Endothelial Nitric Oxide Gene Knockout Mice

Cardiac Phenotypes and the Effect of Angiotensin-Converting Enzyme Inhibitor on Myocardial Ischemia/Reperfusion Injury

Xiao-Ping Yang, Yun-He Liu, Edward G. Shesely, Manohar Bulagannawar, Fang Liu, Oscar A. Carretero

Abstract—We tested the hypothesis that nitric oxide (NO) released by endothelial NO synthase (eNOS) is not only important in blood pressure regulation but also involved in cardiac function and remodeling and in the cardioprotective effect of angiotensin-converting enzyme inhibitors (ACEi). With the use of a 2D Doppler echocardiography system equipped with a 15-MHz linear transducer, we evaluated left ventricular (LV) morphology and function in conscious eNOS knockout mice (eNOS−/−; n=15) and their wild-type littermates (eNOS+/+; n=16). We also studied whether in eNOS−/− mice (1) myocardial ischemia/reperfusion injury is more severe and (2) the cardioprotective effect of ACEi is diminished or absent. In comparison with the wild type, eNOS−/− mice had significantly increased systolic blood pressure (128±3 versus 108±5 mm Hg; P<0.001) and decreased heart rate (531±22 versus 629±18 bpm; P<0.001) associated with increased LV posterior wall thickness (0.80±0.04 versus 0.64±0.02 mm; P<0.001) and LV mass (18.3±0.9 versus 13.1±0.5 mg/g body weight; P<0.01). Despite hypertension and LV hypertrophy, LV chamber dimension, shortening fraction and ejection fraction (indicators of LV contractility), and cardiac output did not differ between the 2 strains, which indicates that LV function in eNOS−/− mice is well compensated. We also found that in eNOS+/+ mice, ACEi decreased the ratio of myocardial infarct size to area at risk from 62.7±3.9% to 36.3±1.6% (P<0.001), whereas in eNOS−/− mice this effect of ACEi was almost abolished: the ratio of myocardial infarct size to area at risk was 67.2±2.9% in the vehicle-treated group and 62.7±3.9% in mice treated with ACEi. Moreover, infarct size in vehicle-treated eNOS−/− mice was not significantly different from eNOS+/+ mice given the same treatment. We concluded that (1) endothelium-derived NO plays an important role in the regulation of blood pressure homeostasis; (2) NO released under basal conditions has no significant impact on cardiac function; and (3) ACEi protect the heart against ischemia/reperfusion injury in mice and that this effect is mediated in part by endothelium-derived NO.

Key Words: nitric oxide ■ myocardial ischemia ■ myocardial reperfusion injury ■ mice, knockout ■ angiotensin-converting enzyme inhibitors ■ echocardiography

Nitric oxide (NO) is formed from L-arginine and oxygen by 3 isoenzymes collectively called nitric oxide synthase (NOS).1-3 Those NO isoenzymes expressed constitutively in endothelial (eNOS) and neuronal cells (nNOS) are Ca2+-dependent and can be activated by elevated intracellular calcium concentration,4,5 whereas the Ca2+-independent isoenzyme (iNOS) can be induced by cytokines in macrophages and vascular smooth muscle cells and produces an excessive amount of NO.6,7 In the vasculature, endothelium-derived NO (EDNO) appears to play an important role in the regulation of blood pressure (BP), regional blood flow, and vascular tone, as well as receptor-mediated vasodilator responses to agonists such as acetylcholine, bradykinin, thrombin, and others.8-10 In addition to its vasodilator properties, NO inhibits platelet aggregation, neutrophil infiltration, and smooth muscle cell proliferation and migration.11-13

In the heart, eNOS is expressed mainly in the coronary vasculature and endocardial endothelium and may function as a tissue protective factor against damage to the heart, such as myocardial ischemia/reperfusion injury. Infusion of the NO precursor L-arginine or NO donors into the dog or cat heart reduced infarct size and preserved endothelial function.14-17 Our group and others18,19 previously found that, in rats and rabbits, NO was involved in the cardioprotective effect of angiotensin-converting enzyme inhibitors (ACEi) on myocardial ischemia/reperfusion injury because the infarct size-limiting effect of ACEi was blocked by either the B2-kinin receptor antagonist icatibant (Hoe 140) or the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). We interpret our data as evidence that ACEi block kinin degradation, which in turn stimulates NO release by eNOS and eventually leads to cardioprotection. However, some results...
have been controversial. Patel et al.20 and Schulz et al.21 showed that inhibition of NO synthase with L-NAMe reduced myocardial reperfusion injury in rabbit hearts and infusion of L-arginine into isolated rabbit hearts potentiated ischemia/reperfusion injury.22 Although the reasons for this discrepancy are unclear, we suspect it may be due to the nonspecificity of L-NAMe and the different species used. The dose of L-NAMe used in rabbits probably blocks not only eNOS but also iNOS. Our group and others23,24 have recently shown that selective inhibition of iNOS reduced infarct size. Thus, it could be that NO produced by eNOS has a cardioprotective effect, whereas the excessive amount of NO released by iNOS may be detrimental to the heart.

In addition, pharmacological probes themselves may alter the cardiovascular response to various pathophysiological events, which emphasizes the complexity of the data and the difficulty of interpretation. Use of genetically altered animals could overcome these limitations. Mice with genetically disrupted eNOS genes (eNOS−/−) have recently been made available with the use of gene targeting in mouse embryonic stem cells (ES).25,26 This allows us to study the specific role of NO produced by eNOS in the regulation of BP and cardiac morphology and function as well as the cardioprotective effect of ACEi.

In the present study, we used a 2D Doppler echocardiography system equipped with a high-frequency transducer (L15-8) to evaluate left ventricular (LV) morphology and function in eNOS homozygous (+/−) mutant mice versus wild-type littersmates (+/+) We also tested the hypotheses that (1) NO released by eNOS has cardioprotective properties (myocardial ischemia/reperfusion injury would be more severe in mice that lack the eNOS gene [eNOS−/−]); and (2) ACEi protect the heart against ischemia/reperfusion injury; this effect is partially mediated by NO released from eNOS. In eNOS−/− mice, the cardioprotective effect of ACEi is diminished or absent.

Methods

Mice
eNOS homozygous mutants (−/−) generated by gene targeting25 were obtained by breeding pairs of eNOS heterozygous (+/−) mice. Wild-type (+/+) littersmates were used as controls. They were maintained as hybrids with a 129/Ola and C57BL/6J genetic background. Mice were bred in our Mutant Mouse Facilities, were housed in an air-conditioned room with a 12-hour light/dark cycle, received standard mouse (or rat) chow, and drank tap water. Experiments were conducted in male mice weighing 25 to 30 g (10 to 12 weeks old). This study was approved by the Henry Ford Hospital Care of Experimental Animals Committee.

BP and Cardiac Morphology and Function

Measurement of Systolic BP and Heart Rate

Systolic BP (SBP) and heart rate (HR) were measured using a noninvasive computerized tail-cuff system (BP-2000, Visitech Systems) as described previously.22,25 Briefly, mice were trained for 7 days by measuring SBP daily, after which SBP and HR were measured and recorded for 5 consecutive days. Each day, 3 sets of 10 measurements were obtained; a set was accepted if the computer identified >6 successful readings of 10 measurements.

Echocardiographic Evaluation of Cardiac Morphology and Function

Echocardiography was performed in conscious mice. We first trained mice on 2 or 3 separate occasions by picking them up by the nape of the neck and holding them firmly in the palm of 1 hand in the supine position, with the tail held tightly between the last 2 fingers. The left hemithorax was carefully shaved, and a prewarmed ultrasound transmission gel (Parker Laboratory) was applied to the precordium. Transthoracic echocardiography was performed using a Doppler echocardiographic system (Acuson c256) equipped with a 15-MHz linear transducer (15L-8) in a phased-array format, which offers real-time digital acquisition, storage, and review capabilities. Generally, the heart was first imaged in the 2D mode in the parasternal long-axis view for measurement of wall thickness and chamber dimensions, as well as aortic dimension and flow velocity. A 2D mode short-axis view of the mid-LV was then obtained at the chordal level by rotating the transducer clockwise 30° to 45° and used to measure LV cross-sectional area. Images obtained during training sessions were not recorded. Once the mice were trained, images were stored in digital format on a magnetic optical disk for review and analysis. All data were obtained by 2 collaborating observers but analyzed by only 1 of them in a blind fashion. Our previous study showed that inter- and intra-observer error was <10% for each parameter mentioned below, and correlation coefficients were >75%, which indicates good inter- and intra-observer agreement (unpublished data). The following parameters were measured.

1. LV chamber dimensions and wall thickness.
2. LV Mass=1.055 [(IVSd+LVDd+PWTd)/2−LVDd], 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. SF=(LVDd−LVDs)/LVDd×100, where SF is LV shortening fraction and LVDs is systolic LV dimension.
4. EF=(LVAd−LVAs)/LVAd×100, where EF is ejection fraction, LVAd is LV diastolic area, and LVAs is LV systolic area.
5. CO=CSA×π×D×VTI (2)/1.055, where CO is cardiac output, CSA is aortic cross-sectional area, AoD is aortic diameter, SV is stroke volume, and VTI is aortic flow velocity-time integral.

All primary measurements were made and digitized by the goal-directed, diagnostically driven software installed within the echocardiograph. Each measurement averaged 3 beats.

Effect of ACEi on Ischemia/Reperfusion Injury

Surgery

Mice were anesthetized with sodium pentobarbital (50 mg/kg IP), intubated and ventilated with room air using a positive-pressure respirator. A polyethylene catheter (PE10 fused to PE50) was inserted into the left carotid artery to measure mean BP (MBP) and HR. A left thoracotomy was performed via the fourth intercostal space, the heart exposed, and the pericardium opened as described previously.29 The left anterior descending coronary artery was ligated with a 9-0 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. The success rate of inducing acute myocardial ischemia was 100%. After 30 minutes of sustained ischemia, the suture was released and the heart reperfused. Successful reperfusion was identified by return of the original color, accompanied by obvious ST-segment elevation. The lungs were then inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed. Animals were kept on a heating pad and remained anesthetized throughout the experiment. The total mortality rate (those that died during surgery and the 2-hour reperfusion period) was 17.7%.

Experimental Protocols

Each eNOS mutant genotype (+/− and −/−) received either vehicle or ACEi (ramiprilat 50 μg/kg given as a bolus 5 minutes before

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Comparison of Cardiac Hemodynamics, Morphology, and Function Between Endothelial NOS Knockout Mice (eNOS+/−) and Wild-Type Controls (eNOS+/+)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>eNOS+/− (n=16)</th>
<th>eNOS+/+ (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>26.9±1.1</td>
<td>26.2±0.9</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>108±5</td>
<td>128±3</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>629±18</td>
<td>531±22</td>
</tr>
<tr>
<td>LVd, mm</td>
<td>2.30±0.07</td>
<td>2.26±0.06</td>
</tr>
<tr>
<td>PWTd, mm</td>
<td>0.64±0.02</td>
<td>0.80±0.04*</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>13.1±0.5</td>
<td>18.3±2.0*</td>
</tr>
<tr>
<td>SF, %</td>
<td>53.4±1.7</td>
<td>54.0±1.8</td>
</tr>
<tr>
<td>EF, %</td>
<td>88.2±0.7</td>
<td>87.0±1.4</td>
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<tr>
<td>CO, ml · min⁻¹ · 10 g⁻¹</td>
<td>7.8±0.4</td>
<td>8.1±0.5</td>
</tr>
</tbody>
</table>

BW indicates body weight.

*P<0.01 vs eNOS+/+ mice.

Measurement of Myocardial Infarct Size
The LV was cut into 3 slices proceeding transversely from base to apex. The slices were incubated with triphenyltetrazolium chloride (10 mg/mL) in 0.2 mol/L phosphate buffer solution for 30 minutes. Noninfarcted myocardium, which contains lactate dehydrogenase, stained brick red by reacting with triphenyltetrazolium chloride, whereas the necrotic (infarcted) tissue remained unstained because of lack of enzymes. Each slice was photographed, magnified, and projected onto a screen; infarcted area (uncolored), area at risk (uncolored and brick red), and nonoccluded areas (blue) were measured with a planimeter and calculated. The following parameters were averaged across the 3 slices for each heart: (1) ratio of infarct size to area at risk (IS/AR); (2) ratio of infarct size to LV (IS/LV); and (3) ratio of area at risk to LV (AR/LV).

Statistical Analysis
Statistical analysis was performed by biostatisticians from the Department of Biostatistics and Research Epidemiology at our institute. Results are expressed as mean±SEM. A 2-sample, 2-sided Student t test was used to compare SBP and cardiac morphology and function between the 2 strains of mice. P≤0.05 was considered significant. Holm’s procedure with adjusted multiple testing was used to compare MBP, HR, and myocardial infarct size in mice with and without ACEi treatment between and within strains. An adjusted level of P≤0.01 indicates statistical significance. P≥0.01 but <0.05 was considered marginally significant.

Results
BP and HR
As expected, SBP in conscious eNOS knockout mice (eNOS+/−) was significantly higher than in wild-type controls (eNOS+/+) (128±3 versus 108±5 mm Hg; P<0.01), whereas HR in eNOS−/− mice was lower than in controls (531±22 versus 629±18 bpm; P<0.01) (Table). In anesthetized mice (ischemia/reperfusion study), there was no difference in basal MBP and HR between the 2 strains (Figure 1). During reperfusion, MBP tended to increase in the vehicle groups (both eNOS+/− and eNOS+/+) and remained unchanged or slightly decreased in the ACEi groups; however, the difference was not statistically significant. HR was similar among all groups.

Cardiac Morphology and Function
PWTd and LV mass were significantly increased in eNOS−/− mice versus eNOS+/+ mice (PWTd, 0.80±0.04 versus 0.64±0.02 mm, P<0.01; LV mass, 18.3±0.9 versus 13.1±0.5 mg per 10 g body wt, P<0.01), indicating concentric LV hypertrophy (Table and Figure 2). LV dimension, SF and EF (both indicators of LV contractility), and CO did not differ from eNOS+/− controls (Table).

Effect of ACEi on Ischemia/Reperfusion Injury
In eNOS+/+ mice, ACEi decreased IS/AR from 63.2±3.4% to 36.3±1.6% (P<0.001) and IS/LV from 40.5±2% to 21.7±1.1% (P<0.001) (Figure 3); whereas in eNOS−/− mice, this effect of ACEi was absent. IS/AR was 67.2±2.9% in the vehicle-treated group and 41.3±4.2% in the vehicle group and 38.3±2.5% with ACEi. Myocardial ischemia/reperfusion injury was no more severe in eNOS−/− mice than in wild-type controls (Figure 3). AR/LV was similar in all groups (Figure 4). No ventricular arrhythmias were observed during either ischemia or reperfusion.

Discussion
In the present study, we confirmed and extended a previous finding22 that in mice that genetically lack EDNO, BP was significantly higher than in wild-type controls. The increase in BP was associated with greater LV wall thickness and mass and well-compensated LV function. Furthermore, we found that ACEi protect the heart against ischemia/reperfusion injury in mice and that this effect is diminished in

Figure 1. MBP and HR during ischemia and reperfusion in eNOS knockout mice (eNOS−/−) and wild-type controls (eNOS+/+) treated with either vehicle or the ACEi ramiprilat.

![Figure 1](http://hyper.ahajournals.org/)

Figure 2. MBP and HR in vehicle- and ACEi-treated groups.

![Figure 2](http://hyper.ahajournals.org/)
eNOS$^{-/-}$ mice, which indicates an EDNO-mediated mechanism.

The role of NO in the regulation of basal vascular tone and BP has been studied extensively by various pharmacological approaches. Typically, chronic inhibition of NO synthesis by the L-arginine analogues $N^O$-monomethyl L-arginine (L-NMMA) and L-NAME produces a hypertensive and hypertrophic response. This effect can be reversed by supplementation with the NO precursor L-arginine. However, these NO inhibitors are nonselective and affect all 3 NOS isoforms, making it difficult to distinguish the role of each isoform in the control of vascular function. Thus, mice with disruption of the gene encoding for eNOS may provide a novel approach to overcoming this limitation. In the present study, we found that SBP in awake eNOS mutant mice was significantly higher than in their littermate controls, which agrees with the previous finding by Shesely et al$^{25}$ that eNOS$^{-/-}$ mice had an increased SBP not seen in iNOS knockout mice. This provides direct evidence that EDNO may play an essential role in maintaining BP homeostasis. On the other hand, we did not find a significant difference in MBP between eNOS$^{-/-}$ and eNOS$^{+/+}$ when mice were anesthetized (ischemia/reperfusion study) in contrast to the findings of Huang et al$^{26}$ and Gödecke et al.$^8$ One reason for this discrepancy might be the use of different anesthetics. We have previously found that pentobarbital has a significant negative inotropic and chronotropic effect on the mouse heart (unpublished observations) that might confound measurements of BP and HR.

In accord with earlier reports by Shesely et al$^{25}$ and Gödecke et al.$^8$ we also found that eNOS$^{-/-}$ mice had a significant decrease in HR that is not normally seen in either hypertensive patients or spontaneously hypertensive rats or mice lacking atrial natriuretic factor.$^{32,33}$ Although we do not
have a good explanation for this, we suspect that eNOS may affect the control of baroreflex resetting \(^3\) or be involved in establishing the baroreceptor set point. \(^3\) Another possibility is that nNOS expression might be enhanced in eNOS knockout mice as a compensatory mechanism. Increased nNOS activity may have a direct effect on cholinergic receptors and decrease heart rate, because a link between cholinergic receptor stimulation and NOS activation has been reported. \(^3\) For example, Balligand et al. \(^3\) showed that the acetylcholine analogue carbachol decreased the spontaneous beating rate in neonatal rat ventricular myocytes, in association with increased release of NO. This effect could be reversed by L-NAME or methylene blue. However, the precise mechanisms responsible for the decreased HR in eNOS mutant mice remain unclear.

Concentric cardiac hypertrophy was seen in eNOS\(^{-/-}\) mice, as indicated by an increase in LV posterior wall thickness and cardiac mass. These cardiac changes follow a pattern similar to those observed in 2-kidney, 1 clip hypertensive mice (unpublished data) and thus may be secondary to hypertension. However, we cannot exclude the possibility that lack of eNOS causes a direct hypertrophic response, because it has been shown that EDNO has antiguangic or antihypertrophic properties. \(^5\) Preexisting cardiac hypertrophy seemed to have no significant influence on either myocardial infarct size or cardiac performance. Infarct size in vehicle-treated eNOS\(^{-/-}\) mice was no different from eNOS\(^{+/+}\) mice receiving the same treatment, and SF, EF, and CO were similar in the 2 strains. Previous reports of the role of NO in cardiac performance have been controversial. In vitro, NO inhibits the contractile response to \(\beta\)-adrenergic stimulation, probably as a result of an increase in cGMP which reduces intracellular Ca\(^{2+}\) and thereby decreases contractility. \(^3\) In vivo, however, inhibition of NOS with L-NAME either decreases cardiac contractility or has no effect. \(^4\) This discrepancy may be related to the experimental preparation, the nonspecific effect of NOS inhibitors, different species, or the use of anesthetics. We believe this is the first report of cardiac morphology and function in intact eNOS mutant mice, particularly conscious mice, that provides potentially important information regarding the role of endogenous NO in the control of cardiac performance.

We found that ACEi decreased myocardial ischemia/reperfusion injury in eNOS\(^{+/+}\) mice and that this infarct-liming effect was almost abolished in eNOS\(^{-/-}\) mice. This indicates that EDNO is an important mediator for the action of ACEi. Linz et al. \(^4\) reported that the ACEi ramiprilat increases NO formation and reduces ischemic injury in the isolated rat heart and that these effects were blocked by the B\(_2\) kinin receptor antagonist icatibant. Our group \(^1\) and Hartman \(^1\) have obtained similar results, which show that ACEi reduced myocardial infarct size in an in vivo model of myocardial ischemia/reperfusion injury and that this effect was blocked by either the B\(_2\)-kinin receptor antagonist icatibant or the NOS inhibitor L-NAME. We interpret these data as evidence that ACEi block kinin degradation, which in turn stimulates NO release and eventually leads to cardioprotection. However, L-NAME has been reported to reduce myocardial reperfusion injury. \(^2\) Although we do not have a good explanation for this discrepancy, we believe it may be related to the nonselectivity of L-NAME. Our group and others \(^2\) have shown that iNOS is activated after myocardial infarction and that inhibition of iNOS with aminoguanidine or S-methylisothiourea (selective inhibitors of iNOS) reduces myocardial infarct size. Thus, if L-NAME protects the heart against ischemia/reperfusion injury, this may be due to inhibition of iNOS, not eNOS.

Mechanisms by which increased EDNO reduces myocardial ischemia/reperfusion injury may involve (1) inhibiting platelet aggregation and neutrophil adhesion to endothelial cells and infiltration of the myocardium, thereby ameliorating endothelial dysfunction and myocardial injury; \(^3\) scavenging oxygen-derived free radicals such as superoxide \(^4\); and (3) improving coronary perfusion and/or myocardial metabolism and decreasing oxygen consumption. \(^4\)
Finally, we found that myocardial ischemia/reperfusion injury in eNOS−/− mice was no more severe than in eNOS+/− mice. These data agree with our previous finding that L-NAME itself did not increase infarct size in rats with ischemia/reperfusion injury but did block the cardioprotective effect of ACEI,18 which indicates that basal release of endogenous EDNO may not participate in the pathophysiology of ischemia/reperfusion injury. In response to stimuli such as increased kinin or prostaglandin levels, eNOS activity increases, and this increased release of EDNO may be an important part of the cardioprotective mechanism.

In summary, we have shown that (1) in mice genetically lacking EDNO, a significant increase in SBP and decrease in HR occurs that is associated with LV hypertrophy and well-compensated ventricular function and (2) ACEI protect the heart against ischemia/reperfusion injury in wild-type mice but not eNOS−/−. We conclude that (1) EDNO plays an essential role in the regulation of BP homeostasis; (2) NO released under basal conditions has no significant impact on cardiac function; and (3) eNOS is an important mediator responsible for the cardioprotective effect of ACEI.

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References


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