Simvastatin Versus Ezetimibe
Pleiotropic and Lipid-Lowering Effects on Endothelial Function in Humans

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Background—Statins may exert important pleiotropic effects, ie, improve endothelial function, independently of their impact on LDL cholesterol. In humans, however, pleiotropic effects of statins have never been unequivocally demonstrated because prolonged statin treatment always results in reduced LDL cholesterol levels. We therefore tested the hypothesis that similar reductions in LDL cholesterol with simvastatin and ezetimibe, a novel cholesterol absorption inhibitor, result in different effects on endothelial function.

Methods and Results—Twenty patients with chronic heart failure were randomized to 4 weeks of simvastatin (10 mg/d) or ezetimibe (10 mg/d) treatment. Flow-dependent dilation (FDD) of the radial artery was determined by high-resolution ultrasound before and after intra-arterial vitamin C to determine the portion of FDD inhibited by radicals (\(\Delta FDD-VC\)). Activity of extracellular superoxide dismutase, a major vascular antioxidant enzyme system, was determined after release from the endothelium by a heparin bolus injection. Endothelial progenitor cells were analyzed with an in vitro assay. Simvastatin and ezetimibe treatment reduced LDL cholesterol to a similar extent (15.6% versus 15.4%; \(P=NS\)), whereas changes in mevalonate, the product of HMG-CoA-reductase, differed between groups (\(\Delta\)mevalonate-simvastatin, \(-1.04\pm0.62\) versus \(\Delta\)mevalonate-ezetimibe, \(1.79\pm0.94\) ng/mL; \(P<0.05\) between groups). Importantly, FDD was markedly improved after simvastatin (10.5±0.6% versus 5.1±0.7%; \(P<0.01\)) but not after ezetimibe treatment (5.6±0.5% versus 5.8±0.6%; \(P=NS\)). \(\Delta FDD-VC\) was substantially reduced after simvastatin but not after ezetimibe treatment. Simvastatin treatment increased the number of functionally active endothelial progenitor cells, whereas ezetimibe had no effect.

Conclusions—Four weeks of simvastatin treatment improves endothelial function independently of LDL cholesterol lowering, at least in part by reducing oxidant stress. Simvastatin may thereby exert important pleiotropic effects in humans. (Circulation. 2005;111:2356-2363.)

Key Words: endothelium • stem cells • heart failure • statins • superoxide dismutase

Statins may exert numerous pleiotropic effects, ie, improve endothelial function, increase vascular nitric oxide (NO) bioavailability, reduce oxidant stress, and improve endothelial progenitor cell (EPC) function, which have largely been proposed on the basis of experimental studies.1–5 In rodents, the improvement in outcome after myocardial infarction or stroke by statins is dependent on endothelial NO synthase but independent of LDL cholesterol because statins have little effect on LDL cholesterol in mice or rats.6–8 It has, however, never been unequivocally demonstrated in humans that prolonged statin treatment exerts effects independently of LDL cholesterol because their application always results in reduced LDL cholesterol levels.

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The observation of pleiotropic statin-induced effects has resulted in the notion that statins are a potential therapeutic option in conditions not related to LDL cholesterol. Of note, in patients with chronic heart failure (CHF), several recent studies have shown that lower serum cholesterol levels are independently associated with an impaired prognosis,9 which raises concerns about whether cholesterol lowering is bene-
ficial in these patients. In contrast, statin treatment has been associated with improved left ventricular (LV) function\textsuperscript{10} and prognosis\textsuperscript{14} in patients with CHF, raising the possibility that statins exert potent beneficial effects in these patients independently of LDL cholesterol.

Interestingly, there is evidence from both experimental and clinical studies that reduced endothelial NO availability may play an important role in the pathophysiology of heart failure. In fact, reduced endothelial NO availability results in impaired myocardial neovascularization and EPC function,\textsuperscript{7,12,13} augmented LV dysfunction and remodeling,\textsuperscript{14,15} and reduced myocardial efficiency.\textsuperscript{16} In mice with heart failure after myocardial infarction, endothelial NO synthase (eNOS) deficiency impairs survival,\textsuperscript{14} but endothelial overexpression of eNOS attenuates LV dysfunction and improves survival in mice after myocardial infarction.\textsuperscript{17} We have recently demonstrated that statin treatment improves LV function and survival in experimental heart failure after myocardial infarction by an eNOS-dependent but LDL cholesterol–independent mechanism.\textsuperscript{7}

In the present study, we tested the hypothesis that similar reductions in LDL cholesterol with simvastatin and ezetimibe, a novel intestinal cholesterol absorption inhibitor, result in different effects on endothelial function, oxidant stress, and EPCs in patients with CHF.

**Methods**

Twenty patients with New York Heart Association functional class III CHF were studied. Characteristics of patients are shown in the Table. Patients with diabetes mellitus, current tobacco use, significant valvular heart disease, heparin therapy within the last 24 hours, or any condition that would preclude the safe withholding of vasoactive medication were excluded from the study.

**Protocol**

Patients were randomized to 4 weeks of treatment with simvastatin (10 mg/d) or ezetimibe (10 mg/d) to have a similar effect on LDL cholesterol levels. Measurements of flow-dependent, endothelium-mediated vasodilatation (flow-dependent dilation [FDD], before and after vitamin C) and blood sampling for determination of endothelium-bound extracellular superoxide dismutase (ecSOD) and cultivation of EPCs were performed the same day before and after 4 weeks of therapy. Before measurements of FDD, vasoactive medications were withheld and alcohol and caffeine were prohibited for ≥12 hours. Blood samples were taken to determine endothelium-bound ecSOD activity and cultivation of EPCs as described in detail below. Then, FDD of the radial artery was determined, the antioxidant vitamin C was infused intra-arterially, and FDD was measured again to determine the portion of FDD inhibited by oxygen free radicals. The local ethics committee approved the protocol.

**Measurement of FDD**

Radial artery diameters were measured with a high-resolution ultrasound system (ASULAB). This method is well established in our laboratory, has an excellent reproducibility and variability, and was used as described in detail previously.\textsuperscript{18–20}

Blood flow velocity was recorded continuously; radial artery diameter was determined every 30 seconds until stable baseline conditions were obtained (~30 minutes). Then, a wrist arterial occlusion (8 minutes) was performed, and FDD in response to reactive hyperemic blood flow was assessed. When radial artery diameter and blood flow had returned to baseline values, the antioxidant vitamin C was infused (25 mg/min for 10 minutes in the brachial artery), followed by determination of FDD.

**Measurement of Endothelium-Bound ecSOD Activity In Vivo**

ecSOD is rapidly released from endothelium into plasma by heparin bolus injection, allowing determination of endothelium-bound ecSOD activity in humans in vivo.\textsuperscript{19–21} Plasma Cu, Zn, and Mn-SOD are not affected by heparin.\textsuperscript{19,21} For measurement of endothelium-bound ecSOD, 2 venous blood samples (antecubital vein) were obtained at baseline. Heparin (5000 U) was then injected into the brachial artery of the same (nondominant) arm, and blood samples were drawn in intervals from the antecubital vein (1, 3, 5, 7, and 10 minutes after heparin injection) as described in detail previously.\textsuperscript{19,20} Tubes were immediately centrifuged (2000g for 15 minutes at 4°C), and plasma was stored at −80°C.

Activity of SOD in plasma was measured at pH 8.2 by a modified nitrite method.\textsuperscript{22} Superoxide generated by hypoxanthine and xanthine oxidase was changed to nitrite ion by hydroxylamine. Nitrite ion was measured by color densitometry at 550 nm with a coloring reagent. The amount of SOD required to inhibit the rate of nitrite ion generation by 50% was defined as 1 U of SOD activity, according to McCord and Fridovich.\textsuperscript{23} Calibrations were performed with known amounts of purified bovine SOD. Endothelium-bound ecSOD activity was calculated as area under the curve of the increase in plasma SOD activity after heparin bolus injection as described previously.\textsuperscript{19,20} Reagents were from Sigma-Aldrich.

**Isolation and Cultivation of EPCs**

Isolation and characterization of EPCs were performed as described previously.\textsuperscript{24} In brief, peripheral blood mononuclear cells were isolated from 14 mL of the patient’s blood using density gradient centrifugation with Bicoll (Biochrome) and seeded 10\textsuperscript{3} cells on 6-well plates coated with human fibronectin (Sigma) in endothelial basal medium-2 (Clonetics). The medium was supplemented with endothelial growth medium-2 (Single Quots, Clonetics) containing FBS, human vascular endothelial growth factor-A, human fibroblast growth factor-B, human epidermal growth factor, insulinlike growth factor-1, and ascorbic acid in appropriate amounts. After 4 days of culture, nonadherent cells were removed by washing the plates with PBS. The remaining adherent cells were trypsinized and reseeded 10\textsuperscript{3} cells on fibronectin-coated 6-well plates. New media were applied, and the cell culture was maintained through day 7.

**Characterization of EPCs**

Fluorescent chemical detection was performed to determine the cell type of attached human peripheral blood mononuclear cells after 7 days in culture. To detect the uptake of 1,1′-dioctadecyl-3,3′,3′,tetramethylindocarbocyanine–labeled acetylated LDL (acLDL-Dil) (Molecular Probes), cells were incubated with acLDL-Dil (6 μg/mL) at 37°C for 2 hours. Cells were then fixed with 1% paraformaldehyde for 10 minutes and incubated with FITC-labeled Ulex europaeeus agglutinin-1 (UEA-1; Sigma) for 1 hour. After staining, samples were viewed and evaluated by light and fluorescence microscopy.
were viewed with an inverted fluorescent microscope (Leica). Cells double stained for both UEA-1 and acLDL-DiI were counted as EPCs. Two investigators in blinded experiments counted ≥4 randomly selected high-power fields.

**Measurement of Mevalonate**

Mevalonate levels were determined in plasma samples by a novel gas chromatogram–mass spectrometry analysis with d₄-mevalonic acid lactone as the internal standard. In brief, mevalonic acid was converted to the corresponding lactone at acidic pH. Aqueous samples were absorbed by anhydrous sodium sulfate, and lactone was eluted in ethyl acetate. After evaporation and redissolution in ethyl ether, samples were purified by silica gel chromatography and eluted with dichloromethane:methanol (95:5 vol/vol). After evaporation of solvent, derivatized by MSTFA (containing 1% TMCS):pyridine (2:1 vol/vol). This derivatization produced a tri-(trimethylsilyl)-mevalonic acid molecule, which could be analyzed by electron ionization gas chromatogram–mass spectrometry in single-ion-monitoring mode. The method was validated in a range from 0.5 to 128 ng mevalonic acid lactone per 1 mL for plasma. Interday and intraday accuracy precision analysis of QC samples was below ±15% at each concentration level. Samples stored below −20°C were stable up to 12 weeks. No degradation occurred during freeze-thaw testing.

**Statistical Analysis**

All data are expressed as mean±SEM. Comparisons of >2 measurements were done by 1-way ANOVA for repeated measures, followed by the Student-Newman-Keuls test (comparisons within one group of patients and between the different groups of patients). The data of functionally active EPCs were analyzed by use of the Wilcoxon test to compare EPC numbers before and after treatment with simvastatin and ezetimibe and to compare changes in EPC numbers in response to both treatments. The comparison of Δmevalonate, ΔFDD, ΔFDD-vitamin C, and ΔeSOD between groups was performed with the Student t test. A value of P<0.05 was considered statistically significant.

### Results

**Patient Characteristics**

There were no significant differences in patient characteristics at baseline between patients randomized to simvastatin or ezetimibe therapy as shown in the Table.

**Change in LDL Cholesterol and Mevalonate Levels After Simvastatin Versus Ezetimibe Treatment**

LDL cholesterol levels were reduced to a similar extent after 4 weeks of treatment with simvastatin and ezetimibe (15.6±5.2% versus 15.4±5.3%; P=NS; Figure 1). In contrast, the changes in plasma levels of mevalonate, the product of HMG-CoA-reductase, after 4 weeks of treatment differed significantly between groups (Δmevalonate after simvastatin, −1.04±0.62 ng/mL; Δmevalonate after ezetimibe, 1.79±0.94 ng/mL; P<0.05 between groups).

**Effect of Simvastatin Versus Ezetimibe Treatment on FDD**

In contrast to similar changes of LDL cholesterol levels, there was a marked difference in the effect of both treatments on FDD, defined as percent increase in vessel diameter after wrist occlusion. FDD was substantially improved after 4 weeks of simvastatin treatment (Figure 2A), whereas no effect of ezetimibe treatment on FDD was observed (Figure 2B). Furthermore, there was a significant difference in ΔFDD after both treatments as shown in Figure 2C. Forearm blood flow at rest (simvastatin versus ezetimibe group, 30±4 versus 34±5 mL/min) and at maximal reactive hyperemia (simvastatin
versus ezetimibe group, 100±27 versus 85±19 mL/min) was similar in patients with CHF after 4 weeks of simvastatin or ezetimibe treatment. Systemic blood pressure and heart rate did not change during the experimental protocol (data not shown).

**Effect of Simvastatin Versus Ezetimibe Treatment on ΔFDD–Vitamin C**

A significant improvement in FDD was seen after intra-arterial infusion of the antioxidant vitamin C in both groups of patients with CHF before treatment (Figure 3). After 4 weeks of simvastatin treatment, there was a marked reduction in the effect of vitamin C on FDD (Figure 3A), whereas no change in the response of FDD to vitamin C was observed after treatment with ezetimibe (Figure 3B). Furthermore, there was a significant difference in the change of the vitamin C effect on FDD after both treatments, as shown in Figure 3C.

**Effect of Simvastatin Versus Ezetimibe Treatment on ecSOD Activity**

Endothelium-bound ecSOD activity (released after heparin bolus injection) was markedly increased after 4 weeks of simvastatin treatment (Figure 4A); however, there was no effect of ezetimibe treatment on ecSOD (Figure 4B). Furthermore, there was a significant difference in the change of endothelium-bound ecSOD activity after both treatments, as shown in Figure 4C.

**Effect of Simvastatin Versus Ezetimibe Treatment on EPCs**

To evaluate the effect of simvastatin versus ezetimibe treatment on EPC number and function, we isolated mononuclear cells from the blood of each patient before and after 4 weeks of statin or ezetimibe treatment. After culturing the cells for 7 days, we identified adherent EPCs by acLDL-DiI uptake and concomitant UEA-1 binding. The absolute number of functionally active EPCs before the start of simvastatin or ezetimibe treatment ranged from 115 to 475 double-positive cells per high-power field and was similar in the statin and ezetimibe group before treatment (252±49 versus 244±38; P=NS). There was a marked increase in the number of functionally active EPCs after 4 weeks of simvastatin treatment (446±79 versus 252±49; P<0.05; Figure 5A), whereas no change was observed after ezetimibe treatment (242±36 versus 244±38; P=NS; Figure 5B). Furthermore, there was a significant difference in the change of the number of functionally active EPCs after both treatments as shown in Figure 5C.

**Discussion**

The present study produced 4 major findings. First, 4 weeks of therapy with simvastatin, an HMG-CoA-reductase inhibitor, but not with ezetimibe, a novel cholesterol absorption inhibitor, improved endothelium-dependent vasodilation in...
patients with CHF despite a similar change in LDL cholesterol, suggesting that prolonged statin treatment improves endothelial function by LDL cholesterol–independent mechanisms in humans. Second, the antioxidant vitamin C improved endothelial function at baseline, an effect that was substantially reduced after simvastatin but not ezetimibe treatment, suggesting that reduced vascular oxidant stress contributes to the beneficial effect of statin treatment on endothelial function. Third, simvastatin treatment increased the activity of endothelium-bound ecSOD, a major vascular antioxidant enzyme system, by >100%, whereas no effect of ezetimibe was observed, suggesting that improved ecSOD activity contributes to the antioxidant effects of statin treatment. Finally, simvastatin treatment increased the number of functionally active EPCs, an effect that was not observed after ezetimibe therapy, suggesting another potentially important LDL cholesterol–independent effect of chronic statin treatment in patients with CHF.

Numerous pleiotropic, LDL cholesterol–independent effects of statins have been proposed largely on the basis of experimental studies. In particular, it has been demonstrated by several in vitro studies that statins enhance endothelial NO bioavailability by both promoting endothelial NO production25–28 and preventing NO inactivation by radicals.3 Furthermore, in rodents, statins improve outcome after myocardial infarction or stroke by LDL cholesterol–independent but eNOS-dependent mechanisms.6–8

The present study has addressed the question of whether prolonged statin treatment exerts effects independent of LDL cholesterol in humans by comparing the effect of simvastatin treatment with ezetimibe. Importantly, there was a marked difference with respect to the effect of both treatments on endothelial function despite similar changes in LDL cholesterol levels. Of note, most pleiotropic effects of HMG-CoA reductase inhibitors are thought to be related to inhibition of mevalonate-dependent isoprenylation of small GTP-binding proteins, ie, Rho, Ras, and Rac.1 In fact, the effects of HMG-CoA-reductase inhibition on endothelial NO synthase and Rac-1–dependent antioxidant properties are reversed after the addition of mevalonate.3,25 The different changes in mevalonate levels observed in the present study after simvastatin and ezetimibe treatment are consistent with the notion that differences in the mevalonate pathway may contribute to different vascular effects of both treatment strategies.

In the present study, ezetimibe therapy did not improve endothelium-dependent vasodilation despite a reduction in LDL cholesterol serum levels. Of note, the impairment of endothelium-dependent vasomotion and vascular oxidant stress in animals on a high-cholesterol diet is reversible by dietary correction of hypercholesterolemia.29 In addition, in...
patients with hypercholesterolemia, acute LDL cholesterol reduction by >60% with apheresis and chronic cholesterol-lowering treatment with diet and cholestyramine resulting in >30% LDL cholesterol reduction have been shown to improve endothelium-dependent vasodilation, suggesting that increased LDL cholesterol levels contribute to endothelial dysfunction in hypercholesterolemia.

Notably, the patients included in the present study had no hypercholesterolemia. Furthermore, the change in LDL cholesterol levels after 4 weeks of ezetimibe treatment was moderate (15% reduction in LDL cholesterol). Therefore, both the absence of hypercholesterolemia and the moderate change in LDL cholesterol levels may explain, at least in part, the lack of effect of ezetimibe therapy on endothelium-dependent vasomotion in the present study. In addition, we cannot exclude the possibility that some effect of ezetimibe on endothelium-dependent vasomotion may have been found if we had analyzed more patients. Importantly, however, the present study suggests a marked difference in the effect of chronic simvastatin and ezetimibe treatment on endothelial function in patients with CHF.

Therefore, we further analyzed mechanisms through which statin treatment may improve endothelial function independently of LDL cholesterol in patients with CHF. Of note, the impairment of endothelium-dependent vasodilatation in experimental and clinical heart failure is reversible by radical scavengers. In the present study, there was a markedly reduced effect of the antioxidant vitamin C on endothelium-dependent vasodilation after simvastatin treatment, compatible with the concept that simvastatin treatment reduced vascular oxidant stress in patients with CHF.

Furthermore, the activity of endothelium-bound ecSOD, a major vascular antioxidant enzyme system, was markedly increased after statin treatment, which may have contributed to reduced vascular oxidant stress after statin treatment in patients with CHF. Although the association of increased ecSOD activity and improved endothelium-dependent vasodilation does not prove a cause-and-effect relationship, there is recent evidence to suggest that ecSOD is critical for the vascular bioavailability of NO. We have recently observed that reduced ecSOD activity in patients with CHF is closely related to impaired endothelium-dependent vasodilation and vascular oxidant stress. Moreover, in mice lacking ecSOD, endothelium-dependent vasodilatation is substantially impaired as a consequence of increased vascular oxidant stress. In addition, ecSOD activity is profoundly regulated by NO, suggesting that it represents an important feed-forward mechanism to enhance the bioactivity of NO. Furthermore, recent in vitro studies suggest that statins increase thioredoxin activity by an NO-dependent pathway, which may represent another mechanism through which improved NO availability promotes endogenous vascular antioxidant defense systems. Statin treatment has been shown to inhibit the activation of the oxidant enzyme system NAD(P)H oxidase, likely by preventing the membrane translocation of the small G protein rac-1, which may contribute to reduced vascular oxidant stress after statin treatment.

Of note, several recent studies in patients with CHF have suggested that reduced serum cholesterol levels are independently associated with an impaired prognosis, raising concerns as to whether cholesterol lowering is beneficial in these patients. In contrast, however, statin treatment has been associated with improved outcome in patients with severe CHF. Thus, it is conceivable that the beneficial effects of statins in CHF are unrelated to cholesterol levels. The findings of the present study suggest a potential mechanism by which statins may exert beneficial effects independently of LDL cholesterol in patients with CHF. There is increasing evidence from both experimental and clinical studies that reduced endothelial NO availability may importantly contribute to the pathophysiology of heart failure. In fact, reduced endothelial NO availability results in impaired myocardial neovascularization and EPC function, augmented LV dysfunction and remodeling, and reduced myocardial efficiency. In mice with experimental heart failure after myocardial infarction, eNOS deficiency impairs survival, and endothelial overexpression of the eNOS attenuates LV dysfunction and improves survival. We have recently demonstrated that statin treatment improves LV function and survival in experimental heart failure after myocardial infarction by an eNOS-dependent mechanism.

Several recent studies have shown that the degree of endothelial dysfunction is a strong and independent predictor of cardiovascular events. It is therefore conceivable that improved endothelial function may have contributed to the beneficial effects of simvastatin observed in the 4S and HPS studies.

In the present study, we have observed a marked increase in functionally active EPCs in patients with CHF after 4 weeks of statin treatment, whereas ezetimibe therapy had no effect on EPCs. Several recent studies have shown that EPCs have the potential to increase endothelial regeneration and improve myocardial neovascularization and function. We and others have previously observed that ENOS-derived NO plays an essential role in the mobilization and function of EPCs. Furthermore, a close relation between endothelial NO-mediated vasodilation and the functional capacity of EPCs has recently been observed in humans. The finding of the present study—that simvastatin but not ezetimibe treatment increased the number of functionally active EPCs—may further support the concept that chronic statin treatment exerts LDL cholesterol–independent effects on vascular function in patients with CHF.

Study Limitations
The present study suggests that chronic simvastatin therapy exerts a beneficial, LDL cholesterol–independent effect on endothelial function in humans. It cannot be concluded that this represents a class effect of statins. However, a rapid effect on endothelium-dependent vasodilation within 1 or 3 days of treatment has been shown for simvastatin, pravastatin, and cerivastatin, consistent with the concept that other statins also may exert pleiotropic effects in humans.

The present study has compared the effect of simvastatin and ezetimibe therapy on endothelial function in patients with chronic heart failure. Therefore, the present findings are not necessarily applicable to other patient populations. As discussed, statin treatment today is considered a potential
therapeutic option in patients with CHF independent of LDL cholesterol levels, and identifying mechanisms by which statins may exert cholesterol-independent, beneficial effects in this patient population is of particular interest.

In summary, the present study provides evidence that prolonged simvastatin treatment exerts important pleiotropic effects that may mediate beneficial, cholesterol-independent effects in patients with CHF. In more general terms, the beneficial effect of simvastatin as observed in large clinical trials (4S, HPS) may be related in part to cholesterol-independent effects, suggesting that how cholesterol is reduced may be important.

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