Statin-Induced Improvement of Endothelial Progenitor Cell Mobilization, Myocardial Neovascularization, Left Ventricular Function, and Survival After Experimental Myocardial Infarction Requires Endothelial Nitric Oxide Synthase

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Background—Endothelial nitric oxide (eNO) bioavailability is severely reduced after myocardial infarction (MI) and in heart failure. Statins enhance eNO availability by both increasing eNO production and reducing NO inactivation. We therefore studied the effect of statin treatment on eNO availability after MI and tested its role for endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular (LV) dysfunction, remodeling, and survival after MI.

Methods and Results—Wild-type (WT) and eNO synthase (eNOS)−/− mice with extensive anterior MI were randomized to treatment with vehicle (V) or atorvastatin (Ator, 50 mg/kg QD by gavage) for 4 weeks starting on day 1 after MI. Ator markedly improved endothelium-dependent, NO-mediated vasorelaxation; mobilization of endothelial progenitor cells; and myocardial neovascularization of the infarct border in WT mice after MI while having no effect in eNOS−/− mice. LV dysfunction and interstitial fibrosis were markedly attenuated by Ator in WT mice, whereas no effect was observed in eNOS−/− mice after MI. Importantly, Ator significantly increased the survival rate during 4 weeks after MI in WT mice (Ator versus V, 80% versus 46%; P<0.01, n=75) but not in eNOS−/− mice (43% versus 48%; NS, n=42).

Conclusions—These findings suggest that increased eNO availability is required for statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, LV dysfunction, interstitial fibrosis, and survival after MI. eNO bioavailability after MI likely represents an important therapeutic target in heart failure after MI and mediates beneficial effects of statin treatment after MI. (Circulation. 2004;110:1933-1939.)

Key Words: endothelium • nitric oxide synthase • statins • myocardial infarction • heart failure

Statins have been suggested to exert beneficial effects on left ventricular (LV) function and remodeling after experimental myocardial infarction (MI),1,2 raising the possibility that statins may aid in the prevention and treatment of heart failure.3 In patients with heart failure, statin therapy is associated with improved LV function4 and survival.5 The underlying mechanisms, however, of how statins may exert these beneficial effects remain to be determined.

Statins enhance endothelial nitric oxide (eNO) bioavailability by both promoting eNO production6–9 and preventing NO inactivation by radicals.10 Interestingly, there is evidence that reduced eNO availability may play an important role in the pathophysiology of heart failure both after experimental MI11–13 and in patients with heart failure.14–17 Inhibition of eNO production results in impaired endothelium-dependent vasodilation, reduced myocardial neovascularization,18 and impaired vascular endothelial growth factor–induced mobilization of endothelial progenitor cells (EPCs).19 Furthermore, eNO synthase (eNOS)–deficient mice develop more severe LV dysfunction and remodeling after MI than do wild-type (WT) mice,20 and vice versa, endothelial overexpression of eNOS has been shown to attenuate LV dysfunction in mice after MI.21

We therefore hypothesized that statin treatment may profoundly increase eNO availability after MI and thereby exert beneficial effects on myocardial neovascularization, mobilization of EPCs, LV dysfunction, remodeling, and survival after MI.

Methods

Animals, MI, and Experimental Protocol
Male C57BL/6 mice and eNOS-deficient (eNOS−/−) mice, aged 14 to 16 weeks, were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were housed under standard laboratory conditions with free access to food and water. All experiments were performed in accordance with guidelines from the local ethics committee (Hannover, Germany) and the American Heart Association.
Me). MI was induced by permanent ligation of the left anterior descending coronary artery as described previously. On day 1 after MI, WT and eNOS−/− mice were randomized to treatment with atorvastatin (Ator, 50 mg/kg QD) or vehicle (V) by gavage for 4 weeks (randomization, 1:2 and 1:1, respectively). This dose of Ator in mice has been shown to correspond to a daily dose of 80 mg Ator in humans (with respect to plasma concentrations of the drug24). Only mice with extensive MI (>30%) were included in the protocol. Ten sham-operated animals served as controls. The local committee on animal research approved all animal experiments.

Studies of Endothelium-Dependent, NO-Mediated Vasorelaxation
Endothelium-dependent, NO-mediated vasorelaxation in response to acetylcholine and endothelium-independent relaxation in response to nitroglycerin were studied in ring segments of thoracic aortas as described previously.25,26 Notably, it has been shown that acetylcholine responses are lacking in eNOS-deficient mice,26,27 which indicates that these responses are a good measure of eN0 bioavailability.

Echocardiographic Measurements
ECG analysis was performed under light anesthesia (ketamine 100 mg/kg, xylazine 1.25 mg/kg, and atropine 0.6 mg/kg IP) and spontaneous respiration with a commercially available ultrasonic system (ATL5000 CV) with a linear 15-MHz high-frequency transducer as described previously. The investigator (A.S.) was blinded to the experimental group.

Histomorphometric Analysis
After fixation, LV tissue slices were embedded in paraffin, cut into 6-μm sections, and stained with collagen-specific Sirius red F3BA as described previously.21 Intersitial collagen volume fraction was quantified by polarized light microscopy of picro Sirius red stained slides and collagen was hydrolyzed with 6N hydrochloric acid (130 °C, 1 hour). In brief, samples of remote LV myocardium were dried and weighed, triturations in remote myocardium, performed as described previously.32 Hydroxyproline concentrations in remote myocardium, performed as described previously.32 Histomorphometry was performed in a blinded fashion with the Quantimet 500MC digital image analyzer. Mean cardiomyocyte cross-sectional area and infarct size were determined in hematoxylin- and eosin-stained sections with a digital image analyzer as described previously.29

Analysis of Capillary Density
For immunohistochemistry analysis of capillary density, frozen mid-LV specimens were obtained and multiple 5-μm sections (≥4) were cut with a cryostat. Immunohistochemical staining of endothelial cells was obtained through 4 representative border-zone high-powered fields (HPFs) by a single investigator blinded to the treatment group. Results are reported as number of endothelial cells per HPF±SEM. In addition, specimens were counterstained with hematoxylin, and endothelial cells of the infarct border zone were related to the total number of nuclei.

EPC Culture Assay
For EPC culture, mononuclear cells were isolated from 1 mL of peripheral blood by density-gradient centrifugation with Histopaque (Sigma),30 seeded on tissue-culture coverslips (9×104 cells) coated with rat vitronectin and 0.5% gelatin (Sigma) in endothelial basal medium (endothelial growth medium-2 MV, Clonetics), and supplemented with EGM-2 MV Single quots (containing fetal bovine serum, human vascular endothelial growth factor-A, human fibroblast growth factor-B, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid in appropriate amounts). After 4 days in culture, nonadherent cells were removed by washing with phosphate-buffered saline. The cell culture was maintained through day 7, and fluorescence chemical detection was performed. To detect the uptake of 1,1′dioctadecyl-3,3′,3′,tetramethyldilocabocya-

Figure 1. A, Endothelium-dependent, NO-mediated vasodilation in response to acetylcholine (Ach) in sham-operated, MI, and MI+Ator WT mice 4 weeks after surgery. B, Endothelium-independent vasorelaxation in response to nitroglycerin (NTG). n=6 to 8; *P<0.05 vs sham, #P<0.05 vs MI. All other abbreviations are as defined in text.

were incubated with acLDL-DiI (6 μg/mL, 37°C, 2 hours). Cells were then fixed with 1% paraformaldehyde for 10 minutes and incubated with fluorescein-labeled Griffonia simplicifolia lectin I (BS-1 lectin, Vector Labs) for 1 hour. After being stained, samples were analyzed with an inverted fluorescence microscope (Leica), and double-stained cells for both BS-1 lectin and acLDL-DiI were counted as EPCs in at least 4 randomly selected HPFs.31

Fluorescence-Activated Cell Sorting Analysis
A volume of 200 μL EDTA-blood was incubated for 15 minutes with a monoclonal antibody against kinase insert domain receptor (Sigma) and the fluorescein isothiocyanate-labeled monoclonal antibody against Sca-1 (Becton Dickinson). Isotype-identical antibodies were used as controls (IgG1-phycoerythrin and IgG2a–fluorescein isothiocyanate, Becton Dickinson). Two blinded observers assessed the number of EPCs.

Hydroxyproline Measurements
In addition to measurements of collagen volume fraction, myocardial fibrosis was assessed by determination of hydroxyproline concentrations in remote myocardium, performed as described previously.32 In brief, samples of remote LV myocardium were dried and weighed, and collagen was hydrolyzed with 6N hydrochloric acid (130°C, 3 hours). Collagen concentration was estimated by a colorimetric method as described by Woessner.32

Blood Pressure and Heart Rate
Systolic blood pressure and heart rate were measured by a computerized tail-cuff system (Visitech Systems) as described previously.25

Statistical Analysis
All data are expressed as mean±SEM. To compare the data between groups, ANOVA was used. Comparison of survival was performed with Kaplan-Meier analysis. A value of P<0.05 was considered statistically significant.

Results
Effect of Ator Treatment on Endothelium-Dependent, NO-Mediated Vasodilation in Mice After MI
In sham-operated mice, acetylcholine produced endothelium-dependent relaxations of 83±3% (Figure 1). These responses were substantially impaired in aortas of WT mice 4 weeks after MI (24±5%, P<0.01, Figure 1). Ator treatment mark-
edly improved endothelium-dependent vasodilation in response to acetylcholine in mice after MI (77% ± 6%, *P* < 0.05; Figure 1). In contrast, endothelium-independent vasodilation in response to nitroglycerin was not impaired in mice after MI and was not changed by Ator treatment (Figure 1). In eNOS−/− mice, no endothelium-dependent vasodilation in response to acetylcholine was observed, indicating that these responses are dependent on NO.

### Role of eNOS for Ator-Induced Effects on LV Function After MI

LV fractional shortening and LV ejection fraction were reduced to a similar extent in WT and eNOS−/− mice 4 weeks after MI compared with sham-operated animals (Figure 2, Table). Notably, Ator treatment significantly improved LV fractional shortening and LV ejection fraction in WT mice after MI, whereas no effect was observed in eNOS−/− mice (Figure 2, Table).

### Role of eNOS for Ator-Induced Effects on Survival After MI

Importantly, the survival rate during 4 weeks after MI was significantly (*P* < 0.01) higher in Ator-treated WT mice than in V-treated mice (80% versus 46%, Figure 3A). In contrast, Ator treatment had no effect on survival after MI in eNOS−/− mice (43% versus 48%, NS; Figure 3B).

### Role of eNOS for Ator-Induced Effects on Myocardial Fibrosis and Cardiomyocyte Hypertrophy

Recent studies have suggested that reduced eNO availability may promote cardiac fibrosis, and NO inhibits extracellular matrix production by cardiac fibroblasts. We therefore determined the effect of Ator treatment on myocardial fibrosis after MI in WT and eNOS−/− mice by using 2 approaches, ie, measurements of LV collagen volume fraction and hydroxyproline content. Both approaches revealed a similar

### Echocardiography, Histomorphometric Analysis, Blood Pressure, and Plasma Biochemical Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type (Sham)</th>
<th>Wild Type (MI)</th>
<th>Wild Type (MI + Ator)</th>
<th>eNOS−/− (Sham)</th>
<th>eNOS−/− (MI)</th>
<th>eNOS−/− (MI + Ator)</th>
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<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
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<tr>
<td>LV fractional shortening (%)</td>
<td>37.1 ± 1.1</td>
<td>39.3 ± 1.3</td>
<td>43 ± 2.1</td>
<td>39.3 ± 1.3</td>
<td>10.1 ± 1.9</td>
<td>11.8 ± 1.9</td>
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<tr>
<td>LV end-diastolic diameter (mm)</td>
<td>3.7 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>5.9 ± 0.2</td>
<td>5.3 ± 0.2</td>
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<td><strong>Histomorphometric analysis</strong></td>
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<tr>
<td>Mean infarct size, %</td>
<td>N/A</td>
<td>44 ± 2</td>
<td>46 ± 2</td>
<td>N/A</td>
<td>47 ± 3</td>
<td>46 ± 2</td>
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<tr>
<td>Myocyte CSA, μm²</td>
<td>259 ± 12</td>
<td>336 ± 25</td>
<td>36 ± 0.1</td>
<td>582 ± 16*</td>
<td>509 ± 32‡</td>
<td>46 ± 0.1</td>
</tr>
<tr>
<td>LV wt/body weight, mg/g</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>4.9 ± 0.3*</td>
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<td><strong>Blood pressure and heart rate</strong></td>
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<td>SBP, mm Hg</td>
<td>108 ± 2</td>
<td>134 ± 3</td>
<td>134 ± 3</td>
<td>131 ± 7</td>
<td>123 ± 4</td>
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<tr>
<td>Heart rate, bpm</td>
<td>608 ± 19</td>
<td>524 ± 13</td>
<td>524 ± 13</td>
<td>562 ± 28</td>
<td>563 ± 19</td>
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<td><strong>Plasma biochemical parameters</strong></td>
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<tr>
<td>Total cholesterol, mmol/dL</td>
<td>3.8 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>3.1 ± 0.3</td>
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<tr>
<td>Creatinine, mg/dL</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.2</td>
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CSA indicates cross-sectional area; SBP, systolic blood pressure. All other abbreviations are as defined in text.

*P* < 0.01 vs sham; †*P* < 0.01 vs MI; ‡*P* < 0.05 vs MI.
increase in myocardial fibrosis after MI compared with sham-operated mice in WT and eNOS−/− groups (Figure 4). With respect to the response to Ator treatment, however, there was a marked difference. Ator treatment substantially reduced myocardial fibrosis in WT mice after MI while having no effect on myocardial fibrosis in eNOS−/− mice (Figure 4).

MI caused a similar increase in cardiomyocyte hypertrophy in WT and eNOS−/− mice compared with sham-operated mice (the Table). Treatment with Ator reduced cardiomyocyte hypertrophy after MI to a greater extent in WT mice compared with eNOS−/− mice (P<0.05, Table).

Role of eNOS for Ischemia and Ator-Induced Mobilization of EPCs
Recent studies have suggested that inhibition of endothelial NO production impairs myocardial neovascularization, and statins have the potential to exert proangiogenic effects. We therefore studied the effect of Ator treatment on capillary density in the infarct border zone in WT and eNOS−/− mice after MI that was assessed in at least 4 representative border-zone HPFs. As shown in Figure 5, Ator treatment resulted in a substantial increase in capillary density in the infarct border zone in WT mice after MI. In contrast, no effect on capillary density was observed in eNOS−/− mice after Ator treatment (Figure 5). These results were confirmed when specimens were counterstained with hematoxylin, and endothelial cells in the infarct border zone were related to the total number of nuclei (data not shown).

Role of eNOS for Ischemia and Ator-Induced Mobilization of EPCs
Increasing evidence suggests that neovascularization critically involves mobilization of EPCs in addition to the local proliferation and growth of endothelial cells. Statin treatment has been shown to augment mobilization of EPCs; however, it is not known whether this effect can be seen in the presence of an ischemic stimulus. In WT mice, circulating EPCs were significantly elevated 4 weeks after MI (by >100%), but EPC mobilization was markedly further augmented by Ator treatment (by >200%, Figure 6A and 6B). In contrast, in eNOS−/− mice, neither ischemia nor statin treatment increased circulating EPCs, indicating that these responses are NO dependent (Figure 6A and 6B). These data were confirmed by directly measuring the number of circulating Sca-1+/KDR+ cells by fluorescence-activated cell sorting analysis (Figure 6C).

Infarct Size and Plasma Biochemical Parameters
Mean infarct sizes did not differ between groups (the Table). After 4 weeks, total cholesterol was not lowered by Ator treatment in WT and eNOS−/− mice (Table), indicating that the observed effects of Ator were cholesterol independent.

Discussion
The present study demonstrates that Ator therapy markedly improves eN0 availability after MI. Increased eN0 availability was required for Ator-induced improvement of LV function and survival after MI, because these effects were observed in WT only and not in eNOS−/− mice. Furthermore, Ator markedly improved myocardial neovascularization at the infarct border and substantially enhanced mobilization of EPCs in WT but not in eNOS−/− mice, suggesting a critical role for restored eN0 production in statin-induced myocardial neovascularization and EPC mobilization. In addition, Ator reduced myocardial interstitial fibrosis and cardiomyocyte hypertrophy after MI to a greater extent in WT mice than in eNOS−/− mice, suggesting that statin-induced eN0 production limits interstitial fibrosis and cardiomyocyte hypertrophy. These findings are consistent with the notion that preservation of eN0 bioavailability is a principal and important mechanism for the beneficial effects of statin treatment after MI.
with heart failure. In addition to the impairment of endothelium-dependent vasodilation, several mechanisms have now been identified whereby reduced eNO bioavailability may contribute to the pathophysiology of heart failure. In particular, inhibition of eNO production reduces myocardial neovascularization, augments myocardial remodeling, and impairs myocardial perfusion and efficiency.

In the present study, we have evaluated the effect of statin treatment and the role of eNOS for myocardial neovascularization after MI. We observed a markedly enhanced neovascularization at the infarct border after statin treatment in WT mice; however, this response was completely abolished in eNOS / mice. Several mechanisms may contribute to improved myocardial neovascularization after restoration of eNO availability. In particular, in the present study we observed a marked enhancement of EPC mobilization after statin treatment in WT mice after MI that was lacking in eNOS / mice. The observed association of improved myocardial neovascularization and mobilization of EPCs does not prove a cause-and-effect relation; however, there is recent evidence to support the concept that mobilization of EPCs is involved in neovascularization. Asahara et al have shown that bone marrow–derived EPCs incorporate into foci of neovascularization at the border zone of MI and that administration of ex vivo expanded EPCs could increase myocardial neovascularization at the infarct border and improve LV function. eNOS-derived NO production has been shown to be critically important for the functional capacity of mobilized EPCs that could partly explain why basal mobilization of EPCs in our V-treated, WT mice with severely reduced eNO availability was insufficient. In addition, statin treatment increases the expression of adhesion molecules on mobilized EPCs and reduces senescence of EPCs that may importantly impact on their functional capacity at sites of ischemia, an effect that would be lacking in V-treated mice after MI. The relative contributions of EPC mobilization to ischemia and statin-induced effects on myocardial neovascularization, LV function, and survival after MI need to be addressed by future studies. Other potential mechanisms whereby restored eNO production may improve myocardial neovascularization include reduced expression of growth inhibitors, ie, angiotatin, and an improved local vascular...
endothelial growth factor expression and activity that have been observed after overexpression of eNOS. 46

Furthermore, in the present study we observed additional mechanisms whereby statin treatment may exert beneficial effects after MI in an eNOS-dependent way. There was a substantial reduction of myocardial interstitial fibrosis and cardiomyocyte hypertrophy after MI after statin treatment that was largely eNOS dependent. The present study was not designed to elucidate underlying mechanisms of how eNOS limits cardiac fibrosis and hypertrophy. Previous studies in cultured cardiac fibroblasts, however, have shown that NO reduces extracellular matrix production. 34 With respect to cardiomyocyte hypertrophy, we have previously shown that NO, by signaling via cGMP-dependent protein kinase type I, inhibits cardiomyocyte hypertrophy and targets the calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway, 47,48 which may at least in part explain eNOS-dependent inhibition of cardiomyocyte hypertrophy after MI after statin treatment. Though diminished, there was still some effect of statin treatment on cardiomyocyte hypertrophy in eNOS−/− mice, suggesting that eNOS-independent mechanisms are also involved in this effect of statin treatment after MI. Inhibition of Rac1 and a subsequent antioxidant effect of statins have been suggested as additional mechanisms whereby statins may attenuate cardiomyocyte hypertrophy. 49 In addition, statin treatment reduced blood pressure in eNOS-knockout mice after MI, which may also have contributed to reduced cardiomyocyte hypertrophy after MI.

In the present study, we did not observe enhanced LV remodeling and reduced survival after MI in V-treated, eNOS−/− mice compared with V-treated, WT mice after MI, as was shown by Scherrer-Crosbie et al. 20 A critical determinant of the effect of eNOS knockout on these end points may be the degree of impairment of eNO bioavailability after MI, so that eNOS knockout would have less effect when eNO availability is more severely reduced. In the present study, there was a dramatically reduced eNO availability in WT mice after MI. It seems likely that in the study of Scherrer-Crosbie et al, eNO availability was better preserved because LV function and survival in WT mice after MI were substantially better than in the present study, partly owing to a smaller infarct size. The present study, however, in line with the findings of Scherrer-Crosbie et al, suggests that eNO availability after MI represents a critical determinant for LV dysfunction and survival. In addition, it is possible that in eNOS-knockout mice with more severe LV dysfunction after MI, there is a more pronounced compensatory response that may reduce differences between eNOS-knockout and WT mice. Of note, myocardial superinfection of inducible NOS has been described in eNOS-knockout mice but not in WT mice after severe myocardial ischemia as an adaptive response mediating protective effects. 50 Furthermore, recent studies suggest a protective role of neuronal NOS in the myocardium. Thus, the potential compensatory mechanisms in eNOS−/− mice with severe LV dysfunction (ie, mediated by inducible or neuronal NOS or other vasodilator systems) need to be addressed in future studies.

It should be noted that other mechanisms whereby restored endothelium-derived NO availability may exert beneficial effects after MI are likely to contribute to eNOS-dependent improvement of LV function and survival after MI, as observed in the present study. Endothelium-derived NO regulates coronary vasomotor tone and myocardial perfusion, 16,51,52 and improved eNO availability may therefore reduce myocardial perfusion abnormalities observed in failing ventricles. 38,39 Furthermore, it has been suggested that eNOS-derived NO may exert beneficial effects by increasing the LV preload reserve and participate in maintaining the Frank-Starling response in the failing ventricle. 53–55 Moreover, recent studies have suggested that eNOS-derived NO production regulates myocardial oxygen consumption, 40,41 and increased eNO availability may thereby improve myocardial efficiency.

Of note, the beneficial effects of statin treatment were observed without a change in serum cholesterol levels in the present study. Previous studies have shown that competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase has no hypocholesterolemic activity in mice, whereas it is effective in lowering serum cholesterol levels in other species such as monkeys and humans. 56 The underlying mechanisms for the lack of hypocholesterolemic activity of HMG-CoA reductase inhibition in mice are not entirely understood but may involve a marked induction of hepatic levels of HMG-CoA reductase. 56 In summary, the present study demonstrates a marked effect of statin treatment on eNO availability after MI, which is required for statin-induced improvement of LV function and survival after MI. These findings are compatible with the concept that preservation of eNO bioavailability is a principal mechanism mediating the beneficial effects of statin treatment after MI and represents an important therapeutic target.

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