Improvement in Left Ventricular Remodeling by the Endothelial Nitric Oxide Synthase Enhancer AVE9488 After Experimental Myocardial Infarction

Daniela Fraccarollo, PhD; Julian D. Widder, MD; Paolo Galuppo, PhD; Thomas Thum, MD, PhD; Dimitrios Tsikas, MD; Michael Hoffmann, MD; Hartmut Ruetten, MD, PhD; Georg Ertl, MD; Johann Bauersachs, MD

Background—Reduced endothelial nitric oxide (NO) bioavailability contributes to the progression of heart failure. In this study, we investigated whether the transcription enhancer of endothelial NO synthase (eNOS) AVE9488 improves cardiac remodeling and heart failure after experimental myocardial infarction (MI).

Methods and Results—Starting 7 days after coronary artery ligation, rats with MI were treated with placebo or AVE9488 (25 ppm) as a dietary supplement for 9 weeks. AVE9488 therapy versus placebo substantially improved left ventricular (LV) function, reduced LV filling pressure, and prevented the rightward shift of the pressure-volume curve. AVE9488 also attenuated the extent of pulmonary edema, reduced LV fibrosis and myocyte cross-sectional area, and prevented the increases in LV gene expression of atrial natriuretic factor, brain natriuretic peptide, and endothelin-1. eNOS protein levels and calcium-dependent NOS activity were decreased in the surviving LV myocardium from placebo MI rats and normalized by AVE9488. The beneficial effects of AVE9488 on LV dysfunction and remodeling after MI were abrogated in eNOS-deficient mice. Aortic eNOS protein expression and endothelium-dependent NO-mediated vasorelaxation were significantly enhanced by AVE9488 treatment after infarction, whereas increased vascular superoxide anion formation was reduced. Moreover, AVE9488 prevented the marked depression of circulating endothelial progenitor cell levels in rats with heart failure after MI.

Conclusions—Long-term treatment with the eNOS enhancer AVE9488 improved LV remodeling and contractile dysfunction after MI. Molecular alterations, circulating endothelial progenitor cell levels, and endothelial vasomotor dysfunction were improved by AVE9488. Pharmacological interventions designed to increase eNOS-derived NO constitute a promising therapeutic approach for the amelioration of postinfarction ventricular remodeling and heart failure. (Circulation. 2008;118:818-827.)

Key Words: endothelium □ heart failure □ myocardial infarction □ nitric oxide □ nitric oxide synthase □ remodeling

Cardiac remodeling after myocardial infarction (MI) involves myocyte hypertrophy, chamber dilation, and interstitial fibrosis. Alterations in cardiomyocytes and collagen matrix lead to contractile dysfunction and contribute to the progression of ventricular enlargement and heart failure.1-2 Nitric oxide (NO) generated by endothelial NO synthase (eNOS) plays an important role in the pathophysiology of postinfarction ventricular remodeling and heart failure.3 NO regulates vascular tone and cardiomyocyte contractility and protects against myocyte apoptosis and hypertrophy.4,5 Moreover, NO acts as an antithrombogenic and antiatherogenic agent by inhibiting vascular smooth muscle proliferation, platelet aggregation, and leukocyte adhesion.4,6

Clinical Perspective p 827

Genetic ablation of eNOS impaired contractile function and promoted left ventricular (LV) hypertrophy and dilatation after infarction.7 The beneficial effects of angiotensin-converting enzyme inhibition and AT1 antagonists on LV remodeling were almost abolished in mice lacking eNOS,8 emphasizing the importance of eNOS-derived NO in the cardioprotective effects of various established pharmacological interventions. On the other hand, infarcted mice with targeted overexpression of the eNOS gene within the vascular endothelium9 had improved survival and reduced heart failure progression. In addition, cardiomyocyte-specific eNOS over-
expression improved cardiac performance and attenuated myocyte hypertrophy after MI.

Therefore, compounds that specifically upregulate eNOS appear to be a useful tool for improving LV remodeling and function after infarction. In the present study, we investigated whether long-term treatment with AVE9488, a low-molecular-weight compound shown to enhance eNOS expression and NO production, improves cardiac remodeling and heart failure after MI in rats.

Methods
All procedures conformed to the guidelines of the American Physiological Society and were approved by the institutional Animal Research Committee.

Pharmacokinetics of AVE9488 in Rats
The pharmacokinetics of AVE9488 was studied in male Wistar rats. After a single oral dose of AVE9488 (2 mg/kg), the serum area under the curve was 4,844 hours, maximum plasma concentration (Cmax) was 0.598 mg/mL, time to reach Cmax (tmax) was 0.5 hours, elimination half-life (t1/2) was 4.8 hours, and bioavailability was 83.8%.

MI and Study Protocols
Left coronary artery ligation was performed in adult male Wistar rats.12 Seven days after coronary ligation or sham operation, rats were randomized to receive either placebo (untreated) or AVE9488 (25 or 250 ppm) for 9 weeks. In preliminary experiments, long-term treatment with AVE9488 at a low (25 ppm) and high (250 ppm) dose led to similar effects on hemodynamics, LV remodeling, and endothelial function. Therefore, we used the treatment with 25-ppm AVE9488 for further analyses. Treatments were given as food additives. Twice weekly, rats were weighed and their food intake was measured. The average drug dose of AVE9488 received by the rats was 1.25 mg per 1 kg body weight per day.

Hemodynamic and Volume Measurements
Hemodynamic and volume measurements were performed after 9 weeks of treatment. Mean arterial pressure, LV systolic and end-diastolic pressures, dP/dt, and heart rate were measured under intraperitoneal pentobarbital anesthesia (30 mg/kg body weight) as described.13 The time constant of LV pressure isovolumic decay (τ, regression of log(pressure)/time) was calculated by the Weiss method. Correlation coefficients for all studies were >0.99. The in vivo LV pressure-volume relationship was analyzed with a conductance catheter (SPR-774, Millar Instruments, Houston, Tex). Furthermore, LV volume was calculated for each rat from the pressure-volume loop data recorded at steady state and analyzed with the curve fitting program (Adobe, San Jose, Calif). Quantitative myocardial collagen assessment was performed as previously described14 with minor modifications. Briefly, 7-μm picrosirius red sections of the interventricular septum were examined under either blue-filtered bright-field or polarized light. Images were analyzed with Scion Image Beta 4.02 program (Scion Corp, Frederick, Md).

LV Gene Expression
Total RNA was isolated from LV samples (noninfarcted LV myocardium) with TRIzol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions. Total RNA samples (1 μg) were reverse transcribed with oligo(dT) primers by use of Superscript II (Invitrogen). Quantification of cardiac gene expression was determined by real-time polymerase chain reaction with the Bio-Rad iCycler iQ system (Bio-Rad Laboratories, Hercules, Calif). Published primer and probe sequences (Table 1) were used to amplify and detect atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), endothelin-1, and GAPDH. For each gene, a standard was constructed by cloning the specific cDNA-amplified fragment into pCR2.1-TOPO vector (Invitrogen). Serial 10-fold dilutions of the generated plasmid were used as the standard curve. A given mRNA level was expressed as a ratio with respect to the level of mRNA for GAPDH.

Western Blot Analysis and Immunohistochemistry
LV samples (noninfarcted LV myocardium) and aortic samples were homogenized in ice-cold Tris buffer. The homogenates were centrifuged at 8,000g for 20 minutes at 4°C, and the supernatants were used as total tissue lysates. To obtain a membrane-enriched fraction for eNOS determination, the supernatants were then centrifuged at 120,000g for 60 minutes at 4°C. The resulting pellets were resuspended in Tris buffer and solubilized with 1% Triton X-100 and 1%

| Table 1. Sequences of PCR Primers and Detection Probes (TaqMan) |
|-----------------------------|-----------------------------|-----------------------------|
| mRNA | Sequence (5′→3′) | Accession |
| GAPDH | (+)-AAAGCTGTATCAGT6GGAAAAC | M17701 |
| | (-)-GAAAGCCGACTGACTCCACAG | |
| FAM-ATCTTCGAGCCGCAGTCGCGC-TAMRA | |
| ANF | (+)-AAATCCGGAATACATGGCG | M27428 |
| | (-)-GAGAGGTATGACATCTCTTC | |
| FAM-TCAGAATCTGACAGCCTGAGAG-GAG-TAMRA | |
| BNP | (+)-CCAGAACATCCACAGATGC | RATBNP |
| | (-)-TGAGAGTCTTCTCGAGCTCC | |
| FAM-AGACGTCTCAAAGCGCCCGCTACA-TAMRA | |
| ET-1 | (+)-CTCTCCTCTGTAGGGAGAAG | RATT1 |
| | (-)-CTGATGCTTGTGCTGACAGTGG | |
| FAM-AGACGTCTGGGAGGAAAGAA-TAMRA | |

(+) Indicates forward primers; (−), reverse primers; FAM-TAMRA, probe; and ET-1, endothelin-1.
sodium cholate. Western blotting was performed as described previously. For low-temperature SDS-PAGE, LV extracts were mixed with 3× SDS sample buffer (187.5 mmol/L Tris-HCl [pH 6.8], 6% wt/vol SDS, 30% glycerol, 0.03% wt/vol bromophenol blue, and 15% vol/vol 2-mercaptoethanol) at 0°C. Samples were loaded on 7.5% polyacrylamide gels and subjected to electrophoresis. Gels and buffers were cooled to 4°C before electrophoresis, and the buffer tank was placed in an ice bath during electrophoresis. eNOS dimer/monomer protein was detected by Western blot analysis.

Immunochemical staining for nitrotyrosine was performed on aortic 5-μm-thick frozen sections. Primary antibodies used recognize eNOS, inducible NOS (BD Biosciences PharMingen, San Jose, Calif), nitrotyrosine (Abcam, Cambridge, Mass), β-actin, phospho-vasodilator-stimulated phosphoprotein (VASP), and VASP (Cell Signaling Technology, Danvers, Mass).

NOS Activity Assay
NOS activity was determined by assessing the conversion of L-[guanidino-15N2]arginine to 15N-nitrate with gas chromatography/mass spectrometry as previously described. LV samples (noninfarcted LV myocardium) were homogenized in ice-cold homogenizing buffer (50 mmol/L Tris, pH 7.4; 2 mmol/L EGTA, 1 mmol/L DTT, 2 μmol/L leupeptin, 1 μmol/L pepstatin, 10 mmol/L CHAPS, and 1 mmol/L phenylmethylsulfonyl fluoride). Samples were centrifuged at 4000g for 10 minutes at 4°C. The supernatant was incubated at 37°C with assay buffer (50 mmol/L Tris, pH 7.4; 10 μmol/L BH4, 1 mmol/L NADPH, 5 μmol/L FAD, 5 μmol/L FMN, 5 mmol/L CaCl2, 500 mmol/L calmodulin, and 5 mmol/L L-[guanidino-15N2]arginine). Calcium-independent activity was determined in the presence of 5 mmol/L EGTA and in the absence of calcium/calmodulin.

Vascular Reactivity Studies and Superoxide Anion Formation
Vascular reactivity studies were performed after 9 weeks of treatment. The descending thoracic aorta was dissected after removal of the heart and cleaned of connective tissue. One section (10 mm) was immediately frozen in liquid nitrogen for Western blot analysis. Another section (10 mm) was used for measurement of superoxide anion production; the remainder was cut into rings (3 mm in length) mounted in an organ bath (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurements. Rings were precontracted with phenylephrine (0.3 to 1 μmol/L) to comparable constrictor levels, and the relaxant response to cumulative doses of acetylcholine (1 μmol/L to 10 μmol/L) and sodium nitroprusside (1 μmol/L to 10 μmol/L) was assessed. Vascular O2- formation was measured with lucigenin-enhanced chemiluminescence.

Endothelial Progenitor Cell Levels
Peripheral blood mononuclear cells were isolated by Ficoll density centrifugation as described. Peripheral blood mononuclear cells (2×107) were cultured on fibronectin-precoated 24-well plates in EBM-2 culture medium supplemented with EGM SingleQuots (Cambrex, Belgium) and 20% FCS for 3 days. To exclude contamination with mature circulating endothelial cells, we carefully removed culture supernatant 8 hours after initial seeding and placed it on new fibronectin-precoated chamber slides. After dilution of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate–labeled acetylated low-density lipoprotein (dil-acLDL; Molecular Probes, Carlsbad, Calif) and FITC-conjugated lectin from Ulex europeus (UEA-1; Sigma, St Louis, Mo) in serum-free EBM2 media, cells were washed twice and incubated for 90 minutes at 37°C in EBM2 medium containing 10 μg/mL dil-acLDL and 10 μg/mL UEA-1. After washing, cells were detached by trypsin/EDTA treatment, and the amount of double-positive (dil-acLDL and UEA-1) cells was investigated by flow cytometry. Unlabeled cells were used as negative controls.

### Table 2. Global Parameters of Placebo Sham-Operated Rats and Rats With MI

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=10)</th>
<th>Placebo MI (n=12)</th>
<th>AVE9488 MI (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI size, %</td>
<td></td>
<td>49.6±1.2</td>
<td>49.5±2.2</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>523±26</td>
<td>490±10</td>
<td>456±11</td>
</tr>
<tr>
<td>LV, g</td>
<td>0.80±0.04</td>
<td>0.86±0.03</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td>LV/body weight, mg/g</td>
<td>1.55±0.05</td>
<td>1.76±0.05</td>
<td>1.77±0.09</td>
</tr>
<tr>
<td>RV, g</td>
<td>0.21±0.01</td>
<td>0.49±0.03*</td>
<td>0.34±0.03†</td>
</tr>
<tr>
<td>RV/body weight, mg/g</td>
<td>0.40±0.02</td>
<td>0.98±0.06*</td>
<td>0.75±0.08‡</td>
</tr>
<tr>
<td>Lung fluid weight, g</td>
<td>1.17±0.07</td>
<td>2.26±0.19*</td>
<td>1.70±0.20‡</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>120±8</td>
<td>102±4</td>
<td>101±7</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>146±3</td>
<td>112±3*</td>
<td>118±5*</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg/s</td>
<td>154±10</td>
<td>106±6*</td>
<td>127±6‡</td>
</tr>
<tr>
<td>dP/dtmin, mm Hg/s</td>
<td>9150±474</td>
<td>4844±302*</td>
<td>6257±569‡</td>
</tr>
<tr>
<td>τ, ms</td>
<td>13.2±0.75</td>
<td>21.1±1.8*</td>
<td>15.8±1.3§</td>
</tr>
<tr>
<td>LVESV, μL</td>
<td>227±12</td>
<td>810±45*</td>
<td>534±75†</td>
</tr>
<tr>
<td>EF, %</td>
<td>55.2±3.3</td>
<td>26.9±2*</td>
<td>34.8±2‡</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>390±14</td>
<td>335±20</td>
<td>348±19</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; LVSP, LV systolic pressure; LVESV, LV end-systolic volume; and EF, LV ejection fraction. Rats were treated with placebo or AVE9488 for 9 weeks. Values are mean±SEM.

*P<0.05 vs sham; †P<0.01, ‡P<0.05 vs placebo MI.

MI and Hemodynamic and Volume Measurements in eNOS-Knockout Mice
Left coronary artery ligations were performed in adult male eNOS−/− mice subsequently randomized to receive either placebo (untreated) or AVE9488 (30 mg · kg−1 · d−1) as a dietary supplement. LV systolic and end-diastolic pressures, dP/dt, heart rate, and LV pressure-volume relationship were analyzed 8 weeks after MI with a miniaturized 1.4F impedance pressure catheter (Millar Instruments) as described. Infarct size was quantified histologically by planimetry.

Statistical Analysis
All results are reported as mean±SEM. Statistical significance was tested with 1-way ANOVA, Kruskal-Wallis, or Mann-Whitney U test as appropriate. Test for normality was performed as reported. The Holm post hoc test was used to adjust for multiple comparisons.

Dose-response curves were compared by use of a 2-way repeated-measure ANOVA with a first-order autoregressive covariance structure. Statistical analysis was performed with R software environment for statistical computing, version 2.6.2. Two-sided P values were used. Values of P<0.05 were considered statistically significant.

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

Results
In rats without coronary ligation, AVE9488 treatment (25 or 250 ppm in chow) for 9 weeks did not significantly affect mean arterial pressure (placebo, 120±8 mm Hg; AVE9488 [25 ppm], 107±5 mm Hg; AVE9488 [250 ppm], 113±5 mm Hg; P=NS), LV systolic pressure (placebo, 146±3 mm Hg; AVE9488 [25 ppm], 140±4 mm Hg; AVE9488 [250 ppm], 138±5 mm Hg; P=NS), LV function, or endothelial-dependent relaxation compared with placebo treatment. Therefore, for further experiments, a dosage of 25
ppm was used, resulting in plasma concentrations of 10 to 13 ng/mL.

Long-term AVE9488 treatment (25 ppm) increased eNOS mRNA and protein levels in the LV myocardium (see the Figure in the online-only Data Supplement).

Global Parameters, Hemodynamics, and LV Dilation

Starting on the seventh postoperative day, sham-operated animals received placebo treatment (sham; n=10), and surviving MI rats (n=33) were randomly allocated to placebo (placebo MI; n=19) and AVE9488 (AVE9488 MI; n=14) treatment. Five MI rats died during the 9 weeks of treatment: 4 placebo and 1 AVE9488. Only rats with extensive infarcts (>40%) were included in the study (placebo MI, n=12; AVE9488 MI, n=12).

Infarct size and body weight were similar among the experimental groups (Table 2). LV weight was unchanged by infarction or treatment. Right ventricular weight and pulmonary fluid accumulation were elevated in placebo-treated MI rats compared with sham-operated animals and were significantly reduced by AVE9488 therapy (Table 2).

LV systolic pressure was reduced in MI rats regardless of treatment (Table 2). MI rats on placebo developed elevated LV end-diastolic pressure, lower LV dP/dtmax (Figure 1B), dP/dtmax divided by instantaneous pressure (dP/dtmax · IP₁), and dP/dtmin, and prolonged τ (Table 2). AVE9488 decreased LV end-diastolic pressure; improved dP/dtmax, dP/dtmax · IP₁, and dP/dtmin; and shortened τ. Chronic MI resulted in a rightward shift of the LV pressure-volume loops to high volumes (Figure 1C). AVE9488 prevented the rightward shift of LV volume and significantly decreased LV end-systolic and end-diastolic volumes compared with placebo. LV ejection fraction was markedly lower in placebo MI rats and significantly improved by AVE9488 treatment (Table 2).

Plasma Norepinephrine

Rats with chronic MI displayed an increase in circulating norepinephrine levels (sham, 224±35 pg/mL; placebo MI, 518±120 pg/mL; P=0.07), which were nearly normalized by AVE9488 treatment (323±62 pg/mL).

Interstitial Fibrosis and Myocyte Size

Interstitial collagen density in the failing LV myocardium was increased in placebo MI rats and nearly normalized by AVE9488 therapy (Figure 2A). Similarly, myocyte cross-sectional area was increased in placebo rats and significantly reduced by AVE9488 treatment (Figure 2B).

LV Gene Expression

Myocardial endothelin-1 gene expression was increased in placebo MI rats and suppressed by AVE9488 (Figure 2C).
RNA expression of ANF and BNP in the noninfarcted LV myocardium was examined as molecular markers of the hypertrophic response to infarction. ANF and BNP mRNAs were increased in placebo MI rats and reduced by AVE9488 treatment (Figure 2C).

LV eNOS Expression and Activity, Tetrahydrobiopterin, and Nitrotyrosine
eNOS protein expression in the failing LV myocardium was decreased in placebo MI rats and significantly enhanced by AVE9488 therapy (Figure 3A). Consistently, AVE9488 also increased eNOS activity, which was lower in placebo MI rats (Figure 3B). Myocardial tetrahydrobiopterin (BH$_4$) levels were similar in sham-operated rats and MI rats treated with placebo or AVE9488 (Figure 3C). LV inducible NOS protein levels did not significantly change after MI (ratio of inducible NOS to β-actin: sham, 0.45±0.09; placebo MI 0.62±0.29; AVE9488 MI 0.54±0.15; P=NS). Calcium-independent NOS activity tended to be enhanced in placebo MI rats compared with sham and to be reduced by AVE9488 (sham, 14±1 nmol/µg; placebo MI, 27±4 nmol/µg; AVE9488 MI, 19±3 nmol/µg protein×1000 [¹⁵N]nitrate formation from [guanidino ¹⁵N$_2$]l-arginine). Myocardial nitrotyrosine levels as determined by Western blot analysis were increased in placebo MI rats compared with sham-operated animals and reduced by AVE9488 (Figure 3E).

Vascular Function, Superoxide Anion Formation, Aortic eNOS, and Nitrotyrosine Levels
Chronic MI increased the aortic contractile response to phenylephrine, which was prevented by long-treatment with AVE9488 (Figure 4A). Aortas from placebo MI rats showed a profound decrease in acetylcholine-induced endothelium-dependent relaxation. Long-term treatment with AVE9488 significantly improved the response to acetylcholine (Figure 4B). Endothelium-independent relaxation induced by sodium nitroprusside did not differ among the groups (Figure 4C). In parallel, to the beneficial effects on vascular reactivity, AVE9488 treatment significantly diminished the increased superoxide anion production in rats with MI (Figure 4D). Aortic eNOS protein expression was reduced in placebo MI rats and markedly increased by AVE9488 treatment (Figure 4E).

Immunohistochemical staining showed increased nitrotyrosine, localized mainly in the endothelium, in placebo MI rats compared with sham-operated and AVE9488 MI rats (Figure 4F). In addition, long-term treatment with AVE9488 significantly increased vascular VASP phosphorylation at serine 239, an index of the integrity of the NO/cGMP pathway, in MI rats (ratio of phospho-VASP to VASP: placebo, 1.43±0.08; AVE9488, 1.72±0.07; P<0.05).
Circulating Endothelial Progenitor Cells

Chronic MI was characterized by a depression of circulating endothelial progenitor cell (EPC) levels, which were normalized by long-term AVE9488 treatment (Figure 5).

Hemodynamic and Volume Measurements in eNOS-Knockout Mice

To define the specific role of eNOS in mediating the beneficial effects of AVE9488 in heart failure, we investigated the effects of long-term AVE9488 treatment on LV dilation and dysfunction in eNOS-deficient mice after MI. In these mice, AVE9488 treatment did not affect LV filling pressure and end-diastolic and end-systolic volumes. Moreover, infarcted eNOS−/− mice treated with placebo or AVE9488 showed similar LV dP/dtmax, dP/dtmin · IP−1, dP/dtmin, τ, and LV ejection fraction (Table 3). Thus, the beneficial effects of AVE9488 on LV remodeling were completely abolished in mice lacking eNOS, emphasizing the importance of eNOS in the cardioprotective effects of AVE9488 pharmacological intervention.

Discussion

Long-term treatment with the pharmacological eNOS enhancer AVE9488 ameliorated LV remodeling and contractile dysfunction in rats with heart failure after infarction. AVE9488 prevented LV dilation, hypertrophy, fibrosis, and molecular alterations; increased circulating EPC levels; and improved endothelial vasomotor dysfunction.

Endothelial NO bioavailability and cardiac NO production are markedly diminished in congestive heart failure.3,4,21–24 Genetic ablation of eNOS impaired contractile function and promoted LV hypertrophy and dilation after infarction.7 On the other hand, targeted overexpression of eNOS within the vascular endothelium ameliorated survival, cardiac function, and pulmonary congestion in mice with heart failure after MI.9 Cardiomyocyte-specific eNOS overexpression improved LV performance and remodeling after infarction,10 suggesting that strategies to increase eNOS-derived NO production may be promising treatments to improve LV remodeling and function in the failing heart. In the present study, treatment with the eNOS enhancer AVE9488 increased eNOS expression and restored eNOS activity in the failing LV myocardium, leading to an improvement in LV remodeling and contractile dysfunction. No indication was found that, in rats with chronic MI treated with either placebo or AVE9488, eNOS was uncoupled, producing superoxide rather than NO as observed, for example, in diabetes mellitus.25 Accordingly,
we did not detect any differences in BH4 levels or changes in the dimer-to-monomer ratio among the groups. In addition to the improvement in total NO bioavailability, one might also speculate that AVE9488 treatment interferes with the intracellular compartmentalization of NO; this needs to be addressed in further studies.26

The cardioprotective effects of AVE9488 were abrogated in infarcted mice lacking eNOS, emphasizing the importance of eNOS in the beneficial effects of AVE9488 pharmacological intervention. Several mechanisms may underlie the beneficial effects of this novel treatment. We suppose that the positive modulation of LV function and remodeling by long-term AVE9488 treatment was mediated in part by the reduction in reactive fibrosis in the remote noninfarcted LV myocardium, a major determinant of ventricular remodeling in ischemic cardiomyopathy.2,27 Alterations in the collagen matrix raise cardiac muscle stiffness and impair LV performance, leading to progressive dysfunction and heart failure.2 NO displays a negative trophic role and attenuates cardiac fibrosis; NO-generating compounds inhibit collagen synthesis in vascular smooth muscle cells.28 NO mediates bradykinin-induced downregulation of extracellular matrix production in cardiac fibroblasts29 and decreases angiotensin II–induced cardiac fibrosis.30 Therefore, increased NO production by AVE9488 may beneficially affect postinfarction LV fibrosis by dual mechanisms: enhanced inhibitory action of bradykinin and reduced promoting effect of angiotensin II. Although cardiomyocyte-specific eNOS overexpression10 did not prevent LV fibrosis after MI, our data suggest that the nonrestricted effect of the eNOS enhancer in the present study confers more pronounced cardiac protection postinfarction. AVE9488 therapy also prevented pathological hypertrophy, as evidenced by the reduction in myocyte cross-sectional area and expression of fetal genes such as ANF and BNP. NO, via cGMP-dependent protein kinase type I, inhibits cardiomyocyte hypertrophy31 and plays an important role in the antihypertrophic effect of bradykinin.32 Enhanced eNOS-derived NO production may thereby protect against detrimental hypertrophy after MI, as also shown by attenuated myocyte hypertrophy in cardiomyocyte-specific eNOS transgenic mice,10 and increased LV hypertrophy in eNOS+/− mice.7 Furthermore, eNOS deficiency abrogated the beneficial effects of statins on LV remodeling after MI, thus proving the essential role of eNOS-derived NO for the attenuation of cardiac fibrosis and hypertrophy in response to statin treatment.33

In addition, the marked reduction in myocardial endothelin-1 expression likely contributed to the benefit of AVE9488 therapy by preventing the adverse effects of endothelin on postinfarction LV fibrosis and dilation. Interactions between the endothelin and NO pathways have been
described.\textsuperscript{34,35} Stimulation of endothelium-derived NO production inhibits the expression and production of endothelin.\textsuperscript{36} Furthermore, NO inhibits the expression of endothelin-1 induced by hypoxia.\textsuperscript{37} Prevention of right ventricular hypertrophy and pulmonary edema by AVE9488 treatment may be mediated through an improvement in LV failure. The hypertrophic growth of the right ventricle after MI is related to LV dilation and pump failure, resulting in increased pressure load on the ventricle and accumulation of pulmonary fluid.\textsuperscript{38} However, a direct attenuation of endothelin-1 synthesis by eNOS-derived NO may contribute to the pronounced improvement in right ventricular remodeling and pulmonary edema,\textsuperscript{39} which was also the most eminent observation in mice with endothelium-targeted eNOS overexpression after MI.\textsuperscript{9} The hemodynamic improvement likely accounted for reflex inhibition of sympathetic activation. The reduction in plasma norepinephrine may have further contributed to the benefit of AVE9488 therapy by preventing the adverse cardiovascular effects of excessive sympathetic stimulation. Chronic heart failure impairs endothelium-dependent NO-mediated dilation.\textsuperscript{40} Decreased NO bioavailability results from augmented vascular superoxide formation but also from diminished eNOS expression as a consequence of lower shear stress at the endothelial surface in heart failure.\textsuperscript{16,41} In the present study, increasing eNOS protein expression by long-term treatment with AVE9488 diminished the vascular contractile response to phenylephrine and restored acetylcholine-induced endothelium-dependent relaxation in rats with heart failure after MI. Furthermore, AVE9488 diminished vascular superoxide, which contributes to enhanced NO bioavailability. Although the sequence of events cannot easily be elucidated in the animal model, these vascular effects of AVE9488 treatment are likely to contribute to the improvement in cardiac function and remodeling by reducing afterload. However, because nearly load-independent measures of LV func-

**Figure 5.** Circulating EPCs in sham-operated rats and rats with MI treated with placebo (PLA) or AVE9488 for 9 weeks. Bottom, fluorescence-activated cell sorter analysis of Dil-acLDL/UEA1-FITC double-positive cells. Values are mean±SEM (n=7 to 10). *P<0.05 vs sham; ‡P<0.05 vs placebo MI.

**Table 3.** Hemodynamic and Volume Measurements in eNOS-Knockout Mice Treated With Placebo or AVE9488 for 8 Weeks After MI

<table>
<thead>
<tr>
<th></th>
<th>Placebo MI (n=6)</th>
<th>AVE9488 MI (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI size, %</td>
<td>29±2</td>
<td>28±4</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>102±8</td>
<td>92±4</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>15±2</td>
<td>15±3</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg/s</td>
<td>5753±757</td>
<td>4976±420</td>
</tr>
<tr>
<td>dP/dtmax IP⁻¹, s⁻¹</td>
<td>103±11</td>
<td>103±10</td>
</tr>
<tr>
<td>dP/dtmin, mm Hg/s</td>
<td>5471±584</td>
<td>5305±512</td>
</tr>
<tr>
<td>τ, ms</td>
<td>10.6±1</td>
<td>10.1±1</td>
</tr>
<tr>
<td>LVESV, μL</td>
<td>44±5</td>
<td>49±5</td>
</tr>
<tr>
<td>LVEDV, μL</td>
<td>59±4</td>
<td>64±4</td>
</tr>
<tr>
<td>EF, %</td>
<td>26±3</td>
<td>24±3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>423±23</td>
<td>455±21</td>
</tr>
</tbody>
</table>

LVEDP indicates LV end-diastolic pressures. Other abbreviations as in Table 2. Values are mean±SEM.
tion such as \( \tau \) and \( dP/dt_{\text{max}} \cdot \text{IP}^{-1} \) were significantly improved by the eNOS enhancer, direct effects on the myocardium seem to mediate at least part of the beneficial effects.

Circulating EPC levels are reduced in patients with severe congestive heart failure and endothelial dysfunction.\(^{17,42} \) Long-term AVE9488 treatment increased circulating EPCs, which were markedly reduced in placebo MI rats. Because EPCs may provide a circulating pool of cells that could replace dysfunctional endothelium,\(^{32} \) amelioration of postinfarction endothelial dysfunction by treatment with the eNOS enhancer may relate to increased circulating EPC. Because eNOS-derived NO plays an essential role in EPC mobilization,\(^{43} \) enhanced NO production induced by AVE9488 likely contributed to the increase in EPC levels. Treatment of bone marrow mononuclear cells from patients with ischemic cardiomyopathy with AVE9488 enhanced NO formation and improved their impaired functional activity.\(^{11} \)

**Conclusions**

The eNOS enhancer AVE9488 substantially improved LV remodeling and function, LV molecular alterations, and endothelial dysfunction after experimental MI. Cardioprotection by AVE9488 was absent in infarcted eNOS-deficient mice. Pharmacological interventions aiming to increase eNOS-derived NO production constitute a promising therapeutic approach to the prevention of postinfarction ventricular remodeling and heart failure.

**Acknowledgments**

We thank Meike Leutke, Susanne Schraut, Sabrina Thum, Bibiana Beckmann, and Frank-Mathias Gutzki for expert technical assistance.

**Sources of Funding**

This work was supported by Deutsche Forschungsgemeinschaft (SFB355, B9, B10, SFB 688, A10), by IZKF Würzburg (D22, E31), by the German Heart Failure Network (TP8), and by Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany. AVE9488 is an investigational drug developed by Aventis. Aventis provided the study drug in an animal chow formulation.

**Disclosures**

Dr. Fraccarollo and Bauersachs received research grant support from Sanofi-Aventis related to AVE9488.

**References**

27. Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblick EH, Olivetti G, Anversa P. Structural basis of...
Improvement in Left Ventricular Remodeling by the Endothelial Nitric Oxide Synthase Enhancer AVE9488 After Experimental Myocardial Infarction

Daniela Fraccarollo, Julian D. Widder, Paolo Galuppo, Thomas Thum, Dimitrios Tsikas, Michael Hoffmann, Hartmut Ruetten, Georg Ertl and Johann Bauersachs

_Circulation_. 2008;118:818-827; originally published online August 4, 2008; doi: 10.1161/CIRCULATIONAHA.107.717702

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/118/8/818

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/08/18/CIRCULATIONAHA.107.717702.DC1
http://circ.ahajournals.org/content/suppl/2008/08/18/CIRCULATIONAHA.107.717702.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ 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_ is online at:
http://circ.ahajournals.org/subscriptions/
Legend to online figure:

(A) eNOS mRNA expression and (B) eNOS protein levels as determined by Western blot analysis in myocardial extracts. (C) Representative immunohistochemical staining of myocardial tissue. Rats were treated with placebo or with AVE9488 for 9 weeks. Values are mean±SEM (n=7-10). *p<0.01.
Legend to online figure:

(A) eNOS mRNA expression and (B) eNOS protein levels as determined by Western blot analysis in myocardial extracts. (C) Representative immunohistochemical staining of myocardial tissue. Rats were treated with placebo or with AVE9488 for 9 weeks. Values are mean±SEM (n=7-10). *p<0.01.