Effect of ACE Inhibitors and Angiotensin II Type 1 Receptor Antagonists on Endothelial NO Synthase Knockout Mice With Heart Failure

Yun-He Liu, Jiang Xu, Xiao-Ping Yang, Fang Yang, Edward Shesely, Oscar A. Carretero

Abstract—The beneficial effects of ACE inhibitors (ACEi) or angiotensin II type 1 receptor antagonists (AT\(_1\)-ant) are reportedly mediated by NO in heart failure (HF). We hypothesized that in the absence of endothelial NO synthase (eNOS), (1) left ventricular (LV) dysfunction and myocardial remodeling would be more severe after myocardial infarction (MI), and (2) the cardioprotective effect of ACEi and AT\(_1\)-ant would be diminished or absent in mice with HF after MI. eNOS knockout mice (eNOS\(-/-\)) and wild-type C57BL/6J (C57) mice (+/+) were subjected to MI by ligating the left coronary artery. One month after MI, each strain was treated with vehicle, ACEi (enalapril, 20 mg/kg per day), or AT\(_1\)-ant (valsartan, 50 mg/kg per day) for 5 months. Echocardiography was performed, and systolic blood pressure was measured before MI and monthly thereafter. Interstitial collagen fraction and myocyte cross-sectional area were examined histologically. We found that (1) compared with C57 mice, eNOS\(-/-\) mice that underwent sham surgery had significantly increased systolic blood pressure (\(P<0.05\)) and increased LV mass both initially and at 1 to 3 months, although cardiac function and histological findings did not differ between strains; (2) the development of HF and myocardial remodeling were similar after MI in both strains; and (3) ACEi improved cardiac function and remodeling in C57 mice, as evidenced by increased LV ejection fraction (LVEF) and LV shortening fraction (LVSF) and decreased diastolic LV dimension, mass, myocyte cross-sectional area, and interstitial collagen fraction, but these benefits were absent or diminished in eNOS\(-/-\) mice (for C57 versus eNOS\(-/-\): increase in LVEF after ACEi, 14.2±2% versus −4.9±2.5%, respectively \([P<0.001]\); increase in LVSF, 8.6±2.1% versus −7.2±2.8%, respectively \([P<0.01]\); and decrease in LV mass, −16.6±15 versus 73±23 mm\(^3\), respectively \([P<0.01]\)). AT\(_1\)-ant had benefits similar to those of ACEi, which were also absent or diminished in eNOS\(-/-\) mice (for C57 versus eNOS\(-/-\): increase in LVEF after AT\(_1\)-ant, 13.5±1.8% versus −9.8±3%, respectively \([P<0.001]\); increase in LVSF, 6.1±1.6% versus −3.8±3.1%, respectively \([P<0.01]\)). Our data suggest that the absence of NO does not alter the development of HF after MI; however, it significantly decreases the cardioprotective effects of ACEi or AT\(_1\)-ant. (Hypertension. 2002; 39[part 2]:375-381.)

Key Words: nitric oxide ■ nitric oxide synthase ■ heart failure ■ angiotensin-converting enzyme inhibitors ■ receptors, angiotensin II ■ mice

Experimental and clinical studies suggest that the vascular endothelium may play an important role in modulating the progression of ventricular and vascular remodeling in heart failure (HF). Impaired endothelium-dependent relaxation caused by decreased endothelial NO has been demonstrated in hypertension and HF in humans and in experimental animals.\(^1\)\(^,\)\(^2\) NO can be produced by essentially all cell types in the heart and is known to have profound effects on cardiac function. It is synthesized from \(\text{L-arginine} \) by the catalytic reaction of 3 different isoforms of NO synthase (NOS): neuronal or type 1 NOS (nNOS), inducible or type 2 NOS (iNOS), and endothelial or type 3 NOS (eNOS). nNOS and eNOS are constitutively expressed and Ca\(^{2+}\)-dependent enzymes, whereas iNOS is essentially expressed in macrophages and leukocytes in response to appropriate stimuli. All 3 NOS isoforms are expressed in the heart.\(^3\) NO is a potent endogenous vasodilator that is responsible for the maintenance of basal vascular tone as well as inhibition and modulation of platelet aggregation, leukocyte adhesion, cell respiration, and apoptosis.\(^1\)\(^,\)\(^4\) The beneficial effects may be the result of eNOS-derived NO. Mechanisms of action of NO include the activation of second messengers such as cGMP,\(^5\) direct effects on redox-sensitive regulatory proteins, and interaction with reactive oxygen species. A deficiency of eNOS that results in less NO production could have a detrimental effect. Long-term blockade of NO synthesis produces not only hypertension but also structural changes of the coronary vasculature and myocardium.\(^6\) In addition,
inhibition of NO synthesis with N^\text{\textdegree}-nitro-l-arginine methyl ester (L-NAME) has been shown to induce marked inflammation associated with myocardial interstitial fibrosis, myocardial hypertrophy, and vascular smooth muscle hyperplasia. However, the major limitation of using NO inhibitors is that they nonselectively inhibit all isoforms of NO, so that it is hard to distinguish which isoform is responsible for specific changes. Recently, knockout mice for each of the 3 NOS genes have been generated, and their phenotypes reflect the roles of each NOS isoform in various physiological and pathological processes, which allows us to study the specific role of NO. Blood pressure is higher and the wall of the left ventricle (LV) is thicker in mice genetically lacking eNOS (eNOS−/− mice) than in wild-type control mice. It has been well documented that ACE inhibitors (ACEi) and angiotensin II (Ang II) type 1 receptor antagonists (AT_1-ant) inhibit cardiac remodeling, preserve myocardial function, and prolong survival in patients as well as in experimental animals. These benefits have been attributed to improvement of vascular endothelial function and, thereby, increased NO release. We found that the cardioprotective effect of ACEi in ischemia/reperfusion injury was diminished in eNOS−/− mice compared with wild-type control mice, however, we could find no data regarding the effects of ACEi or AT_1-ant on chronic HF in eNOS−/− mice. The present study was designed to determine (1) whether lack of eNOS aggravates myocardial remodeling and LV dysfunction and (2) whether the cardioprotective effects of ACEi and AT_1-ant are diminished or absent in eNOS−/− mice.

Methods

Animals

eNOS knockout mice (eNOS−/−) were derived from a breeding pair of homozygous (−/−) mice and are currently being bred in our Mutant Mouse Facilities. C57BL/6J mice (C57) purchased from Jackson Laboratories (Bar Harbor, Me) served as wild-type controls, because the eNOS−/− mice were bred on a C57 genetic background. Animals were housed in an air-conditioned room with a 12-hour light/dark cycle; they received standard mouse chow and drank tap water. The present study was approved by the Henry Ford Hospital Care of Experimental Animals Committee.

Surgical Procedures

Male mice aged 12 to 16 weeks were anesthetized with sodium pentobarbital (50 mg/kg IP), intubated, and ventilated with room air by use of a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space, the heart was exposed, and the pericardium was opened as described previously. The left anterior descending coronary artery (LAD) was ligated with an 8-0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was deemed successful when the anterior wall of the LV became cyanotic and the ECG showed obvious ST-segment elevation. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed. Sham-operated mice were subjected to the same procedure except that the suture around the LAD was not tied. Animals were kept on a heating pad until they were awake.

Measurement of Blood Pressure and Cardiac Function

Systolic Blood Pressure

Systolic blood pressure (SBP) was measured in conscious mice by use of a noninvasive computerized tail-cuff system (BP-2000, Visitech Systems), as described previously. Mice were trained for 7 days by measuring SBP daily, after which SBP was recorded weekly for 1 month and monthly thereafter. Three sets of 10 measurements were made for each recording.

Echocardiography

Cardiac geometry and function were evaluated with a Doppler echocardiographic system equipped with a 15-MHz linear transducer (Acuson c256). All studies were performed on awake mice before MI and monthly thereafter. The following parameters were examined: (1) LV chamber dimensions and wall thickness; (2) LV mass = 1.055 [(IVSd + LVDd + PWtd)− 2(LVAd)]^0.5, where 1.055 is the specific gravity of the myocardium, IVSd is interventricular septum thickness, LVDd is diastolic LV dimension, and PWtd is diastolic posterior wall thickness; (3) LV ejection fraction (LVEF) = [(LVAd−LVa)/LVA×100, where LVAd is LV diastolic area and LVA is LV systolic area; and (4) LV shortening fraction (LVSF) = [(LVDd−LVD)/LVDd]×100. All primary measurements were traced manually and digitized by goal-directed, digitally driven software installed within the echocardiograph. Three beats were averaged for each measurement.

Histopathological Study

Heart Weight and Infarct Size

Mice were killed after 6 months of MI. The heart was stopped during diastole by injecting 15% potassium chloride solution, then excised and weighed. The LV was sectioned transversely into 3 slices from the apex to the base, rapidly frozen in isopentane precooled in liquid nitrogen, and then stored at −70°C. For infarct size, 1 section was cut from each LV slice and stained with Gomori trichrome. The infarcted portion of the LV was measured as described previously.

MCSA and ICF

Sections (6 μm) were cut from each slice and double-stained with 1 fluorescein-labeled peanut agglutinin (to delineate the myocyte cross-sectional area [MCSA] and the interstitial space) and 2 rhodamine-labeled Griffonia simplicifolia lectin I (to show the capillaries). Four radially oriented microscopic fields from each section of the noninfarcted area were photographed at a magnification of ×100. MCSA was measured by computer-based planimetry (Jandel) and averaged using data obtained from all photographs. The interstitial collagen fraction (ICF) was measured with computer-assisted videodensitometry (JAVA, Jandel).

Experimental Protocols

The first protocol was to determine whether the deterioration of cardiac remodeling and function in eNOS−/− mice is more severe than that in wild-type control mice. Each strain of mice was divided into (1) sham ligation and (2) MI-vehicle groups.

The second protocol was to determine whether the effect of ACEi or AT_1-ant is diminished or absent in eNOS−/− mice. One month after MI, each strain was separated into 2 groups: (1) MI-ACEi (enalapril, 20 mg/kg per day in drinking water), and (2) MI-AT_1-ant (valsartan, 50 mg/kg per day in drinking water), with treatment continued for another 5 months. Our pilot studies showed that enalapril at 20 mg/kg per day inhibited ~70% of the vasopressor effect of exogenous Ang I (12.5, 25, and 50 ng per mouse) and that valsartan at 50 mg/kg per day inhibited 80% of the vasopressor effect of exogenous Ang II (12.5, 25, 50, and 100 ng per mouse).

Data Analysis

Data are expressed as mean±SE. Two-way repeated-measures ANOVA was used to detect differences within each strain and between strains. To compare drug effects between strains, repeated-measures ANOVA was used together with a test of interaction to determine whether the changes after treatment (from month 1 to 6) were different between C57 and eNOS−/− mice. A value of P<0.05 was considered significant. The Student t test was used for heart weight and histopathological data. The Hochberg method was used to adjust for multiple comparisons. The Fisher exact test was used for
TABLE 1. Mortality of C57 and eNOS−/− Mice After MI

<table>
<thead>
<tr>
<th></th>
<th>C57</th>
<th>eNOS−/−</th>
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<tbody>
<tr>
<td>Surgery</td>
<td>12% (8/69)</td>
<td>22% (20/91)</td>
</tr>
<tr>
<td>Heart rupture (within 1 wk)</td>
<td>34% (21/61)</td>
<td>31% (22/71)</td>
</tr>
<tr>
<td>Died of HF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>25% (3/12)</td>
<td>31% (5/16)</td>
</tr>
<tr>
<td>ACEi</td>
<td>8% (1/12)</td>
<td>38% (6/16)</td>
</tr>
<tr>
<td>AT1-ant</td>
<td>0% (0/12)</td>
<td>29% (4/14)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of deaths divided by the number of animals in the experimental groups.

comparisons of mortality rate for heart surgery, heart rupture, and before and after drug treatment between strains.

Results

Early and Late Mortality
Table 1 shows the mortality rate from coronary artery ligation, cardiac rupture, or HF after MI. Among the MI-vehicle, MI-ACEi, and MI-AT1-ant groups, the mortality rate was higher in the eNOS−/− group than in the C57 group, but the differences were not significant. None of the mice that underwent sham ligation died either during or after the operation.

Body Weight, Heart Weight, and Infarct Size
There was no significant difference in body weight, LV weight, right ventricular (RV) weight, and heart weight between strains in the sham-ligated groups (Table 2). Neither coronary artery ligation nor drug treatment had any effect on body weight. Among the MI-vehicle groups, LV, RV, and heart weights increased similarly in both strains. ACEi or AT1-ant tended to reduce these parameters in both strains, but the differences were not significant. There was no significant difference in infarct size among groups or between strains in mice with HF treated with vehicle, ACEi, or AT1-ant (Table 2).

SBP and Heart Rate
SBP was significantly higher in the eNOS−/− strain than in the C57 strain in the sham-MI, MI-vehicle, and MI-AT1-ant groups (P<0.05) (Figure 1). After MI, SBP was significantly decreased in the eNOS−/− vehicle, ACEi, and AT1-ant groups (P<0.01, P<0.05, and P<0.01, respectively) but not in the C57 strain. Neither ACEi nor AT1-ant had any effect on SBP in either strain. Basal heart rate was significantly lower in the eNOS−/− strain compared with the C57 strain (P<0.001) (Figure 1). After MI, HR was significantly increased in eNOS−/− sham-operated, vehicle-treated, and AT1-ant groups (P<0.01, P<0.01, and P<0.001, respectively).

Cardiac Function and Remodeling
There was no difference between sham-operated C57 and eNOS−/− mice regarding LVEF, LVSF, or LVDD. However, LV mass was significantly greater both initially and at 1 to 3 months in sham-ligated eNOS−/− mice compared with C57 mice (P=0.03, P=0.02, P=0.004, and P=0.003, respectively). No increase in LV mass was seen at 4 and 6 months (Figure 2). After MI, LVEF and LVSF decreased significantly by 1 month and progressed similarly over time in both strains. LV chamber dimension and mass increased significantly by 1 month after MI and increased slowly but progressively thereafter, with no significant differences between strains (Figure 2). Figure 3 shows changes in LVEF, LVSF, LV mass, and LVDD after ACEi or AT1-ant. ACEi significantly increased LVEF and LVSF and decreased LV mass in C57 mice, but these effects were not seen in

<table>
<thead>
<tr>
<th></th>
<th>eNOS+/+ (n=9)</th>
<th>eNOS−/− (n=6)</th>
</tr>
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<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>110±1</td>
<td>110±1</td>
</tr>
<tr>
<td>1m</td>
<td>115±2</td>
<td>120±2</td>
</tr>
<tr>
<td>2-6m</td>
<td>120±3</td>
<td>125±3</td>
</tr>
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Figure 1. In C57 (eNOS+/+) and eNOS−/− strains, SBP (top) and HR (bottom) values are shown for sham-operated mice (sham) and mice with HF treated with vehicle (HF-Veh), ACEi (HF-ACEi), or AT1-ant (HF-AT1-ant). Basal indicates before surgery; 1m, 1 month after surgery; 2-6m, mean value over 2 to 6 months of treatment; and CL, coronary ligation or sham.

TABLE 2. Effects of ACEi or AT1-Ant on BW, HW, and Infarct Size 6 mo After MI

<table>
<thead>
<tr>
<th></th>
<th>C57</th>
<th>eNOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=5)</td>
<td>HF-Vehicle (n=9)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.9±1</td>
<td>31.7±1</td>
</tr>
<tr>
<td>LVW, mg/10 g BW</td>
<td>35.1±0.4</td>
<td>51.7±5.4*</td>
</tr>
<tr>
<td>RVW, mg/10 g BW</td>
<td>7.8±0.7</td>
<td>11.7±1.5*</td>
</tr>
<tr>
<td>HW, mg/10 g BW</td>
<td>45.6±1.0</td>
<td>69.6±7.6*</td>
</tr>
<tr>
<td>IS, %</td>
<td>...</td>
<td>38.6±2.7</td>
</tr>
</tbody>
</table>

LW indicates LV weight; BW, body weight; RVW, RV weight; HW, heart weight; and IS, infarct size. Values are mean±SE.

*P<0.001 vs sham.
Role of eNOS in the Development of HF After MI

eNOS−/− mice reported to have hypertension; they fail to show vasorelaxation in response to acetylcholine and exhibit increased ischemic and inflammatory injury.\cite{1, 18} All of these events may accelerate myocardial remodeling after MI. On the basis of these findings, we anticipated that cardiac remodeling and function might be more severe in eNOS−/− with HF due to MI. Instead, we found that 6 months after MI, myocardial remodeling and cardiac function were equally advanced in eNOS−/− mice and their wild-type controls, although SBP was higher in eNOS−/− mice. This agrees with our previous finding that MI size and cardiac function were similar in eNOS−/− and C57 mice. This suggests that the compensatory capacity of the residual noninfarcted myocardium reaches a maximum after a large MI; therefore, functional and histological changes would not differ further between knockout mice and wild-type control mice. It is also possible that in the absence of NO, other vasodilators, such as prostacyclin or nNOS, may be upregulated or increased in eNOS−/− mice as a compensatory adaptation, thereby preventing further worsening of cardiac remodeling and function.\cite{20} Kanno et al\cite{21} recently reported that ex vivo eNOS−/− mouse hearts exhibited a paradoxical increase in NO production accompanied by superinduction of iNOS during ischemia/reperfusion, which was responsible for the cardioprotection and most likely due to a compensatory adaptation.\cite{21, 22} The adaptive mechanism of nNOS, iNOS, or other vasodilator systems in the development of chronic HF in eNOS−/− needs to be studied further. Our results suggest that eNOS is not an important determinant of the progression and development of HF due to MI. It has been reported that iNOS activity and expression were significantly elevated in the failing heart.\cite{23} It might be that the release of NO derived from iNOS is a more important determinant of pathophysiological events in the development of myocardial remodeling and dysfunction.

Role of eNOS in Cardioprotective Effect of ACEi or AT₁-Ant

Our present data agree with our previous finding that ACEi and AT₁-ant improved LVEF and attenuated cardiac fibrosis and hypertrophy in C57 mice with HF after MI.\cite{11} Furthermore, we believe that we now have the first evidence that this

Discussion

Although SBP was higher in sham-operated eNOS−/− mice than in wild-type control mice, cardiac function and histology were similar except for LV mass, which was greater in sham-operated eNOS−/− mice. Progression and development of HF were also similar in C57 and eNOS−/− mice, suggesting that lack of eNOS does not aggravate myocardial remodeling and cardiac dysfunction after MI. ACEi and AT₁-ant were far less effective against myocardial remodeling and cardiac function in eNOS−/− mice than in C57 mice, suggesting that the beneficial effect of both drugs is mediated by NO. To our knowledge, this is the first demonstration that chronic HF after MI is similar in eNOS−/− and C57 mice, whereas the beneficial effect of ACEi or AT₁-ant is almost entirely abolished.

Myocyte Size and Interstitial Fibrosis

Among the sham-ligated groups, ICF and MCSA were similar initially, and they increased significantly after MI in both strains. ACEi and AT₁-ant significantly reduced interstitial collagen deposition and myocyte size in C57 mice, and these effects were diminished in eNOS−/− mice (Figures 5 and 6).

Role of eNOS in the Development of HF After MI

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Figure 4. Two-dimensional M-mode echocardiographs of C57 (eNOS+/+) and eNOS−/− mice with sham coronary artery ligation (sham) or HF treated with vehicle, ACEi, or AT₁-ant. IS indicates interventricular septum; DD, LV diastolic dimension; and PW, LV posterior wall.
ACEi or AT1-ant. The renin-angiotensin system is activated during HF, and blocking it with ACEi or AT1-ant is an important mediator in the cardioprotective mechanism (see Figure 5). There is evidence that ACEi substantially improve beneficial effect of ACEi or AT1-ant is almost abolished in eNOS−/− mice with HF, supporting the hypothesis that NO is an important mediator in the cardioprotective mechanism of ACEi or AT1-ant. The renin-angiotensin system is activated during HF, and blocking it with ACEi or AT1-ant significantly improves cardiac function, regresses myocardial remodeling, and prolongs survival in patients with HF; however, the underlying mechanism has not been well defined. There is evidence that ACEi substantially improve endothelial dysfunction, thereby increasing blood flow in HF. This effect may be related to the increased bioavailability of NO. We found that ACEi or AT1-ant failed to protect the heart in eNOS−/− mice with HF, confirming that NO is a very important mediator of both types of treatment. ACE is an integral component of the circulating and vascular renin-angiotensin and kallikrein-kinin systems. ACE inhibition reduces Ang II formation and enhances bradykinin activity, and studies have shown that potentiation of kinins may be largely responsible for the therapeutic effect of ACEi, thus, kinins potently stimulate endothelial cells to release NO and vasodilator prostaglandins. AT1-ant have a favorable effect similar to that of ACEi, acting on cardiac hypertrophy, remodeling, and function in patients with HF as well as in experimental animals. Inhibition of Ang II reduces superoxide generation from the myocardium and blood vessels and may increase the bioavailability of NO.

The effect of AT1-ant may also be related to an increase in Ang II by a feedback mechanism, which activates Ang II type 2 receptors and thereby leads to the release of kinins and NO. Although the pharmacological profiles of ACEi and AT1-ant are different, they may act through a common mediator, NO. ACEi or AT1-ant counteract the disproportionate balance between the vasodilator/antihypertrophic properties of kinins and the vasoconstrictor/hypertrophic properties of Ang II by enhancing the release of NO from the endothelium.

In summary, we found that (1) MI caused myocardial remodeling and decreased cardiac function in a similar fashion in eNOS−/− and C57 mice, suggesting that NO derived from eNOS may not play an important role in the pathophysiology of heart failure, and (2) in eNOS−/− mice, the cardioprotective effects of ACEi and AT1-ant were almost abolished, suggesting that NO is an important mediator in the cardioprotective effects of ACEi and AT1-ant.

Acknowledgment

This work was supported by National Institutes of Health grant HL-28982.

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