Modulation of mouse cardiac function in vivo by eNOS and ANP

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Gyurko, Robert, Peter Kuhlenordt, Mark C. Fishman, and Paul L. Huang. Modulation of mouse cardiac function in vivo by eNOS and ANP. Am. J. Physiol. Heart Circ. Physiol. 278: H971–H981, 2000.—To study the role of endothelial nitric oxide synthase (eNOS) in cardiac function, we compared eNOS expression, contractility, and relaxation in the left ventricles of wild-type and eNOS-deficient mice. eNOS immunostaining is localized to the macro- and microvascular endothelium throughout the myocardium in wild-type mice and is absent in eNOS−/− mice. Whereas blood pressure is elevated in eNOS−/− mice, baseline cardiac contractility (dP/dtmax) is similar in wild-type and eNOS−/− mice (9.673 ± 2.447 and 9.928 ± 1.566 mmHg/s, respectively). The β-adrenergic agonist isoproterenol (Iso) at doses of ≥1 ng causes enhanced increases in dP/dtmax in eNOS−/− mice compared with wild-type controls in vivo (P < 0.01) as well as in Langendorff isolated heart preparations (P < 0.02). β-Adrenergic receptor binding (Bmax) is not significantly different in the two groups of animals (Bmax = 41.4 ± 9.4 and 36.1 ± 5.1 fmol/mg for wild-type and eNOS−/− ). Iso-stimulated ventricular relaxation is also enhanced in the eNOS−/− mice, as measured by dP/dtmin in the isolated heart. However, baseline ventricular relaxation is normal in eNOS−/− mice (t = 5.2 ± 1.0 and 5.6 ± 1.5 ms for wild-type and eNOS−/−, respectively), whereas it is impaired in wild-type mice after NOS inhibition (t = 8.3 ± 2.4 ms). cGMP levels in the left ventricle are unaffected by eNOS gene deletion (wild-type: 3.1 ± 0.8 pmol/mg; eNOS−/−: 3.1 ± 0.6 pmol/mg), leading us to examine the level of another physiological regulator of cGMP. Atrial natriuretic peptide (ANP) expression is markedly upregulated in the eNOS−/− mice, and exogenous ANP restores ventricular relaxation in wild-type mice treated with NOS inhibitors. These results suggest that eNOS attenuates both inotropic and lusitropic responses to β-adrenergic stimulation, and it also appears to regulate baseline ventricular relaxation in conjunction with ANP.

CARDIAC CONTRACTILE FUNCTION is modulated by many systems, including the β-adrenergic and muscarinic cholinergic systems. There has been recent interest in whether nitric oxide (NO), an important determinant of systemic, pulmonary, and coronary regional blood flow (36), also plays a role in the contractile function of the heart. The data in this regard have been controversial. Whereas some studies suggest a negative inotropic effect of NO, other studies do not (28, 31, 35, 47). Fewer studies have addressed the role of NO in diastolic relaxation (18, 41). Because all three NO synthase (NOS) isoforms are expressed in the heart (4, 5, 27, 32), the particular NOS isoform(s) that may be involved in the physiological regulation of cardiac contractility and relaxation has not been defined.

In this study, we use endothelial NOS (eNOS) mutant (eNOS−/−) mice as a tool to study the role of eNOS in cardiac function. eNOS−/− mice lack vascular endothelium-dependent relaxation in response to acetylcholine and are hypertensive compared with wild-type littermates, indicating the importance of eNOS to vascular tone and blood pressure regulation (22). We define the expression and localization of eNOS in the myocardium by immunohistochemistry, Western blot analysis, and NOS catalytic assay. To assess cardiac function in vivo, we monitor left ventricular pressure using a Millar catheter at baseline and in response to the β-adrenergic agonist isoproterenol (Iso). The maximum value of the first derivative of pressure with respect to time (dP/dtmax) is used as a measure of left ventricular contractility (systolic function). The time constant of isovolumetric relaxation (t) is used as a measure of left ventricular relaxation (diastolic function).

To confirm these results using a less load-sensitive technique, we measure contractile indexes in isolated hearts perfused in the Langendorff mode.

The dose-response curve of ventricular contractility to Iso is shifted to the left, with differences noted at doses of 1 ng and higher, in eNOS−/− mice compared with that of wild-type mice in vivo as well as in isolated heart preparations. These results are consistent with a role for eNOS-derived NO in blunting the response to Iso. N”-nitro-L-arginine (L-NNA) treatment of wild-type and NOS−/− mice has the same effect. We find no differences in β-adrenergic receptor binding between wild-type and eNOS−/− mice that would account for these changes. These results indicate that the eNOS isoform plays a physiological role in modulating cardiac systolic function. Baseline ventricular relaxation is similar in eNOS−/− mice and wild-type mice, whereas L-NNA treatment of wild-type mice markedly increases the time constant of diastolic relaxation. These results suggest that the eNOS−/− mice demonstrate compensatory mechanisms that maintain diastolic relaxation. To study whether these mechanisms are cGMP dependent, we measured cardiac cGMP levels. Surprisingly,
the cardiac cGMP level is the same in the eNOS−/− mice as in wild-type mice, despite the absence of eNOS. This led us to examine whether other stimulators of guanylyl cyclase might be upregulated. We find that the expression of prepro-atrial natriuretic peptide (prepro-ANP) is upregulated in the eNOS−/− mice, suggesting a molecular mechanism by which diastolic relaxation is maintained in the absence of eNOS.

MATERIALS AND METHODS

Animals. These experiments were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. eNOS−/− mice were generated as previously described (22). Adult female and male mice between 8 and 12 wk of age, weighing 24–30 g, were used. Three sets of wild-type control animals were included: wild-type littermates of the eNOS−/− mice, 129/SvJb wild-type mice, and C57BL6 wild-type mice. The latter two strains were used because the eNOS−/− mice were derived from 129/SvJb embryonic stem cells (J1) and C57BL6 blastocysts. They were included to confirm that the observed phenotypes were due to eNOS gene disruption and not to genetic background effects. For the isolated heart experiments, eNOS−/− mice backcrossed to the C57BL6 background for 10 generations were used. All animals were housed in a dedicated barrier facility in microisolator cages and received autoclaved food and water ad libitum.

eNOS immunohistochemistry. Wild-type (n = 3) and eNOS−/− mice (n = 3) were euthanized, and the heart from each mouse was removed. The hearts were washed in ice-cold phosphate-buffered saline (PBS) and frozen on dry ice. Cryostat sections (16-µm thick) were air dried, fixed in 4% paraformaldehyde, and treated with 10 mM citrate buffer at 100°C for 10 min. After the sections were preincubated in 2% horse serum, monoclonal eNOS antibody (anti-ECNOS, N30020, Transduction Laboratories, Lexington, KY) was added to 1:250 dilution of horse serum and 1% bovine serum albumin (BSA). After 30-min incubation, the sections were rinsed, and transferred to nitrocellulose. A 1:250 dilution of antimouse secondary antibody (goat antimouse, Amersham, Buckinghamshire, UK) was used. Bands were visualized with ECL reagents (Amersham, Buckinghamshire, UK).

Hemodynamic measurements in vivo. Mice were anesthetized with intraperitoneal injection of 2,2,2-tribromoethanol (Avertin, Aldrich Chemicals, Milwaukee, WI), 2% solution in PBS, at a dose of 0.022 ml/g body wt. Anesthesia was considered adequate when no muscle response or change in blood pressure was observed to a tail pinch test. For physiological recordings, animals were placed on a heat-controlled operating table, and body temperature was maintained at 36.5–37°C using a rectal thermometer probe and a DC temperature control module (FHC, New Brunswick, ME). Under these experimental conditions, physiological pH (7.30 ± 0.04), PCO2 (31.4 ± 4.1 mmHg), and PO2 (146.7 ± 5.9 mmHg) remained within normal limits without artificial ventilation, as determined using a pH/blood gas analyzer (model 178B, Ciba-Corning, Medfield, MA) in selected animals at the end of the experiments (n = 6). For continuous blood pressure measurements and blood gas sampling, a catheter made of pulled polyethylene-10 tubing was inserted into the left femoral vein. A second polyethylene-10 catheter was inserted into the right femoral vein for intravenous delivery of saline or Iso (Sigma Chemicals, St. Louis, MO). A midline incision was made over the trachea down to the xyphoid bone. A 1.8-Fr Micro-Tip Catheter Transducer (Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle of the heart for continuous left ventricular pressure measurements. After the placement of the catheters, each animal was allowed to stabilize for at least 10 min or until stable blood pressure, heart rate, and maximal rate of pressure development (dP/dtmax) were observed. Baseline values of heart rate, blood pressure, and left ventricular pressure were then recorded. Intravenous bolus doses of Iso (0.1–10 ng in 20 µl saline) were injected, and arterial and cardiac pressures were recorded continuously. To assess the effect of NOS inhibition, L-NNA (Sigma Chemicals) was injected intraperitoneally at a dose of 12 mg/kg. This amount is sufficient to block vascular responses to cholinergic stimulation (24). After 30 min, baseline measurements and dose response to Iso were measured again. In some animals, saline was given instead of L-NNA to ensure that anesthesia and physiological parameters were stable in the animals throughout the experiment. To elevate blood pressure in wild-type animals to levels observed in eNOS−/− mice, 1 mg/kg phenylephrine (Sigma Chemicals) was administered intraperitoneally (n = 6). To test the role of ANP in ventricular relaxation, 10 µg ANP (Sigma Chemicals) was given intravenously (10 µg/33 µl saline, n = 4).

Isolated heart preparation. Krebs-Henseleit buffer (KHB) was prepared fresh on the day of the experiment (containing in mM: 118 NaCl, 4.7 KCl, 1.75 CaCl2, 1.2 MgSO4, 1.2 K2HPO4, 25 NaHCO3, 0.5 EDTA, and 11 glucose) and equilibrated with 5% CO2-95% O2, resulting in a pH of 7.4. Wild-type and eNOS mutant mice (n = 10 for both groups) were anesthetized with Nembutal (80 mg/kg), and the heart and the lungs were excised. The aortic arch was dissected in ice-cold KHB, and the heart was retrogradely perfused via the aorta in the Langendorff mode under constant pressure (75 mmHg) with KHB at 37°C. For left ventricular pressure measurements, a water-filled balloon connected to the pressure transducer was introduced into the left ventricle through an incision of the left atrium. The balloon was inflated to set the left ventricular end-diastolic pressure (EDP) to 6–8 mmHg. Iso was dissolved in KHB (0.1–10 ng/20 µl). LV pressure was recorded at a sampling rate of 1 kHz, and dP/dt and heart rate were calculated online.
Receptor binding. Left ventricles were washed in ice-cold saline and homogenized in 1 ml of binding buffer (100 mM Tris·HCl, 2 mM EGTA, 1 mM MgCl2, pH 7.2) for 1 min with Tissue-Tearor (Biospec) on ice. Homogenates were centrifuged at 14,000 g for 20 min, and the pellet was homogenized again in 1 ml of binding buffer with a ground glass homogenizer. Samples were centrifuged as above, and the pellet was resuspended in binding buffer and frozen at −70°C. β-Adrenergic agonist binding studies were performed in Multi-Screen FB glass fiber plates (Millipore, Bedford, MA) in binding buffer containing [125I]iodocyanopindolol (6–486 pM) and membrane homogenate (20 µg protein/well) in a total volume of 200 µl. Iso (100 µM) was used to determine nonspecific binding. Assays were performed at 37°C for 40 min and terminated by filtration through the glass fiber membrane. Bound and free [125I]iodocyanopindolol was determined using a gamma counter. Assays were performed in triplicate.

cAMP and cGMP determination. Left ventricles were washed in ice-cold PBS, homogenized in 10% trichloroacetic acid, centrifuged, and extracted with water-saturated ether. The aqueous phase was transferred to a fresh tube and vacuum dried. The resulting pellet was resuspended in sodium acetate buffer and used for cAMP and cGMP radiommunoassay. Aliquots of cyclic nucleotide standards and samples were incubated overnight at 4°C with cAMP or cGMP antiserum in the presence of [3H]cAMP or [3H]cGMP. After the addition of excess acetate buffer, the tubes were centrifuged, and the radioactivity in the pellets was counted. Cyclic nucleotide levels were determined from the standard curve. [3H]cAMP, [3H]cGMP, cAMP, and cGMP antisera, and cyclic nucleotide standards were obtained from Biomedical Technologies (Stoughton, MA).

Northern blotting. RNA was extracted from the left ventricle using Ultraspec RNA isolation system (Biotex, Houston, TX) according to the manufacturer’s specifications. Briefly, ventricles were homogenized, extracted with chloroform, and precipitated with isopropanol. RNA was washed in ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. RNA (10 µg) was run on a 1% agarose gel and transferred to Hybond-N nylon membrane (Amersham). A 0.4-kb fragment of prepro-ANP cDNA, kindly supplied by K. D. Bloch, was radiolabeled using random primers (PrimeIt, Stratagene, LaJolla, CA) and hybridized with the membrane in the presence of formamide under standard conditions (2).

Membranes were washed in 0.2× SSC (20× SSC is 3 M salt, 0.3 M sodium citrate)/0.1% SDS at 42°C and exposed with Kodak XAR film overnight at −80°C using an intensifying screen.

Data analysis. Left ventricular and arterial pressures were recorded and analyzed with a MacLab/8S System and a Power Macintosh 7100/66 computer using Chart version 3.5.1b software at 0.2 or 1-kHz sampling rate (AD Instruments, Milford, MA). τ was calculated by the derivative method (7) as indicated by the following. dp/dt was plotted against the left ventricular pressure (P), and the slope (A) of the portion of the plot corresponding to isovolumetric relaxation (between dp/ dtmin and P min + 5 mmHg) was used to determine the time constant τ, where −1/A equals τ. Statistical analysis was performed using StatView 4.51 (Abacus Concepts, Berkeley, CA). Two-way ANOVA for repeated measures followed by Scheffé’s F procedure was used, and a probability value of P < 0.05 was considered significant.

RESULTS

Cardiac NOS catalytic activity is markedly reduced in eNOS−/− mice. We measured NOS activity in the heart, lung, and aorta by assaying the conversion of [3H]arginine to [3H]citrulline in tissue homogenates. Wild-type heart, lung, and aorta all contain detectable amounts of NOS catalytic activity that is blocked by Nω-nitro-L-arginine methyl ester (L-NAME) (Table 1). No activity is seen in the absence of calcium. The tissue from eNOS−/− mice shows markedly reduced NOS catalytic activity. eNOS catalytic activity in the heart of wild-type mice is 3.7 ± 0.1 pg mg−1·min−1 compared with a value of 0.6 ± 0.1 pg mg−1·min−1 in the eNOS−/− mice. The residual activity in the eNOS−/− heart can be inhibited by L-NAME and likely represents other NOS isoforms such as neuronal NOS (nNOS). Thus the bulk of NOS catalytic activity in the heart is due to eNOS.

Western blot analysis of NOS isoform expression. Western blot analysis seen in Fig. 1 confirms that the heart, lungs, and aorta of wild-type mice contain immunoreactive eNOS protein, whereas the same tissues from eNOS−/− mice do not. Western blot analysis shows no inducible NOS (iNOS) expression in either wild-type or eNOS−/− mouse hearts, and there are no detectable differences in nNOS expression between wild-type and eNOS−/− mice (data not shown).

eNOS immunohistochemistry. We examined the expression and localization of eNOS in mouse cardiac tissue by immunohistochemistry. Intense staining is detected in the endothelium of cardiac vessels of wild-type mice (Fig. 2A). Similarly strong immunostaining is present in the endothelium of the aorta and in the endocardium of the ventricles and atria. In the myocardium, eNOS staining is detected in the capillaries but not in the myocytes (Fig. 2C). No eNOS immunoreactivity is found in the vessels within the myocardiun of eNOS−/− mice (Fig. 2, B and D).

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<th>Table 1. NOS catalytic activity in wild-type and eNOS mutant mice</th>
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Values are means ± SD for 5 animals per group. Nitric oxide synthase (NOS) catalytic activity in solubilized membrane extracts from tissues is determined by [3H]arginine to [3H]citrulline conversion (expressed as pg citrulline·mg protein−1·min−1). eNOS, endothelial NOS.

Fig. 1. Western blot on heart, lung, and aorta extracts of wild-type (WT) and endothelial nitric oxide synthase (eNOS) mutant (MUT) mice showing absence of eNOS immunoreactivity in mutants. Protein (10 µg) was subjected to SDS-PAGE, transferred to Hybond-N membrane, and probed with mouse monoclonal eNOS antibody.
Fig. 2. Immunohistochemical detection of eNOS protein in the myocardium. A: WT mice, intense staining is shown in microvascular and arterial endothelium (A, C). In the eNOS−/− mice, no similar staining is observed (B, D). Hematoxylin counterstaining shows cell nuclei in blue. Magnification: ×100 (A, B) and ×200 (C, D).

Baseline hemodynamic parameters. To ensure that variations between mouse strains did not confound our results, we studied three groups of wild-type mice: 1) wild-type littermates of eNOS−/− mice, which are derived from both C57BL/6 and 129/SVJae backgrounds; 2) wild-type C57BL/6 mice; and 3) wild-type 129/SVJae aemice. Blood pressure, heart rate, and contractility do not differ among these three groups, as shown in Table 2. The eNOS−/− mice are hypertensive, with a mean arterial pressure (MAP) of 102.1 mmHg, compared with wild-type mice, with a MAP of 71.5 mmHg. L-NNA treatment raises the blood pressure of wild-type mice. The baseline heart rate of wild-type animals (564 beats/min) is similar to that of eNOS−/− mice (547 beats/min). Systolic contractility at baseline, reflected by dP/dt_max of eNOS−/− mice (9,928 mmHg/s), is similar to that of the wild-type littermates (9,673 mmHg/s), as shown in Table 2.

Cardiac contractility response toIso in vivo. Increasing doses of the β-adrenergic agonist Iso (0.1–10 ng) were administered intravenously to obtain a dose-response curve of left ventricular dP/dt_max. Figure 3A shows a sample tracing from a wild-type mouse before Iso treatment. After Iso injection, dP/dt_max increases, reaches its peak value within 10–20 s, and returns to baseline within 3 min. The next dose is given only after dP/dt_max returned to baseline. When the same dose of Iso is given twice in this fashion, similar dP/dt_max responses are observed, demonstrating that tachyphylaxis does not occur in this system. Figure 4A shows the Iso dose-response curve for the three strains of wild-type and eNOS−/− mice. The dose-response curve of eNOS−/− mice to Iso lies to the left of the curves for wild-type animals, with differences at Iso doses of 1 ng and higher. At these doses, Iso has a greater effect on contractility in eNOS−/− mice than in wild-type mice (P < 0.01). After the Iso dose-response curve was recorded, mice were injected with L-NNA or saline. Thirty minutes later, an Iso dose-response curve was measured again. The effect of L-NNA was confirmed by an increase in blood pressure (Table 2). Thirty minutes after the L-NNA injection, MAP reached a plateau and remained at this level for at least 3 h. When wild-type mice are injected with L-NNA, the Iso dose-response curve shifts to the left compared with the pretreatment curve, overlapping that of the eNOS−/− mice (Fig. 4B). L-NNA treatment of eNOS−/− mice does not alter the dP/dt_max response. The heart rate response to Iso is not

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<th>MAP, mmHg</th>
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<td>129/SVJ ae</td>
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<td>92.1 ± 7.4*</td>
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<td>eNOS−/−</td>
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<tr>
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<td>94.4 ± 17.7†</td>
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Values are means ± SD; n = number of mice. Baseline hemodynamic parameters of wild-type mice of wild-type littermates and eNOS (eNOS−/−) mice before and after N-nitro-L-arginine (L-NNA) treatment. Mean arterial pressure (MAP), heart rate (HR), and contractility (dP/dt_max) values before and after L-NNA treatment are listed. †P < 0.05 compared with corresponding untreated controls.

†P < 0.05 compared with wild-type littermates.
significantly different between wild-type and eNOS−/− (Fig. 4C). Iso decreases blood pressure temporarily in all groups to a similar extent (Fig. 4D). Iso improves emptying of the left ventricle, as demonstrated by slight decreases in left ventricular EDP. At each dose of Iso, EDP values were similar in all treatment groups (Fig. 4E). In mice injected with saline instead of L-NNA, the second Iso dose-response curve is unchanged from the original curve, showing that contractility is not altered by the period of anesthesia or the intraperitoneal injection (Fig. 4F).

To estimate the effect of increased afterload in eNOS−/− mice in vivo, blood pressure was raised in wild-type mice to levels seen in the eNOS−/− mice using phenylephrine. Intraperitoneal injection of phenylephrine (1 mg/kg) causes a prolonged increase in MAP from 73.1 to 100.6 mmHg and baseline dP/dtmax from 7,406 to 10,920 mmHg/s (n = 6). This degree of afterload increase, however, does not affect the contractility response to Iso (Fig. 5), demonstrating that the increased contractile response in the eNOS−/− mice is not due to higher afterload.

Ventricular relaxation. Ventricular relaxation was assessed in vivo using the Millar catheter, by determining the time constant of the speed of the ventricular relaxation (τ) by the derivative method (7). τ, an index of lusitropy, represents the time required for the left ventricular pressure to decline to 1/e of any initial value during isovolumetric relaxation. The baseline value for τ in wild-type mice is 5.2 ± 1 ms (Fig. 6A). Inhibition of NO by L-NNA pretreatment increases τ to 8.3 ± 2.4 ms, a 60% increase. In the eNOS−/− mice, however, τ is 5.6 ± 1.5 ms, similar to the values measured in wild-type mice. L-NNA has no effect on τ in eNOS−/− mice. In addition, τ also remains unchanged after phenylephrine treatment (Fig. 6B).

Isolated heart experiments. To further confirm the enhanced contractile response of the eNOS−/− mice independent of their increased afterload, we studied isolated hearts perfused at constant pressure in Langendorff mode. Baseline contractility is similar in wild-type and eNOS−/− mice (2,813 ± 199 and 2,692 ± 415 mmHg/s, respectively). After the administration of Iso, eNOS−/− hearts show significantly increased contractile response at Iso doses of 1 ng and higher, in agreement with the in vivo findings (P < 0.02, Fig. 7A). Baseline heart rate (wild-type: 347 ± 31 beats/min, eNOS−/−: 348 ± 30 beats/min) and the positive chronotropic response to Iso is similar in the two groups (Fig. 7B). In the Langendorff preparation, relaxation was assessed using dP/dtmin. Baseline dP/dtmin is unchanged in the eNOS−/− hearts, but they showed enhanced relaxation in response to Iso stimulation (Fig. 7C).

β-Adrenergic receptor binding. Because changes in β-receptor number or affinity can also alter myocardial contractility (1, 34), we compared β-adrenergic receptor binding characteristics in the left ventricles of wild-type and eNOS−/− mice (Fig. 8). Scatchard analysis reveals no difference in maximum binding capacity (Bmax = 41.4 ± 9.4 fmol/mg protein, and 36.1 ± 5.1 fmol/mg for wild-type and eNOS−/−, respectively). Similarly, the apparent dissociation constants (Kd) are not significantly different between the two groups of animals (Kd: 72.4 ± 10.4 and 50.1 ± 14.5 pM for wild-type and eNOS−/−, respectively). Thus we do not detect changes in β-receptor binding that would account for the differences observed in response to Iso between wild-type and eNOS−/− mice.

Cyclic nucleotides and ANP expression. There are no significant differences in cAMP (69.7 ± 14.7 and 78.9 ± 10 pmol/mg in wild-type and eNOS−/−, respectively) and cGMP (3.1 ± 0.8 and 3.1 ± 0.6 pmol/mg in wild-type and eNOS−/−, respectively) levels, determined by RIA, between eNOS−/− and wild-type mice. Because NO and ANP are the major physiological stimuli for soluble and membrane-bound guanylyl cyclases in the heart, we considered whether ANP could
compensate for the absence of eNOS in the eNOS−/− mice. We determined expression levels of prepro-ANP by Northern blotting of RNA extracted from the left ventricle. A single 0.9-kb band is detected in each lane. eNOS−/− mice show strong upregulation of prepro-ANP expression compared with wild-type mice (Fig. 9).

To test whether ANP plays a physiological role in modulation of ventricular relaxation, we assessed ventricular relaxation in wild-type mice after intravenous administration of ANP (10 µg). Whereas ANP has no effect on baseline, it restores ventricular relaxation of L-NNA-treated mice to normal levels, demonstrating that ANP is sufficient to compensate for NO in ventricular relaxation (Fig. 10). ANP administration to L-NNA-treated wild-type mice also restores blood pressure acutely to normal levels (L-NNA: 81.8 ± 5.9 mmHg, L-NNA + ANP: 67.1 ± 8.3 mmHg, P < 0.05) but does not change heart rate (L-NNA: 458 ± 49 beats/min, L-NNA + ANP: 446 ± 28 beats/min, n = 4).
DISCUSSION

The role of NO in modulating cardiac function has been studied using several approaches, including administration of NOS inhibitors, administration of NO donors, and use of genetically altered animals. It has been studied at several levels, including cardiac ventricular myocytes, papillary muscle preparations, isolated perfused hearts, and intact animals. Despite many studies, the precise role(s) of NO in modulating cardiac function is still the topic of much debate (for example, see Ref. 21) because of conflicting results.

The earliest studies to report a negative inotropic effect of NO show that N²-monomethyl-L-arginine (L-NMMA) blocks the effect of cytokines in isolated hamster papillary muscle (14) and that L-NNA increases the inotropic effect of Iso in isolated rat ventricular myocytes (3). In humans with left ventricular dysfunction, the effect of intracoronary infusion of dobutamine is augmented by L-NMMA (20), demonstrating that NO attenuates myocardial contractility in the failing heart.

In dogs, some studies show that NOS inhibition attenuates the response to β-agonists (29, 39), but others find no effect (10, 28). Still other studies (31, 35) suggest the opposite effect, that NO may augment cardiac contractility. Part of the reason for these conflicting results may lie in differences between the experimental preparations and differences among the NOS inhibitors, the routes of their administration, or effects on more than one NOS isoform. For example, the inhibition of vasodi-
iation to acetylcholine in coronary vessels by NOS antagonists is frequently used to verify NO inhibition, whereas myocardial NOS inhibition may require longer perfusion time after intracoronary drug administration.

In this report, we study cardiac contractility and relaxation in homozygous mice in which the gene for eNOS has been disrupted. These animals have been useful tools to study the roles of eNOS in various physiological and pathophysiological processes (12, 13, 19, 23, 24, 37, 38, 42–44, 46). Our results establish the presence of eNOS protein in the wild-type mouse heart by Western blot and NOS catalytic activity and its localization by immunohistochemistry. The eNOS\(^{−/−}\) mouse heart shows no detectable eNOS protein and marked reduction in NOS catalytic activity. The majority of eNOS in the heart is localized to the macro- and microvascular endothelium present throughout the myocardium, providing access of nearly all myocardial cells to NO.

The eNOS\(^{−/−}\) mice show similar basal systolic contractility as the wild-type mice in both intact animals and in the isolated perfused heart, suggesting that basal NO production does not influence contractility in the resting heart. On \(β\)-adrenergic stimulation, however, NO antagonizes the effect of ISO at doses of 1 ng or higher. The eNOS isoform is responsible for the inotropic effect, because the ISO dose-response curve of eNOS\(^{−/−}\) mice is indistinguishable from L-NNA-treated wild-type mice, and treatment of eNOS\(^{−/−}\) mice with L-NNA has no effect. These results suggest that eNOS modulates the systolic response to \(β\)-adrenergic stimulation in the intact animal.

In vivo studies using eNOS\(^{−/−}\) mice are potentially complicated by hypertension, because \(dP/dt_{\text{max}}\) is influenced by loading conditions. In the current study, preload, heart rate, and blood pressure response to ISO are comparable between animal groups. To control for increased afterload, we increased the blood pressure in a group of wild-type animals to levels seen in the eNOS\(^{−/−}\) mice. We observed no change in the contractile response to ISO, demonstrating that acute increases in afterload do not explain the increased contractility seen in the eNOS\(^{−/−}\) mice. However, the use of phenylephrine may be complicated by direct \(α\)-adrenergic effects on the coronary circulation or on the myocardium. The phenylephrine experiment also does not account for potential chronic effects of hypertension, such as molecular or structural adaptations to increased load or upregulation of \(β\)-adrenergic receptors secondary to baroreflex-mediated sympathetic withdrawal. In this regard, we did not detect anatomic or

Fig. 8. \(β\)-receptor binding in membrane homogenates from left ventricle. Saturation curve shows specific binding of \([^{125}\text{I}]\)iodocyanopindolol (I-Cyp) in WT (○) and eNOS\(^{−/−}\) mice (□). Scatchard plot (inset) shows no difference in \(β\)-adrenergic receptor number or binding affinity between WT and eNOS\(^{−/−}\) mice. A representative graph is shown from one of three experiments. B/F, bound I-Cyp/free I-Cyp.

Fig. 9. Northern blot of prepro-atrial natriuretic peptide (ANP) mRNA from left ventricular myocardium. RNA (10 µg) from WT and eNOS\(^{−/−}\) mice were run on agarose gel in duplicates, blotted to nylon membrane, and probed with a radiolabeled prepro-ANP probe. A single band of expected size (0.9 kb) was detected. Bottom: ethidium bromide staining of 28S and 18S ribosomal RNAs as an indication of equal loading of RNA samples.

Fig. 10. Effect of ANP on ventricular relaxation in L-NNA-treated WT mice (n = 4). L-NNA (12 mg/kg) was injected intraperitoneally. Thirty minutes later ANP (10 µg in 33 µl saline) was injected through femoral vein.
histologic changes suggestive of left ventricular hypertrophy at the age of animals used in this study (8–12 wk). We also found no changes in β-receptor binding in the eNOS−/− hearts compared with those in the wild-type hearts. In vivo studies might also be complicated by effects of anesthesia on blood pressure and respiration. For example, slightly decreased blood pH is observed in many experimental settings in mice (11).

To confirm the increased contractility seen in the eNOS−/− mice using an independent, less load-sensitive technique, we studied isolated hearts perfused in the Langendorff mode at constant pressure. The data obtained from the Langendorff hearts yielded similar results to the in vivo findings, confirming the role of eNOS in modulating cardiac contractility. Because the hearts were perfused at constant pressure (75 mmHg), and EDP was maintained between 6 and 8 mmHg, this method is not subject to differences in afterload, preload, or differences in endogenous sympathetic tone.

These results differ from those reported by Vanderschaele et al. (47), who found that β-adrenergic regulation of heart rate, force of contraction, and calcium current are preserved in the papillary muscle of eNOS−/− mice. Explanations for this discrepancy might include different responsiveness of isolated papillary muscle and the intact ventricular myocardium to NO and their use of 3- to 6-mo-old mice with modestly hypertrophied ventricles.

Our results indicate that basal NO production is important to ventricular relaxation, because L-NNA treatment of wild-type mice increases \( \tau \) significantly. These results are in agreement with previous data in humans (41) and isolated guinea pig hearts (18). In contrast to the response of wild-type mice treated with L-NNA, eNOS−/− mice demonstrate normal ventricular relaxation, which is unaffected by L-NNA. The lack of effect of L-NNA argues against a role for other NOS isoforms. These results suggest alternative mechanisms that compensate for absence of eNOS in the mutant mice. Similar physiological compensation has been demonstrated for physiological processes in nNOS mutant mice (9, 25, 26, 33). The baseline dP/d t \text{min} values obtained from the Langendorff hearts confirm that ventricular relaxation is normal in the eNOS−/− hearts. In addition, the dP/d t \text{min} response to ISO indicates that NO antagonizes the β-adrenergic stimulation of ventricular relaxation, similar to its effect on contractility. This suggests that NO antagonizes the β-adrenergic pathway at a point that is common to relaxation and contractility. In vivo measurements of \( \tau \) are less reliable at higher ISO doses, therefore they cannot be directly compared with the dP/d t \text{min} data.

To better define the molecular mechanisms of compensation for ventricular relaxation in the eNOS−/− mice, we measured cyclic nucleotide levels in the heart. Cardiac cGMP levels are the same in the eNOS−/− mice as wild-type mice, despite the absence of functional eNOS. This observation led us to look for other physiological stimulators of cardiac guanylyl cyclase activity, such as ANP. We found that ANP expression is upregulated in the left ventricle of eNOS−/− mice, providing a mechanism to maintain baseline cGMP levels despite the absence of eNOS. Indeed, when ANP is injected intravenously into L-NNA-treated wild-type mice, it restores ventricular relaxation. ANP also lowers blood pressure elevated by L-NNA. However, the phenylephrine experiments suggest that blood pressure changes within this range are unlikely to alter \( \tau \) (Fig. 8B). ANP by itself enhances ventricular relaxation in humans (8, 49) and may work in conjunction with NO (6, 40). Our data do not indicate whether increased cardiac ANP levels are the direct consequence of eNOS gene disruption or whether they are due to secondary changes such as increased afterload. It is apparent, however, that increased levels of ANP are present and sufficient to restore left ventricular relaxation in the eNOS−/− mice.

The overall inotropic effect of ISO appears to reflect a balance between β1- and β2-adrenoceptors and the β3-adrenoceptor (17). β1- and β2-adrenoceptors mediate the positive inotropic effect, whereas activation of the β3-adrenoceptor results in a negative inotropic effect. L-NMMA inhibits the negative inotropic effect of β3-adrenoceptor activation, suggesting that the β3-adrenoceptor acts by stimulating NO production (17). This hypothesis fits well with our observation that baseline contractility is not influenced by NO, but β-adrenergic stimulation is attenuated by increased NO production. Because changes in the number and/or affinity of adrenergic receptors could affect the inotropic response to ISO, we measured β-receptor binding in membrane preparations of wild-type and eNOS−/− mice. We found no difference in the maximum binding capacity and binding affinity for [125]iodocyanopindolol, suggesting that changes in β-receptors do not account for the observed differences between wild-type and eNOS−/− mice. Changes in downstream signaling pathways could be involved in the effects of NO on cardiac contractility and relaxation. Elevated cGMP levels stimulate cAMP phosphodiesterase, promoting degradation of cAMP. cGMP-activated protein kinase G blocks activation of sarcocellular L-type calcium channels, resulting in decreased calcium influx. NO directly regulates the ryodine-sensitive calcium channel of the sarcoplasmic reticulum (15, 45, 48). NO may also decrease contractility by reducing myocardial phosphocreatine and ATP pools (30). During ventricular relaxation, cGMP-activated protein kinase G phosphorylates phospholamban, which disinhibits the sarcoplasmic reticulum Ca2+ ATPase calcium channel thus increasing calcium reuptake into the sarcoplasmic reticulum.

The time scale on which NO production modulates cardiac function appears to differ between contractility and relaxation. Because cardiac cGMP levels are the same in eNOS mutant mice as in wild-type mice, changes in the systolic response to ISO cannot be due to differences in basal, resting cGMP level but rather are due to transient increases during contraction. Diastolic relaxation, on the other hand, may depend on cytoplasmic cGMP levels on a much longer time scale, so that
ANP upregulation is sufficient to maintain cGMP levels and restore ventricular relaxation.

In conclusion, we provide evidence in intact animals and in the isolated Langendorff heart preparation that the eNOS isoform attenuates systolic contractility response to ISO. Our results also suggest that eNOS-derived NO blunts the lusitropic effect of β-adrenergic stimulation. In the absence of sympathetic stimulation, NO does not influence baseline contractility, but it appears to modulate ventricular relaxation in conjunction with ANP.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-33335 and National Heart, Lung, and Blood Institute Grant HL-52818. P. L. Huang is an Established Investigator of the American Heart Association.

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Received 5 May 1999; accepted in final form 14 October 1999.

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