Dysfunctional Regulation of Endothelial Nitric Oxide Synthase (eNOS) Expression in Response to Exercise in Mice Lacking One eNOS Gene

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Background—Previous data suggest that 1 endothelial NO synthase (eNOS) gene is sufficient to allow normal expression and function of eNOS under basal conditions. We hypothesized that this might not hold true for conditions known to increase eNOS gene expression, such as exercise.

Methods and Results—Male mice heterozygous for a disruption of the eNOS gene (eNOS<sup>+/−</sup>) and normal C56Bl/6J mice (eNOS<sup>+/+</sup>), 3 to 4 months of age, underwent exercise training for 3 weeks. Nontrained mice were exposed to the exercise environment (noise and vibration of the treadmill) without exercise for an identical period. In eNOS<sup>+/−</sup> mice (n=7), exercise increased aortic eNOS protein expression by 3.4±0.4-fold (P<0.002). This was associated with a greater vascular cGMP accumulation on stimulation with acetylcholine (P<0.05). Furthermore, exercise training increased eNOS mRNA (1.78±0.4-fold) and protein (1.76±0.17-fold) in left ventricular tissue, as determined by competitive reverse transcription–polymerase chain reaction and Western analysis (P<0.05 for both). In striking contrast, exercise had no effect on aortic eNOS expression and cGMP accumulation in eNOS<sup>+/−</sup> mice (P>0.05). Thus, although eNOS expression appears to be normal in eNOS<sup>+/−</sup> mice under basal conditions, these mice are unable to increase eNOS expression during exercise.

Conclusions—These findings show that regulation of eNOS expression during exercise requires the presence of both alleles of the gene and may have implications for conditions in which polymorphisms of eNOS are present in only 1 allele in humans. These individuals may have a normal vascular reactivity under basal conditions but may be unable to adapt their vascular reactivity in response to exercise training. (Circulation. 2001;103:2839-2844.)

Key Words: exercise ■ endothelium ■ nitric oxide synthase ■ genes ■ cardiovascular disease

Exercise training is associated with a variety of physiological adaptations involving skeletal muscle, cardiac muscle, circulating blood volume, and a variety of metabolic modifications.1–5 Recently, it has become apparent that there are also vascular adaptations to exercise training.6–9 A particularly interesting adaptation is an increase in expression of the endothelial nitric oxide synthase (eNOS) gene in both coronary conductance and resistance vessels.10 Increased eNOS expression may afford greater vasodilation of vessels perfusing exercising muscle and thus may enhance exercise capacity.

In addition to the potentially beneficial effects of eNOS upregulation on exercise capacity, increased NO production may also reduce vascular disease.4,5 It is known that NO not only produces vasodilation but also inhibits platelet aggregation and has antioxidant, antiproliferative, and antiapoptotic properties.11 Pharmacological inhibition of NOS or disruption of the eNOS gene has been shown to accelerate the atherosclerotic process.12–14 Likewise, treatment with exogenous NO has been shown to reduce lesion formation in cholesterol-fed rabbits.15

Recently, there has been substantial interest in the role of polymorphisms of eNOS as they might contribute to cardiovascular disease. Indeed, 1 such polymorphism has been associated with an increased incidence of atherosclerosis and another with coronary vasospasm.16,17 In both of these cases, homozygotes with the polymorphism were affected, whereas heterozygotes were not. These results are of interest in light of recent observations we have made regarding the phenotype of mice heterozygous for the eNOS gene.18 These eNOS<sup>+/−</sup> mice were found to have normal blood pressure, heart rate, and soluble guanylate cyclase activity. Furthermore, vascular responses to a variety of endothelium-dependent and -independent vasoactive agents were identical to those observed in normal mice.

The above findings suggest that 1 eNOS gene provides sufficient eNOS protein expression and activity under basal...
conditions and raise questions about the significance of a heterozygous polymorphism of eNOS. A caveat in this regard is that various genetic alterations do not manifest themselves under normal circumstances but become apparent under conditions of stress. For example, individuals with sickle-cell trait do not suffer from sickle-cell crises unless exposed to high altitudes or severe hypoxia. Thus, it is conceivable that a loss of 1 eNOS gene might not be apparent under basal conditions but might become evident when eNOS gene expression should normally increase, for example, during exercise training. Thus, the purpose of this study was to determine whether eNOS+/− mice can increase vascular eNOS expression in response to exercise training.

Methods

Permission for this study was provided and the experiments were performed according to the guidelines for the use of experimental animals as given by the Deutsches Tierschutzgesetz and to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Animals Studied

Mice heterozygous for the eNOS gene (eNOS+/−) were obtained by mating eNOS wild-type mice (eNOS+/+, C57BL/6J) and mice lacking both eNOS alleles (eNOS−/−) mice. All mice studied were males. The eNOS+/− mice were obtained from Jackson Laboratories and had been backcrossed 10 times to the C57BL/6J strain. Genotypes of the offspring were identified from tail clippings by polymerase chain reactions (PCR) as described previously. Briefly, the offspring were used to identify wild-type mice: sense, 5'-GCATCACCA-AGGAAAGAGCC-3' and antisense, 5'-GAGGCTACAGATGGTGG-3', and primers complementary to the neomycin-resistance cassette were used to identify the presence of the disrupting genetic insert (sense, 5'-TCAGCGTTGTCACTGAGC-3' and antisense, 5'-TCAGAGGGCATAGAAGGC-3'). In addition to these animals, C57BL/6J mice were included as controls. Studies were performed when the mice were 12 to 16 weeks of age. Mice were euthanized by CO2 inhalation.

Determination of eNOS Protein Levels

Aortas were immersed in iced Tris buffer (5 mmol/L, pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aprotinin, PMSF, and antipain (10 μg/mL). The tissues were homogenized for 30 seconds in a Polytron homogenizer. The homogenates were then centrifuged for 10 minutes at 100 g to remove particulate matter and unbroken cells. Total protein levels were determined by the Bradford method. Western blot analysis was performed as described previously. A specific cDNA fragment of 468 bp (position 3010 to 3477) was amplified by use of the following primers: eNOS sense primer: 5'-GGCTCCCTCCTTCCGGCTG-3', and eNOS antisense primer: 5'-TCCCCAGAGCAGCCGGAT-3'. The eNOS-specific cDNA fragment was subsequently cloned into the pCR-Script Amp SK(+) Cloning Vector (Stratagene), and its identity was confirmed by DNA sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS, Perkin-Elmer Co; ABI 373 DNA Sequencer). The DNA sequence was analyzed with Gene Runner software (Hastings Software, Inc). Database searches of GenBank were performed with BLASTN. An internal deleted cRNA standard of 331 bp was constructed by linker primer PCR and identified by DNA sequencing. The internal deleted eNOS cDNA standard was transcribed in vitro into cRNA (RNA Transcription Kit, Stratagene). The cRNA was quantified spectrophotometrically. In competitive RT-PCR experiments, equal amounts of total RNA (50 ng) were incubated in separate reactions with defined amounts of eNOS standard cRNA, reverse transcribed into cDNA (random hexamer primers), and amplified by PCR. Reaction products were separated by agarose gel electrophoresis. The optical density of each PCR fragment was estimated with Biorad GelDoc 1000, and the log of the quotient of normalized standard and sample-specific PCR fragment density was used to calculate the equivalence point. A standard calibration curve is given in Figure 1.

Figure 1. Quantitative evaluation of eNOS mRNA expression in mouse heart. Top, Representative gel comparing mRNA expression in hearts of sedentary controls and exercised C57BL/6J mice. Bottom, Quantification of mRNA expression after densitometric analysis. Ratios of optical density values for native and standard bands as calculated for each standard concentration are plotted against amount of standard mRNA (r=0.976, slope = −1.2993, P<0.0001).

Determination of eNOS mRNA Expression

Expression of eNOS mRNA was measured with a competitive reverse transcription–PCR (cRT-PCR). In aortic rings of C57BL/6J and eNOS−/− mice, a competitive RT-PCR specific for the eNOS gene was performed. Total RNA from mouse aortas was isolated. A specific cDNA fragment of 468 bp (position 3010 to 3477) was amplified by use of the following primers: eNOS sense primer: 5'-GGCTCCCTCCTTCCGGCTG-3', and eNOS antisense primer: 5'-TCCCCAGAGCAGCCGGAT-3'. The eNOS-specific cDNA fragment was subsequently cloned into the pCR-Script Amp SK(+) Cloning Vector (Stratagene), and its identity was confirmed by DNA sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS, Perkin-Elmer Co; ABI 373 DNA Sequencer). The DNA sequence was analyzed with Gene Runner software (Hastings Software, Inc). Database searches of GenBank were performed with BLASTN. An internal deleted cRNA standard of 331 bp was constructed by linker primer PCR and identified by DNA sequencing. The internal deleted eNOS cDNA standard was transcribed in vitro into cRNA (RNA Transcription Kit, Stratagene). The cRNA was quantified spectrophotometrically. In competitive RT-PCR experiments, equal amounts of total RNA (50 ng) were incubated in separate reactions with defined amounts of eNOS standard cRNA, reverse transcribed into cDNA (random hexamer primers), and amplified by PCR. Reaction products were separated by agarose gel electrophoresis. The optical density of each PCR fragment was estimated with Biorad GelDoc 1000, and the log of the quotient of normalized standard and sample-specific PCR fragment density was used to calculate the equivalence point. A standard calibration curve is given in Figure 1.
Training Protocol
Exercise was performed on a treadmill specially designed for mice. Six animals were studied simultaneously. Mice were initially trained 3 times for 10 minutes every other day. The velocity of the treadmill was 15 m/min. After this training, mice were exercised for 3 weeks 5 times a week for 30 minutes at 15 m/min. The training was executed after 7 PM to ensure that the exercise took place in their daily active cycle. Nonexercised controls were exposed to the same noise and the vibration of the environment. All mice completed the exercise protocol without signs of exhaustion. There was no obvious difference in exercise performance between the different genotypes. Within 16 to 20 hours after termination of the last training, mice were euthanized by inhalation of carbon dioxide, and their aortas and hearts were immediately frozen in liquid nitrogen. These tissues were taken to prepare either total protein for Western blotting or total RNA for RT-PCR.

Statistical Analysis
Data are presented as mean±SEM. Data were compared between groups of animals by ANOVA (Graph Pad Prism, 3.0). A value of P<0.05 was considered significant.

Results
Effect of Exercise on Expression of eNOS Protein
To examine the effect of exercise training on aortic eNOS protein expression, Western blots were performed on aortic homogenates. Three weeks of exercise training strongly increased eNOS protein expression in aortic segments of C57BL/6J mice (Figure 2). A similar phenomenon was observed in the left ventricles of hearts from these mice (Figure 3). These results suggest that exercise can increase eNOS protein expression in both conduit and resistance arteries. As shown by a direct comparison between both tissues, the increase of eNOS expression after exercise was greater in the aorta than in the heart (P=0.0013).

In striking contrast, the same exercise protocol had no effect on aortic eNOS protein expression in eNOS<sup>−/−</sup> mice. As shown in Figure 4, expression of eNOS in aortic segments of eNOS<sup>−/−</sup> mice was unaffected by the exercise protocol. Similarly, there was no exercise-induced change in eNOS protein expression in the left ventricles of these mice (Figure 5). These results indicate that exercise training does not increase eNOS expression in eNOS<sup>−/−</sup> mice and suggest that both alleles of the eNOS gene are required for this physiological adaptation.

Effect of Exercise on Expression of eNOS mRNA
To further investigate exercise-induced changes of the eNOS gene expression, we quantified eNOS mRNA in the left cardiac ventricle. As shown in Figure 6A, exercise induced a significant 1.75±0.24-fold increase in mRNA content in the heart of normal C57BL/6J mice (P<0.05). There was no significant difference between this effect and the increase in eNOS protein expression as measured in separate sections of the same cardiac tissue (1.76±0.18-fold, Figure 4, P=0.9732). In accordance with the Western blot results obtained from eNOS<sup>−/−</sup> mice, we found no effect of exercise on eNOS mRNA expression (Figure 6B). This further supports the conclusion that disruption of 1 eNOS gene results in impaired regulation of eNOS expression in response to exercise training.

Effect of Exercise on the Activity of Aortic eNOS
The effect of exercise on eNOS activity was evaluated by determination of cGMP content in isolated mouse aortic
segments after stimulation with 1 μmol/L acetylcholine. As shown in Figure 7, cGMP content after stimulation with acetylcholine was significantly increased by exercise in aortic segments of normal C57BL/6J mice but not in aortic segments from eNOS<sup>1/2</sup> mice. These results indicate that the effect of exercise on eNOS expression results in an enhanced production of a functionally active eNOS protein and that this is absent in eNOS<sup>1/2</sup> mice.

**Discussion**

Recently, we found that under normal conditions, eNOS protein levels, endothelium-dependent vasodilatation, and the vasoconstrictor response to phenylephrine and serotonin are identical between eNOS<sup>+/+</sup> and eNOS<sup>1/2</sup> mice. Furthermore, these animals also exhibited no differences in their blood pressures and heart rates. Thus, loss of 1 eNOS gene does not seem to affect total eNOS expression under normal conditions. This is in marked contrast to the results of our present study showing that whereas exercise training increases eNOS mRNA and protein expression up to 3-fold in normal mice, there is no increase in eNOS expression in the eNOS<sup>1/2</sup> mice.

The mechanisms whereby exercise training increases eNOS expression have not been fully defined. The high cardiac output that occurs during exercise may expose the endothelium to increased levels of shear. In cultured endothelial cells, shear stress potently increases eNOS mRNA and protein expression. Exercise is also associated with an increase in oxidative stress. Recently, we found that even brief exposure of endothelial cells to H<sub>2</sub>O<sub>2</sub> increases eNOS mRNA, protein, and function for up to 72 hours via both transcriptional and posttranscriptional mechanisms. Other humoral factors released during exercise may also contribute. It is conceivable that the effect of exercise depends on a combination of several factors.

It is interesting to consider how the absence of 1 eNOS gene may affect eNOS gene expression during exercise training. One interpretation of our results is that when 2 alleles are present, under basal conditions both are being transcribed at a submaximal rate. Exercise training may thus
increase transcriptional activity of both genes. In this scenario, one might speculate that in eNOS<sup>−/−</sup> mice, the absence of 1 allele results in the opposite allele functioning at near peak transcription rate, such that it cannot be further activated by exercise training.

Another possibility is that in normal mice, 1 gene is silenced and becomes activated during exercise training. Such a phenomenon would involve methylation of 1 eNOS gene, and demethylation during exercise training. It has been shown that DNA methylation patterns are important for regulating gene expression during development and tumorigenesis. This process involves methylation of cytosines in 5′-CpG islands near the promoter of certain genes, changing interactions with repressive chromatin structures. Recently, it has become evident that the expression of certain genes is regulated by methylation and demethylation in physiological and pathophysiological states. If 1 eNOS allele were suppressed by methylation and desuppressed during exercise training, it would follow that this could not occur in mice with only 1 functional eNOS gene. Studies of the methylation status of the eNOS gene may be useful in understanding this process.

It has recently become apparent that mRNA stability plays an important role in regulation of eNOS gene expression. For example, H<sub>2</sub>O<sub>2</sub> stimulates eNOS expression in part via mRNA stabilization, as does shear stress. How this might relate to altered expression of eNOS in eNOS<sup>−/−</sup> mice remains unclear.

Altered mRNA and protein expression of eNOS induced by exercise was associated with altered function as assessed by accumulation of aortic cGMP in response to acetylcholine. The exercise protocol we used did not lead to exhaustion of the mice, and there was no obvious difference between the exercise capacities of the different genotypes. It is quite possible, however, that mice lacking 1 eNOS gene cannot exercise as much as normal animals. Further investigations are needed to clarify this question.

Several polymorphisms of the eNOS gene have been reported, including intron and coding sequence polymorphisms such as the 894G→T polymorphism resulting in an exchange of amino acid 298 from glutamate to aspartate. In some cases, these have been linked to hypertension and coronary spasm, whereas in others, they are not associated with any obvious phenotype or disease process.

To date, it is not clear whether these polymorphisms alter eNOS expression or protein function. The 894G→T polymorphism, however, has recently been associated with decreased eNOS protein stability. Our present results, taken with our prior report, suggest that a polymorphism of 1 gene may not affect eNOS expression or function under basal nonstressed conditions but may alter gene expression during physiological stresses such as exercise training. It therefore may be useful to examine the effect of various eNOS polymorphisms on the ability to adapt to exercise. Finally, a loss or polymorphism of 1 eNOS gene may abrogate the beneficial effects of exercise training in humans in terms of enhanced expression of NO.

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