Control of myocardial oxygen consumption in transgenic mice overexpressing vascular eNOS

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Walsh, E. K., H. Huang, Z. Wang, J. Williams, R. de Crom, R. van Haperen, C. I. Thompson, D. J. Lefer, and T. H. Hintze. Control of myocardial oxygen consumption in transgenic mice overexpressing vascular eNOS. Am J Physiol Heart Circ Physiol 287:H2115–H2121, 2004.—Our objective was to investigate the potential role of selective endothelial nitric oxide (NO) synthase (eNOS) overexpression in coronary blood vessels in the control of myocardial oxygen consumption (MVO$_2$). Transgenic (Tg) eNOS-overexpressing mice (eNOS Tg) (n = 22) and wild-type (WT) mice (n = 24) were studied. Western blot analysis indicated greater than sixfold increase of eNOS in cardiac tissue. Echocardiography in awake mice indicated no difference in cardiac function between WT and eNOS Tg; however, systolic pressure in eNOS Tg mice decreased significantly (Δ26 ± 2.3 to 109 ± 2.3 mmHg; *P* < 0.05), whereas heart rate (HR) was not different. Total peripheral resistance (TPR) was also decreased (9.8 ± 0.8 to 7.6 ± 0.4 4 mmHg·ml⁻¹·min⁻¹; *P* < 0.05) in eNOS Tg. Furthermore, female eNOS Tg mice showed even lower TPR (7.2 ± 0.4 mmHg·ml⁻¹·min⁻¹) compared with male eNOS mice (8.6 ± 0.5, mmHg·ml⁻¹·min⁻¹; *P* < 0.05). Left ventricular slices were isolated from WT and eNOS Tg mice. With the use of a Clark-type oxygen electrode in an airtight bath, MVO$_2$ was determined as the percent decrease in myocardial respiration through nitrolysis of aconitate in the tricarboxylic acid cycle, complexes I and II of the electron transport chain, and cytochrome c oxidase (4, 5, 12).

In recent years, we (28, 38) and others (3) have demonstrated that NO plays a significant role in modulating myocardial O$_2$ consumption (MVO$_2$) during exercise, heart failure, and in response to various agonists. Furthermore, the control of MVO$_2$ by NO was established in genetically altered mice in which the B$_2$-kinin receptor as well as inducible NO synthase (iNOS) and endothelial NOS (eNOS) have been knocked out (27, 29).

Loke et al. (29) found that in mice deficient in iNOS, the regulation of MVO$_2$ was due to stimulation of NO synthesis and this is confirmed by the ability of l-arginine analogues to inhibit NOS. A transgenic (Tg) mouse overexpressing the constitutive eNOS gene in blood vessels has been engineered (23, 42). Studies in these transgenic mice have added to the notion that NO plays a key role in regulating the redox state of cardiac tissues. The overexpression of eNOS attenuates congestive heart failure by decreasing pulmonary and cardiac dysfunction, protects against ischemia-reperfusion injury, inhibits lesion formations in blood vessels, reverses diminished vasorelaxation during ischemic heart failure, and allows for increased oxygenation to skeletal muscle due to vasodilation (1, 2, 9, 19, 20, 32, 34). The goal of our study was to determine whether increased production of NO subsequent to vascular overexpression of eNOS in the mouse heart results in tighter control of MVO$_2$. In part, the goal of this study was to substantiate the contention that blood vessel-derived NO can regulate MVO$_2$.

**MATERIALS AND METHODS**

Tg eNOS-overexpressing mice. A DNA fragment containing the human eNOS gene was isolated from a homemade human genomic cosmid library (18, 23) using eNOS cDNA (kindly donated by Dr. S. Janssens, Leuven, Belgium) as a probe. In addition, the DNA fragment contained −6 kb of the 5′-natural flanking sequence, including the native eNOS promoter, and −3 kb of the 3′-sequence to the gene. Vector sequences were removed by restriction endonucleases. A solution of 1–2 μg/ml DNA was used for microinjection into fertilized oocytes from FVB donor mice and transplanted into the oviducts of pseudopregnant B10 × CBA mice. Founder mice and offspring were genotyped by PCR using DNA isolated from tail biopsies. The primers used were sense, 5′-GTCCTGCAGACCGTGCAAGC-3′, and antisense, 5′-GGCTGTGTTGTCGTGACCG-3′. Mice were backcrossed to C57B/6 for at least five generations (>96%) C57B/6. All eNOS transgenic mice were hemizygous (18, 23, 42).

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Hemodynamics. Male and female (n = 22) Tg eNOS and WT (C57B/6X129) (n = 24) mice (12–16 wk old) were used to measure blood pressure, cardiac function, and MVO₂. All mice were housed in the same type of cage and supplied with standard mouse chow and had free access to water. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western blot analysis. The right ventricle was harvested from anesthetized eNOS Tg mice and from C57B/6 WT controls. The tissue was pulverized in liquid nitrogen and resuspended in lysis buffer containing protease inhibitors (14). After sonification for 60 s, samples were centrifuged at 5,000 rpm for 10 min, and protein concentration was determined as previously described (14). Briefly, 100 μg of protein were separated on a 7.5% SDS-PAGE, followed by an electronic transfer to a polyvinylidene fluoride membrane (Amer sham; Piscataway, NY). Human umbilical vein endothelial cell protein was loaded as a positive control for eNOS. The transferred proteins were incubated with a 1:500 dilution of a monoclonal anti-eNOS antibody (Affinity BioReagents; Golden, CO), a monoclonal 1:1,000 dilution anti-iNOS antibody, or a monoclonal 1:500 dilution anti-neuronal NOS (nNOS) antibody (BD Transduction Labs; San Diego, CA) at 4°C overnight. The bound primary antibody was detected by a peroxidase-coupled anti-mouse antibody (dilution 1:2,000; Amersham), followed by a chemiluminescent reaction using luminol (SuperSignal West Pico, Pierce; Rockford, IL). Afterward, the membrane was exposed to a film, and bands were analyzed by densitometry, as previously published (14, 26).

Tail-cuff blood pressures. Blood pressures (systolic and diastolic) were measured by an automated tail-cuff method (model NIBP-8; Columbus Instruments) along with the heart rate in unanesthetized mice. Each mouse was trained in the morning for five sessions in holding chambers warmed to 37–39°C to maximize arterial dilatation. Five consecutive stable readings were averaged in each mouse.

Transthoracic two-dimensional Doppler echocardiography studies for cardiac morphology and function. Transthoracic two-dimensional echocardiography was performed in awake mice trained for 3 days with the use of an electrocardiograph (model Sequoia 256, Acuson) equipped with a 15-MHz linear transducer (15L8) in a phased-array format. Aquasonics ultrasound transmission gel (model 100, Parker Labs; Fairfield, NJ) was centrifuged at 1,000 rpm for 15 min to release any resolution artifacts. The gel was then warmed for 30 min in a water bath to avoid inducing hypothermic bradycardia. Generally, the heart was first imaged by placing the probe parallel to the sternum to obtain a long-axis view. From this image, we traced the left ventricular (LV) area during diastole (LVDA) and systole (LVSA) and calculated the ejection fraction (EF) over three consecutive cardiac cycles. The probe was then placed to obtain the parasternal short-axis view at the level of the pulmonary valve to measure the velocity time index. The parasternal short-axis view also allowed for an additional calculation of EF using B-mode. In the short-axis view, the heart was imaged with the two-dimensionally guided M-mode at the level of the papillary muscles. End-diastolic (LVEDD) and systolic (LVESD) LV chamber dimensions as well as posterior wall thickness were measured using the American Society of Echocardiography leadingedge techniques (16, 17, 43).

The equations used for hemodynamic measurements are the following:

\[ \text{SF} (%) = \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100 \]

\[ \text{EF} (%) = \left( \frac{\text{LVDA} - \text{LVSA}}{\text{LVDA}} \right) \times 100 \]

\[ \text{LV}_{\text{max}} = 1.055 \left( \text{IVST} + \frac{\text{LVEDD} + \text{PWT}}{3} - \text{LVEDD}^3 \right) \text{ (mg)} \]

\[ \text{LV}_{\text{cavity}} = \text{LVEDD}^3 \text{ (mm}^3) \]

where SF is shortening fraction, IVST is interventricular septum thickness, and PWT is posterior systolic wall thickness, LV_{cavity} refers to LV chamber dimension, and LV_{max} is LV weight.

Measurement of MVO₂. A Clark-type platinum O₂ electrode system was placed (model 5331, Yellow Springs Instruments; Yellow Springs, OH) with isolated cardiac tissue slices to measure MVO₂. The O₂ electrode was inserted into a small glass reaction chamber (3–8 ml) maintained at a constant 37°C temperature bath (model 5301, Yellow Springs Instruments). A built-in magnetic stirrer (480 rpm at 60 Hz) located in the bath assembly was used to prevent clumping of the tissue in the incubation medium path of the Teflon electrode membrane.

Preparation of cardiac tissue slices. Mice were anesthetized with pentobarbital sodium (65 mg/kg), and 0.5 ml of plasma were immediately removed from the left ventricle for nitrate measurements (38). The heart was removed immediately. The atria, large coronary arteries, connective tissue, and fat were discarded. A piece of the right ventricle was dissected and frozen in liquid nitrogen for Western blot analysis. The left ventricle was then bisected such that each piece of muscle contained a portion of the septum, free wall, and apex.

Myocardial tissues were incubated in Krebs bicarbonate solution containing (mmol/l) 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.1 MgSO₄, and 5.6 C₆H₁₂O₆ at 37°C, bubbled with 21% O₂-5% CO₂-74% N₂ (pH 7.4) to equilibrate for at least 2 h.

At the end of each incubation period, each piece of tissue was placed in a stirred bath with 3 ml of air-saturated Krebs bicarbonate solution containing 10 mmol/l HEPES (pH 7.4). The bath was sealed using a Clark-type platinum O₂ electrode. Succinate (5 × 10⁻⁴ mol/l), a substrate for complex II, and sodium cyanide (10⁻³ mol/l), an inhibitor of complex IV of the electron transport chain, were added in succession at the completion of the concentration-response curve to each agonist to confirm that the changes in O₂ uptake were effects due to mitochondrial respiration. The 100% increase in O₂ consumption in the presence of succinate suggest that O₂ is not limiting in the bath, and the abolition of O₂ consumption after the addition of sodium cyanide confirms that changes in MVO₂ were of mitochondrial origin (24, 27–29, 37, 40, 48).

Effect of bradycardin, carbachol, and N⁶-nitro-1-arginine methyl ester on MVO₂. Cumulative concentrations of the B₂ kinin receptor agonist bradykinin (BK; 10⁻¹⁰–10⁻⁴ mol/l, Sigma) and the muscarinic receptor agonist carbachol (CCh; 10⁻¹⁰–10⁻⁴ mol/l, Sigma) were given alone or in the presence of the NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME) (10⁻⁴ mol/l, Sigma).

Data and statistical analysis. Myocardial tissue respiration was calculated as the rate of decrease in P O₂, assuming an initial O₂ tension in tissue in the incubation medium path of the Te electrode membrane. Data were then analyzed with the Student’s t-test.

RESULTS

Western blot analysis. Western blot analysis of eNOS Tg (n = 6) mice showed an approximate sixfold increase in eNOS...
protein (Fig. 1). There was no significant difference in protein expression of iNOS (70 ± 9.8 vs. 74 ± 12) or nNOS (108 ± 19 vs. 106 ± 32) in WT and eNOS Tg mice, respectively.

Assessment of blood pressure by tail cuff. Table 1 summarizes hemodynamic measurements from five successive blood pressure measurements taken from each WT (n = 24) and eNOS Tg (n = 22) mouse. Systolic, diastolic, and mean arterial pressure (MAP) in eNOS Tg mice versus WT were significantly lower (126 ± 2.3 to 109 ± 2.3, 84 ± 1.3 to 70 ± 1.9, and 97 ± 1.3 to 82 ± 1.8 mmHg, P < 0.05, respectively). Total peripheral resistance (TPR), calculated as MAP divided by cardiac output (CO), normalized for body weight, was also significantly decreased in eNOS Tg mice (7.64 ± 0.35 vs. 9.81 ± 0.80 mmHg·ml⁻¹·min⁻¹; P < 0.05). Heart rate (HR) was not significantly different between the two groups.

Echocardiographic assessment of hemodynamics. Two successive echo measurements were taken from each individual mouse in the morning. There was no significant difference between WT and eNOS Tg mice (Table 1). There was an increase in plasma nitrate from 5.6 ± 0.4 to 8.1 ± 1.0 μM in eNOS Tg mice.

Gender differences in WT and eNOS mice. When each of the two groups, WT and eNOS Tg, were separated into male and female there were some significant differences in hemodynamics (Table 1). Most importantly, TPR was even further reduced in female eNOS Tg (7.17 ± 0.41 mmHg·ml⁻¹·min⁻¹) compared with male eNOS Tg (8.59 ± 0.53 mmHg·ml⁻¹·min⁻¹), whereas the WT females had a TPR of 8.83 ± 0.52 mmHg·ml⁻¹·min⁻¹ and the male WT had a TPR of 11.54 ± 1.58 mmHg·ml⁻¹·min⁻¹; P < 0.05.

Baseline MV O 2 in eNOS myocardial tissue. Baseline MV O 2 was not significantly different between WT (181 ± 13 nmol·g⁻¹·min⁻¹) and eNOS Tg (188 ± 14 nmol·g⁻¹·min⁻¹) or WT (153 ± 12 nmol·g⁻¹·min⁻¹) and eNOS Tg (165 ± 11 nmol·g⁻¹·min⁻¹) and in the presence of L-NAME.

Effect of bradykinin on MV O 2. BK (10⁻¹⁰–10⁻⁴ mol/l; Sigma, n = 6) caused a concentration-dependent decrease in MV O 2 in WT (17 ± 1.1%), with enhanced inhibition of MV O 2 in eNOS Tg mice (33 ± 2.7%) (Fig. 2). In the presence of L-NAME, the responses to BK were attenuated to 14 ± 1.3% and 15 ± 5.5% in both WT and in eNOS Tg mice, respectively.

Effect of CCh on MV O 2. Cumulative doses of CCh (10⁻¹⁰–10⁻⁴ mol/l, Sigma, n = 6) also caused a dose-dependent decrease in MV O 2 in WT by 20 ± 1.7% and an even larger decrease in eNOS Tg by 31 ± 2.0% (Fig. 3). The addition of L-NAME attenuated the reduction in MV O 2 (18 ± 2.7% and 23 ± 4.6%) in both WT and eNOS Tg, respectively.

| Table 1. Changes in hemodynamics in WT and eNOS Tg mice |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | eNOS Tg         |                |                |                |
|                                | Male            | Female         | Male            | Female         |
| Weight, g                      | 30 ± 0.6        | 24 ± 0.4       | 29 ± 0.5        | 23 ± 0.3       |
| HR, beats/min                  | 628 ± 14        | 609 ± 28       | 652 ± 16        | 613 ± 10       |
| EF, %                          | 78 ± 1.4        | 76 ± 1.0       | 76 ± 1.1        | 76 ± 1.1       |
| FS, %                          | 55 ± 1.3        | 54 ± 1.0       | 54 ± 0.7        | 54 ± 1.3       |
| IVSD                           | 0.85 ± 0.02†    | 0.74 ± 0.01    | 0.80 ± 0.02     | 0.75 ± 0.01    |
| IVSS                           | 1.63 ± 0.04†    | 1.49 ± 0.03    | 1.56 ± 0.28*    | 1.45 ± 0.03    |
| LVEDD, mm                      | 2.87 ± 0.06     | 2.83 ± 0.05    | 2.84 ± 0.05     | 2.73 ± 0.05    |
| LVESD, mm                      | 1.29 ± 0.05     | 1.3 ± 0.04     | 1.3 ± 0.03      | 1.25 ± 0.05    |
| LPWD                           | 0.95 ± 0.03     | 0.9 ± 0.02     | 1.05 ± 0.02†    | 0.87 ± 0.02    |
| LPWS                           | 1.24 ± 0.05     | 1.23 ± 0.04    | 1.4 ± 0.04†     | 1.14 ± 0.03    |
| CO, ml/10 g                    | 10.6 ± 0.88     | 11.9 ± 0.7     | 9.8 ± 0.99      | 11.2 ± 0.64    |
| LVCAV, mcl                     | 24 ± 1.4        | 23 ± 1.2       | 23 ± 1.1        | 21 ± 1.2       |
| LV mass, mg/10 g               | 28 ± 1.2        | 30 ± 1.0       | 29 ± 1.0        | 29 ± 1.2       |
| Mass/volume                    | 0.81 ± 0.009†   | 0.79 ± 0.005   | 0.82 ± 0.006*   | 0.79 ± 0.007   |
| SBP, mmHg                      | 108 ± 1.9†      | 108 ± 3.3†     | 129 ± 2.1       | 122 ± 2.4      |
| DBP, mmHg                      | 68 ± 2.4†       | 70 ± 2.5†      | 81 ± 2.3        | 78 ± 2.9       |
| MBP, mmHg                      | 81 ± 2.0†       | 81 ± 2.4†      | 97 ± 2.0        | 92 ± 2.6       |
| HR, beats/min                  | 681 ± 26        | 704 ± 12       | 685 ± 15        | 679 ± 13       |
| TPR, mmHg·ml⁻¹·min⁻¹           | 8.59 ± 0.53†‡   | 7.17 ± 0.41†   | 11.54 ± 1.58*   | 8.83 ± 0.52    |
| HW, mg/g                       | 0.149 ± 0.007   | 0.117 ± 0.005  | 0.150 ± 0.009   | 0.101 ± 0.005  |
| HW-to-BW ratio                 | 0.49 ± 0.007    | 0.48 ± 0.001   | 0.49 ± 0.008    | 0.47 ± 0.018   |

Values are means ± SE. WT, wild type; eNOS, endothelial nitric oxide synthase; HR, heart rate; EF, ejection fraction; FS, fractional shortening; IVSD, interventricular septum thickness at end diastole; IVSS, interventricular septum thickness at end systole; LVEDD, left ventricular (LV) end-diastolic dimension; LVESD, LV end-systolic dimension; LPWD, LV posterior wall thickness in diastole; LPWS, LV posterior wall thickness in systole; CO, cardiac output; LVCAV, LV cavity volume; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; TPR, total peripheral resistance; HW, heart weight; BW, body weight. Shown are echocardiographic measurements, blood pressures, TPR, and HW-to-BW measurements in male and female eNOS transgenic (Tg) mice and WT controls (C57Bl/6). Echocardiograms showed no significant difference in cardiac function in eNOS Tg mice. SBP, DBP, and MBP were significantly decreased in male and female eNOS Tg mice (P < 0.05). Both male and female mice had decreased TPR; however, female eNOS Tg mice had significantly lower TPR compared with male eNOS Tg (P < 0.05). HR were measured both during echocardiography and by tail-cuff blood pressure measurements. Hemodynamic measurements were analyzed with Student’s unpaired t-test. *P < 0.05 vs. male WT; †P < 0.05 vs. WT; ‡P < 0.05 vs. male eNOS Tg.
Effect of forskolin on MV O$_2$. Forskolin (10$^{-12}$-10$^{-6}$ mol/l, n = 7), caused a dose-dependent decrease in MV O$_2$ in WT by 24 $\pm$ 2.8% and in eNOS Tg by 31 $\pm$ 1.8% (Fig. 4). The addition of L-NAME attenuated the reduction of MV O$_2$ in both WT (17 $\pm$ 2.0%) and eNOS Tg (24 $\pm$ 2.8%).

Effect of SNAP on MV O$_2$. SNAP (10$^{-7}$-10$^{-4}$, n = 4), also caused a dose-dependent decrease in MV O$_2$. WT showed a 36 $\pm$ 1.7% decrease, whereas eNOS mice showed a 37 $\pm$ 3.6% decrease (Fig. 5). The addition of L-NAME did not alter the actions of SNAP in WT (39 $\pm$ 0.6%) or eNOS Tg (34 $\pm$ 1.1%).

**DISCUSSION**

In this study, we have demonstrated that in heart from mice with coronary vascular overexpression of eNOS, NO plays an enhanced role in the regulation of MV O$_2$. These findings support our previous studies indicating that vascular NO is coupled to the control of tissue respiration. Initial phenotypic and hemodynamic measurements of eNOS Tg mice showed approximately a sixfold increase in eNOS protein as seen by Western blot analysis, whereas systolic and diastolic pressures decreased, and there were no changes in ventricular contractile function by echocardiography. Furthermore, the overexpression of eNOS leads to an increased ability of drugs which stimulate NO production to regulate MV O$_2$. BK and CCh reduced oxygen consumption to an even greater degree in heart from eNOS Tg compared with wild-type controls. Similarly, forskolin and SNAP administration reduced oxygen consumption in eNOS Tg mice. L-NAME reversed the inhibitory response of BK, CCh, and forskolin, but not that of SNAP. Vascular eNOS overexpression resulted in an enhanced inhibition in tissue respiration; hence, we conclude that vascular NO can control MV O$_2$ and that introduction of an extra copy of the eNOS gene can further enhance the regulation of the redox state in myocardial tissue by NO.

Decreased blood pressures in eNOS Tg mice are consistent with data from several laboratories (22, 30, 32–34, 36). Other studies (2, 19), however, did not find significant hypotension; this could be due to either the depressive effects of anesthesia and/or differences in the transgenic model. We calculated TPR by normalizing MAP with our CO measurements from the echocardiograms. Overall, eNOS Tg mice had significantly lower resistance compared with WT. Interestingly, there were

**Fig. 2.** Effect of cumulative doses of bradykinin (BK) on myocardial O$_2$ consumption (MV O$_2$) in eNOS Tg (n = 6) and WT (C57B/6) mice (n = 6). BK caused a dose-dependent decrease of MV O$_2$ in WT mice, and this effect was significantly increased in eNOS Tg mice. *P < 0.05, two-way ANOVA, followed by multiple comparison using Student-Newman-Keuls test.

**Fig. 3.** Effect of cumulative doses of carbachol (CCh) on MV O$_2$ in eNOS Tg (n = 6) and WT (C57B/6) mice (n = 6). CCh caused a dose-dependent decrease of MV O$_2$ in WT mice, and this effect was significantly increased in eNOS Tg mice. *P < 0.05, two-way ANOVA, followed by multiple comparison using Student-Newman-Keuls test.

**Fig. 4.** Effect of cumulative doses of forskolin on MV O$_2$ in eNOS Tg (n = 7) and WT (C57B/6) mice (n = 6). Forskolin caused a dose-dependent decrease of MV O$_2$ in WT mice, and this effect was significantly increased in eNOS Tg mice. *P < 0.05, two-way ANOVA, followed by multiple comparison using Student-Newman-Keuls test.

**Fig. 5.** Effect of cumulative doses of S-nitroso-N-acetyl penicillamine (SNAP) on MV O$_2$ in eNOS Tg (n = 4) and WT (C57B/6) mice (n = 4). SNAP caused a dose-dependent decrease of MV O$_2$ in WT mice, and this effect was not significantly increased in eNOS Tg mice. *P < 0.05, two-way ANOVA, followed by multiple comparison using Student-Newman-Keuls test.
gender-specific decreases in TPR as well, for example, there was a further significant decrease in resistance in female mice (Table 1). Gender-specific differences in eNOS Tg mice are not surprising due to the fact that we have recently found a 50% increase in mortality of male eNOS–/– at 21 mo (25). In our calculations, the decrease in TPR was due to a significant decrease in MAP, and we assumed central venous pressure (CVP) was negligible. Even if one uses an estimate of CVP of a few mmHg, the marked reduction in MAP from 97–92 mmHg in WT mice to 81 mmHg in eNOS Tg mice, a difference of 11–16 mmHg, make it unlikely that a change in CVP would be great enough to alter our conclusions. In fact, we probably underestimated the change in TPR in eNOS Tg mice.

Our methods for echocardiographic assessment are those described by Yang et al. (46). We measured EF% from three different planes of the heart. Two measurements were taken parallel to the parasternal short axis of the left ventricle. With B-mode tracings, 5 were calculated from circumferential changes during systole and diastole, values ranged between −55% and 65%. In an M-mode tracing, the values ranged from 80% to 90%. Even though our measurements for CO seem physiologically high, Janssen et al. (17) surveyed different methods of measuring CO in conscious mice with flow probes and echocardiography and determined an acceptable ranges of 12–27 ml/min. Baseline oxygen consumption was not different between WT and eNOS Tg hearts showing that although eNOS is upregulated, the production of NO during basal conditions remains small. In our buffer solution there are no agonists and no flow (shear) to stimulate eNOS until we add an agonist. Concurrently, in our in vitro preparation eNOS is not constitutively active, in contrast we have previously shown in tissues treated with lipopolysaccharide, where iNOS is constitutively active, there is a reduction in baseline MVO2, which is reversed by 1-NAME (7). When stimulated with BK, however, the reduction in MVO2 is significantly greater in eNOS Tg than in WT controls. This shows that kinin/Ca2+-dependent mechanisms competitively inhibit mitochondrial respiration. Loke et al. (27), using B2-kinin receptor–/– mice, found that MVO2 was not decreased by BK. Later, Loke et al. (29) used eNOS(−/−) mice and stimulated the heart with BK. Not surprisingly, MVO2 was not altered in the eNOS knockouts showing that the coupling of kinin receptors to eNOS and the formation of NO was abolished. Muscarinic receptors are also expressed in coronary blood vessels. Shen et al. (37) and Xie et al. (44) demonstrated that CCh, a muscarinic agonist, stimulates eNOS to decrease MVO2 in the canine heart in vitro. Furthermore, in vivo we speculate the basal decrease in blood pressure is mostly due to Ca2+-independent shear stress mechanisms rather than Ca2+-dependent agonist activation of eNOS.

eNOS is thought to be activated by several Ca2+ and phosphorylation-dependent mechanisms, which allow for subsequent electron flux from the NH2 terminus reductase to the COOH terminal oxygenase, where l-arginine is converted to l-citrulline and NO. Various kinases drive activation of eNOS through phosphorylation, including; Akt, which phosphorylates Ser1177; AMPK, which phosphorylates Ser1177, and Thr495; PKA, which phosphorylates Ser1177 and Ser633; and PKG, which also phosphorylate Ser1177 and Ser633 (8). PKC and mitogen-activated protein kinases are also involved in phosphorylation of eNOS, but they negatively regulate eNOS activity (8). Zhang et al. (47) demonstrated that adenylate cyclase is involved in phosphorylation of eNOS. They showed that stimulated cAMP signaling in canine coronary microvessels caused an increase in nitrite production during administration of an activator of adenylate cyclase, forskolin. To test whether the decrease in MVO2 is proportional to the increase in eNOS protein through phosphorylation, we stimulated the myocardium in eNOS Tg with forskolin. Our results indicated there was enhanced inhibition of MVO2 in eNOS Tg, showing that cAMP-dependent eNOS phosphorylation causes activation of the enzyme.

SNAP caused a dose-dependent decrease in MVO2 in eNOS Tg mice. 1-NAME was given in the presence of BK, CCh, or forskolin, and there was inhibition of tissue respiration. 1-NAME did not reverse the actions of SNAP.

It is important to note that there are different techniques to overexpress eNOS either constitutively or selectively; however, none of the previous studies addressed the role of NO in the control of cardiac oxygen consumption. Early studies [i.e., Ooboshi et al. (31)] involving eNOS overexpression with adenoviral transfections in rats, found that vasodilation was increased and that blood flow/oxygenation increased after hindlimb ischemia. Microinjection of eNOS into the rostral ventrolateral medulla and the nucleus tractus solitarius of the brain caused bradycardia and hypotension (21, 22, 36). Brunner et al. (2) found an attenuation of myocardial/reperfusion injury in myocyte-specific overexpression of eNOS using human cDNA amplified by PCR and then subcloned into vectors containing α-myosin heavy chain. The next step was to genetically modify mice to constitutively overexpress eNOS. One group used a bovine eNOS gene with a preproendothelin-1 promoter to produce a heterozygous strain with vascular-specific eNOS overexpression (20, 32–34). These mice exhibit hypotension, inhibition of lesion formation, attenuation of cardiac hypertrophy during isoproteronol perfusion, and consistent with Brunner et al. (2), protection against ischemia-reperfusion injury (20, 30, 32, 34). Finally, the transgenic mouse used in our experiments was created using a human cDNA eNOS promoter. Van Haperen et al. (42) performed initial phenotypic studies on two founder strains of eNOS Tg mice (eNOS Tg2 and eNOS Tg3). Immunohistochemical staining was performed to determine overexpression patterns in the aorta, heart, liver, kidney, adrenal, and testis. Sections from WT showed little or no staining in sections. Staining in eNOS Tg mice was clearly present in capillaries between myocytes, in sinusoids of the liver, and in peritubular capillaries and glomeruli of the kidney. In the adrenal gland, cortical capillaries and medullary capillaries sinusoids were also stained as well as only blood vessels in the testis. All sections sampled did not exhibit staining in the parenchyma cells in eNOS Tg2 and eNOS Tg3 mice. This supports the conclusion that eNOS overexpression in these mice is specific to endothelial cells in blood vessels (42). With the use of these mice, Jones et al. (19) reported a decrease in the progression of congestive heart failure. Our studies have focused on the role of eNOS to control NO production and regulate mitochondrial respiration. To address the role of mitochondrial NOS selectively controlling MVO2, the most recent studies by Giulivi et al. (6, 10) suggest that a truncated nNOS protein is mitochondrial NOS (11). In our study, nNOS protein was not different in the WT or eNOS Tg mice, where eNOS protein was markedly elevated.
in eNOS Tg mice. Furthermore, BK and carbachol stimulate eNOS, not nNOS, providing evidence to suggest that upregulated eNOS is primarily responsible for the enhanced regulation of MVO\(_2\) in eNOS Tg mice.

In our previous studies (25), eNOS\(^{(-/-)}\) male mice developed severe heart failure at 21 mo of age. Recent studies by Pacher et al. (35) also support age-related cardiac dysfunction eNOS\(^{(-/-)}\) mice. In Pacher’s study, they report 10-mo-old iNOS\(^{(-/-)}\) mice showed no change in LV function, where eNOS\(^{(-/-)}\) mice at 4 mo had increased TPR, increased contractility, and moderate diastolic dysfunction, and at 10 mo of age, eNOS\(^{(-/-)}\) developed severe cardiac dysfunction characterized by decreased CO and EF (35). The use of adenosine transfection or genetic modification suggests that if eNOS is restored, a disease process may be alleviated or reversed. There is, however, one important caveat to mention. If there is an abundance of eNOS and subsequent excessive NO generation then this may cause deleterious effects on lesion formation and cardiac function. In the presence of excess NO and low PO\(_2\), the formation of superoxide (O\(_2^·\)) and peroxynitrite (ONOO\(^-\)) may effect tissue respiration. In our study, l-NAME acutely (in 30 min) inhibited the effects of BK, carbachol, or forskolin suggesting that the accumulation of nitrosylated proteins, with some long half-life, from ONOO\(^-\) were likely not responsible for the significant reduction in MVO\(_2\). Our previous studies (40) also show that increased endogenous NO generation regulates tissue respiration independent of ONOO\(^-\). Xie et al. (45), showed that tiron, an intracellular scavenger of O\(_2^·\), or uric acid, a scavenger of ONOO\(^-\) did not modify the effects of SNAP on tissue oxygen consumption. Our hemodynamic and echocardiographic assessments also show that the relative vascular overexpression of eNOS does not cause deleterious effects subsequent to altered cardiac function or decreased blood pressures.

In summary, there is enhanced control of mitochondrial respiration to BK, carbachol, and forskolin in hearts from blood pressures.

**References**


CONTROL OF MYOCARDIAL OXYGEN CONSUMPTION IN MICE


