in sham animals despite increased myocardial CD133<sup>+</sup> and cKit<sup>+</sup> BMPCs after pravastatin.
lineage because the percentage of GATA-4$^+$ cells was unchanged.

**Lack of Effect of Pravastatin on Perfusion in Hibernating Myocardium**

Mobilization of EPCs can salvage tissue in rodent hind limb ischemia models via increases capillary density, suggesting small vessel proliferation. The ability of statins to increase myocardial perfusion is less clear. Indeed, this question provided the initial rationale for our study, where pravastatin was anticipated to improve perfusion to collateral-dependent hibernating myocardium. Surprisingly, there was no difference in resting or vasodilated flow in LAD or remote myocardium after pravastatin (online data supplement). Likewise, relative perfusion differences between LAD and remote regions were unchanged. Although we did not assess anatomic variables such as capillary density, the failure of coronary flow to increase in ischemic myocardium supports the notion that statins do not have any meaningful effect on coronary collateral resistance vessels. Dissociations between flow and capillary density can occur because the latter vessels contribute negligibly to coronary vascular resistance.24 Nevertheless, because there were small increases in end-diastolic wall thickness after pravastatin (<15%), we cannot exclude the possibility that angiogenesis matched to changes in regional mass occurred.

A potential explanation for the failure of pravastatin to increase perfusion may relate to a biphasic dose-dependent effect on angiogenesis. Weis et al found that low-dose cerivastatin or atorvastatin potentiated endothelial cell proliferation, whereas higher doses inhibited it.25 In vivo, Boodhwani et al demonstrated that high-dose atorvastatin (1.5 mg/kg per day) impairs collateral development in normocholesterolemic swine.26 Our findings are similar using a less potent HMG-CoA reductase inhibitor. In disease states where endothelium-dependent vasodilation of coronary resistance vessels is impaired, such as atherosclerosis, statins may have important beneficial effects in improving endothelium-dependent vasodilation of resistance arteries, including collateral resistance vessels.24 These functional effects rather than de novo arteriogenesis or vasculogenesis likely contribute to the well-known improvement in symptomatic myocardial ischemia after administering statins to patients.27

**Effect of Statins on Myocardial Function**

Previous animal studies have demonstrated beneficial effects of statins on global myocardial function in a variety of models of heart disease. In general, all have used high doses of statins that greatly exceed the pharmacological range used in humans (eg, 20 mg/kg simvastatin per day). These are also even higher than doses demonstrated to impair angiogenesis in vivo and vitro. In rodent models of infarction, statins improved global LV function by preventing deleterious LV remodeling without reducing infarct size.28–31 Statins have also been demonstrated to prevent the progression of hypertrophy following angiotensin infusion and aortic banding and to prevent heart failure in genetic models of cardiomyopathy.32 Studies evaluating the effect of statins on heart failure in large animals are limited. In dogs with heart failure,
Statins attenuate the rate of decline in LV function in pacing-induced cardiomyopathy and cardiomyopathy attributable to coronary microembolization. In general, mechanistic insight has largely focused on the ability of statins to improve NO signaling and reduce circulating markers of inflammation. None of the previous studies has elucidated a quantitative contribution of statins to myocyte regeneration.

Our results demonstrate that pravastatin increases regional function in hibernating myocardium in the absence of infarction or heart failure. Although functional improvement was not related to improved perfusion, it was accompanied by an increase in regional end-diastolic wall thickness and an increase in myocyte nuclear density. Resolution of myocyte cellular hypertrophy in LAD myocardium could also contribute improved wall thickening. Thus, the mechanism of improvement appears to be related to myocyte regeneration that restores regional myocyte loss from apoptosis during the development of hibernating myocardium. Although clinical trials in hibernating myocardium are lacking, Gheorghiade et al reported a dramatic serial functional improvement in a patient with hibernating myocardium from a chronic LAD occlusion after simvastatin.

Statins Increase Myocytes in the Growth Phase of the Cell Cycle in Diseased Hearts

We found that statins increased myocytes in the proliferative phase of the cell cycle in a fashion similar to that which we have reported after intracoronary administration of AdvFGF-5. Although this was accompanied by a global increase in myocardial tissue CD133+ and cKit+ BMPCs, increases in myocyte Ki67 and phospho–histone-H3 staining were restricted to hibernating hearts. The possibility that increased circulating CD133+/cKit+ BMPCs leads to increased myocyte regeneration is unlikely because pravastatin increased CD133+/cKit+ BMPCs but did not cause myocyte proliferation in normal hearts. Thus, additional myocardial factors are probably required to stimulate myocyte proliferation. Because Ki67 increased in the remote myocardial region of hibernating hearts, as well as the dysfunctional LAD region, the stimulus is global. Circulating factors related to neurohormonal activation such as elevated catecholamines, angiotensin, endothelin, and cytokines are plausible but seem unlikely in this model because global function is normal and hemodynamic evidence of neurohormonal activation is absent. A more likely possibility is that a factor released following transient myocardial ischemia recirculates to normal as well as ischemic myocardium. Alternatively, it is possible that myocardial stretch related to increased preload stimulates the expression of preload-induced myocardial growth factors and genes that facilitate myocyte proliferation in conjunction with the increase in tissue CD133+ and cKit+ cells. Additional studies will be required to address these possibilities directly. Finally, although statins could have increased myocyte number by inhibiting apoptosis, we have previously demonstrated that apoptosis has returned to baseline 4 months after instrumentation in this model, which is the time frame over which the present studies were conducted.

The actual origin of cycling myocytes cannot be unambiguously determined from our study. One possibility is that the CD133+ and cKit+ cells differentiate directly into cardiac myocytes. This is supported by the observation that a portion of them express GATA4 and troponin I. Another possibility is that CD133+ and cKit+ cells stimulate resident cardiac stem cells (CSCs) to proliferate with Ki67+ myocytes arising from CSCs, as has been suggested after administration of mesenchymal stem cells. It is also plausible that CD133+ and cKit+ cells fuse with cardiac myocytes and facilitate their reentry into the growth phase of the cell cycle. Finally, paracrine factors released from CD133+ and cKit+ cells could stimulate CSC proliferation or facilitate myocytes to reenter the cell cycle. Additional studies using models using genetic fate mapping and stem cell tracking will be required to address these possibilities. Although the precise signaling is unknown, the increase in myocyte nuclear density, smaller myocyte size, and improvement in regional function demonstrated in vivo all suggest an effect of pravastatin on myocyte regeneration.

Clinical Implications

Our results support the hypothesis that the improvement in ejection fraction in patients with heart failure previously demonstrated with simvastatin and atorvastatin may result from stimulation of endogenous cardiac repair mechanisms. Nevertheless, this may not be a class effect, as evidenced by the failure of the extremely potent drug rosuvastatin to improve ejection fraction. In concert with the latter study, the recently completed large placebo controlled clinical trials of rosuvastatin in patients who have predominantly class II and III heart failure with depressed ejection fraction (CORONA and GISSI-HF) have failed to show any impact of this drug on cardiovascular mortality. Results of rosuvastatin on serial function in these studies have not yet been reported. Individual statins likely vary in their ability to affect myocardial function through differential potency (or dose range), as well as their ability to affect relevant pleotropic pathways (eg, upregulation of Akt or inhibiting myocardial proliferative pathways such as Rho, Rac, and Ras). Finally, age-related impairment in cardiac stem cell repair mechanisms may be important, and the clinical trials have focused on elderly patients with heart failure (mean ages of 68 to 73 years) versus younger patients in small positive studies using myocardial function as an end point.

In summary, our study demonstrates functional improvement in hibernating myocardium after pravastatin that is independent of angiogenesis or atherosclerotic plaque regression that occurs in the absence of a proinflammatory state or neurohormonal activation. Beneficial effects may be absent when infarction rather than hibernating myocardium predominates because residual perfusion to scar is low. Nevertheless, because patients with ischemic cardiomyopathy usually have patchy fibrosis averaging ∼20% of LV mass, our observations may be very relevant to understanding statin-mediated functional improvement in nonischemic cardiomyopathies, as well as settings where dysfunction from reversible ischemia and LV remodeling play major roles. Finally, the ability of statins to mobilize circulating BMPCs may be an important variable to consider in understanding the independent effects.
of exogenous cell-based therapies administered in clinical trials examining cardiac repair.

Acknowledgments

We thank Anne Coe, Deana Gretch, Elaine Granica, and Amy Johnson for technical assistance.

Sources of Funding

Supported by the Veterans Affairs Administration; American Heart Association; Buswell Fellowship; National Heart, Lung, and Blood Institute; Albert and Elizabeth Rekate Fund; and Empire State Stem Cell Board.

Disclosures

None.

References


Pravastatin in Hibernating Myocardium


33. 2006;132:1299–1306.
34. 2003;112:2951–2958.
44. 2003;103:839–843.
47. 2003;307:332–337.


Pravastatin Improves Function in Hibernating Myocardium by Mobilizing CD133\(^+\) and cKit\(^+\) Bone Marrow Progenitor Cells and Promoting Myocytes to Reenter the Growth Phase of the Cardiac Cell Cycle

Gen Suzuki, Vijay Iyer, Thomas Cimato and John M. Canty, Jr

Circ Res. 2009;104:255-264; originally published online December 18, 2008;
doi: 10.1161/CIRCRESAHA.108.188730

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/2/255

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/12/18/CIRCRESAHA.108.188730.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Online Supplement

Pravastatin Improves Function in Hibernating Myocardium by Mobilizing CD133+ and cKit+ Bone Marrow Progenitor Cells and Promoting Myocytes to Reenter the Growth Phase of the Cardiac Cell Cycle

Gen Suzuki
Vijay Iyer
Thomas Cimato
and
John M. Canty, Jr.

From the VA WNY Health Care System and the Departments of Medicine and Physiology&Biophysics and the Center for Research in Cardiovascular Medicine at the University at Buffalo.

Supported by the VA, AHA, Buswell Fellowship NHLBI, Albert and Elizabeth Rekate Fund and the Empire State Stem Cell Board.

Correspondence:

John M. Canty, Jr.
Division of Cardiology
University at Buffalo
Biomedical Research Building, Room 345
3435 Main Street
Buffalo, NY 14214

Phone: 716-829-2663
canty@buffalo.edu

[7]Chronic Ischemic Heart Disease, [130] Animal Model of Human Disease,
Materials and Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

Flow Cytometry and Mobilization of Bone Marrow Progenitor Cells with Pravastatin

We initially assessed the dose-dependency of pravastatin to mobilize bone marrow progenitor cells in normal swine (n=11). Baseline measurements were compared to those after 2-weeks of treatment with low dose (20 mg/day) or high dose (160 mg/day) pravastatin. Mononuclear cells were isolated from peripheral blood (30 ml) and bone marrow (30ml) using the Becton Dickinson CPT cell separation system. White cell and monocyte counts were performed with a hemocytometer. Approximately 10-20x10⁶ mononuclear cells were analyzed by FACS after staining for CD133 (PE conjugated, Miltenyi Biotech) and cKit (FITC conjugated, eBioscience). Isotype controls were used as negative controls. Single stains were performed to determine quality control and multichannel compensation. Data are expressed as progenitor cells (CD133+ or cKit+) per million mononuclear cells. All counts were corrected for the absolute mononuclear cell count.

Effects of Pravastatin (160 mg/day) on Flow and Function in Hibernating Myocardium

Hibernating myocardium was produced as previously described¹. Briefly, pigs were sedated (Telazol; tiletamine 50mg/ml and zolazepam 50 mg/ml)/xylazine (100 mg/ml, 0.022 mg/kg i.m.), intubated and ventilated with a 0.5–2% isoflurane-oxygen mixture. Through a limited pericardiotomy, the proximal LAD was instrumented with a Delrin occluder (1.5 mm i.d.). Antibiotics (cefazolin, 25 mg/kg and gentamicin, 3 mg/kg i.m.) were given 1-hour before surgery and repeated after closing the chest. Following analgesia was performed by using intercostal nerve block (0.5% Marcaine), intramuscular doses of butorphanol (2.2 mg/kg q6h) and flenixin (1-2 mg/kg q.d.).
**Serial Physiological Studies** - Studies began 4-months after instrumentation (n=22) at which time reductions in flow, function and coronary flow reserve indicative of hibernating myocardium were present\(^2\). Sedation was initiated with Telazol/xylazine and maintained with propofol (5-10 mg/kg/hr i.v.). Under sterile conditions, we inserted a 6-Fr introducer into the left brachial artery. A Millar Micro-Tip pressure catheter was inserted into the left ventricle (LV) apex for microsphere injection. The introducer side port was used to monitor aortic pressure and perform blood withdrawal for microspheres. Animals were heparinized (100 U/kg), and hemodynamics allowed to equilibrate for at least 30-minutes. Regional wall-thickening was assessed with transthoracic echocardiography from a right parasternal approach\(^3\). All pigs showed anterior dysfunction but dyskinesis was not present under any condition. Systolic wall-thickening (ΔWT=ESWT-EDWT) was measured in LAD and remote regions. Ventricular dimensions and LV mass were calculated using ASE criteria. This was followed by LV microsphere injection to assess resting perfusion. Finally, pharmacological vasodilation was produced using adenosine (0.9mg/kg/min i.v.) with phenylephrine infused and titrated to maintain mean blood pressure ~100 mmHg and microsphere flow repeated. At the end of the study, catheters were removed and pigs were brought back to the animal facility. Animals were treated with pravastatin (160 mg/day for five weeks, n=12) and compared to untreated controls (n=10). We also studied the effects of pravastatin on normal control animals without a stenosis or LV dysfunction (pravastatin treated n=10, untreated n=5).

Animals were brought back for repeat physiological studies five weeks later after which they were euthanized under anesthesia. The LV was immediately excised, weighed and sectioned into 1-cm rings parallel to the AV groove from apex to base. Thin rings between each major ring were incubated in TTC to quantify infarction. Central areas of the ischemic LAD and remote region were taken for flow and histology as previously demonstrated\(^4\) and outlined below.
**Microsphere Perfusion** - Resting and vasodilated myocardial perfusion were assessed using fluorescent microspheres (15μm) as previously described\(^1\),\(^3\). We injected \(~3\times10^6\) microspheres into the LV over 10 seconds while an arterial reference sample was withdrawn at 6 ml/min (90-seconds). Midventricular rings were divided into twelve circumferential wedges with each cut into three transmural layers\(^5\). Dyes were extracted using standard techniques and fluorescence quantified at selected excitation wavelengths\(^6\). In each animal the circumferential flow distribution during adenosine was analyzed to identify the hibernating risk region\(^5\). From this we eliminated border regions and derived weighted averages from central hypoperfused or normally-perfused remote regions. Relative perfusion was determined by dividing the flow in each individual piece by the corresponding average full-thickness value from normal myocardium.

**Myocardial Histopathology**

**Myocyte Nuclear Density and Morphometry** - Samples adjacent to LAD (hibernating) and posterior descending arteries (normal) were fixed (10% formalin) and paraffin-embedded. Point-counting of trichrome-stained sections was used to quantify connective tissue\(^1\). PAS stained sections were used to quantify myocyte diameter and nuclear length (100 longitudinal myocytes per region) in subendocardial and subepicardial thirds of the left ventricle\(^5\). Myocyte diameter from the pravastatin group was compared to age-matched animals with untreated hibernating myocardium and sham controls. We also assessed regional myocyte nuclear density as previously described\(^7\).

**Quantitative Immunohistochemistry of Progenitor Cells and Myocyte Proliferation** - To identify myocyte proliferation, frozen tissue sections (5μm) were incubated with anti-Ki67 (clone MIB-1, Dako, 1:200) to detect myocytes in the growth phase of the cell cycle. An anti-phospho-Histone H3 antibody (upstate, 1:1000) was used to detect cells in the mitotic phase of the cell cycle\(^7\). Co-staining with anti-cTnI (Santa Cruz, 1:200) was used to confirm these changes were in cardiac myocytes.
Hematopoietic progenitor cells were quantified using the cell surface marker CD133 (immature HSC epitope, Miltenyi Biotec, 1:100)\textsuperscript{8}, cKit (CD117, Santa-Cruz, 1:200) and CD45 (BD biosciences, 1:200). Samples were post-treated with FITC and TRITC conjugated secondary antibody (Zymed). Nuclei were stained with TO-PRO-3 (Invitrogen, 1µM). To quantify the percentage of CD133+/cKit+ cells expressing cardiac lineage markers, we co-stained sections with GATA-4 (Santa Cruz, 1:100) and cTnI (Santa Cruz, 1:100). All of these antibodies have previously been used in swine\textsuperscript{5,8}. Images were acquired with a confocal microscope (Bio-Rad MRC 1024). CD133+/cKit+ cells as well as myocytes in the proliferative phase of the cell cycle were expressed in relation to myocyte nuclear density measurements. We assessed expressed the frequency of Ki67 positive myocyte nuclei per million myocyte nuclei as we have previously escibed.\textsuperscript{5} An average of $745\pm74$ fields ($\sim116\text{ mm}^2$) were examined per slide for each stain.

Statistics

Data are expressed as mean±standard error. Our primary analysis focused upon differences in hemodynamics, flow, function and quantitative immunohistochemical parameters between pravastatin and untreated animals using a two-way ANOVA with post-hoc difference assessed using the Holm-Sidak test (Sigma Stat 3.0). We also assessed serial physiological changes in animals studied at five weeks as compared to baseline measurements using paired t-tests with each animal used as its own control. A similar approach was used to assess differences in perfusion between vasodilation and rest as well as between LAD and remote regions of the same heart. Differences of $p<0.05$ were considered significant.
Results

Serial hemodynamics in each of the treatment groups are presented in Online Table 1. Online Table 2 summarizes serial measurements in myocardial function assessed using echocardiography. Baseline findings at the initial study confirmed dysfunctional hibernating myocardium. Animals in each treatment group had similar resting hemodynamics and systolic function as summarized in the Tables and the following presents the average baseline data for the entire group. Regional systolic LAD wall thickening (ESWT-EDWT) was reduced in comparison to remote normally perfused regions (3.7±0.3 mm vs. 6.6±0.3 mm, p<0.001). There were reductions in resting full-thickness perfusion (LAD 0.72±0.06 vs. 0.94±0.05 ml/min/g in remote, p<0.01) with the greatest reduction in the subendocardium (0.66±0.06 vs. 1.05±0.06 ml/min/g in remote, p<0.001). Full-thickness flow during adenosine was severely attenuated (LAD 1.22±0.22 vs. 4.47±0.25 ml/min/g in remote, p<0.001) and subendocardial flow did not increase above the resting value (adenosine LAD flow 0.68±0.15 ml/min/g, p-ns. vs. rest).
References


Online Table I. Effects of Pravastatin on Hemodynamics.

<table>
<thead>
<tr>
<th></th>
<th>Systolic Pressure (mm Hg)</th>
<th>Mean Aortic Pressure (mm Hg)</th>
<th>Heart Rate (bpm)</th>
<th>LVEDP (mm Hg)</th>
<th>LV dP/dt Max (mm Hg/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REST</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Initial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>120±3</td>
<td>94±4</td>
<td>94±8</td>
<td>24±2</td>
<td>2055±197</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>114±4</td>
<td>83±3</td>
<td>106±4</td>
<td>18±2</td>
<td>2013±103</td>
</tr>
<tr>
<td><strong>Final</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>122±7</td>
<td>96±7</td>
<td>96±7</td>
<td>24±3</td>
<td>2024±111</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>126±5†</td>
<td>93±4†</td>
<td>102±5</td>
<td>16±2*</td>
<td>2538±125*†</td>
</tr>
<tr>
<td><strong>ADENOSINE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Initial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>125±5</td>
<td>95±4</td>
<td>95±4</td>
<td>36±3</td>
<td>2149±278</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>127±4</td>
<td>90±4</td>
<td>102±5</td>
<td>30±5</td>
<td>1945±134</td>
</tr>
<tr>
<td><strong>Final</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>129±7</td>
<td>92±7</td>
<td>92±4</td>
<td>32±2</td>
<td>2022±199</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>128±6</td>
<td>88±4</td>
<td>96±6</td>
<td>24±2*</td>
<td>2281±183</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM; *p<0.05 vs. Untreated, †p<0.05 vs. Initial; LVEDP – Left Ventricular End Diastolic Pressure; LV dP/dt – first derivative of LV pressure.
Online Table II. Effects of Pravastatin on Echocardiographic Measurements of Regional Function.

<table>
<thead>
<tr>
<th></th>
<th>LAD</th>
<th>Remote</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDWT (mm)</td>
<td>ΔWT (mm)</td>
</tr>
<tr>
<td><strong>Initial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>10.4±0.4</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>9.8±0.6</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td><strong>Final</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>10.8±0.6</td>
<td>2.9±0.4</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>11.1±0.7†</td>
<td>5.2±0.5*†</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *p<0.05 vs. Untreated; †p<0.05 vs. Initial; LAD – Left Anterior Descending Artery; LV – Left Ventricular; EDWT – End-Diastolic Wall Thickening; WT – Wall Thickening