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Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training

Michael E. Davis,[1,2] Hua Cai,[1] Louise McCann,[1] Tohru Fukai,[1] and David G. Harrison[1,2]
[1]*Division of Cardiology and* [2]*Molecular and Systems Pharmacology Program, Emory University, and Veterans Hospital Medical Center, Atlanta, Georgia 30322*

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Davis, Michael E., Hua Cai, Louise McCann, Tohru Fukai, and David G. Harrison. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. *Am J Physiol Heart Circ Physiol* 284: H1449–H1453, 2003. First published December 5, 2002; 10.1152/ajpheart.00918.2002.—We have shown that c-Src plays a role in shear stress stimulation of endothelial nitric oxide synthase (eNOS) expression in cultured cells. To examine the role of c-Src in vivo, we exercised C57Blk/6 and c-Src heterozygous (c-Src$^{+/-}$) mice on a treadmill for 3 wk. Western analysis demonstrated that c-Src$^{+/-}$ mice express less than one-half the normal amount of c-Src. Exercise increased heart rate and blood pressure to identical levels in both strains as determined using radiotelemetry. Exercise training increased eNOS protein >2-fold in the aorta and 1.7-fold in the heart in C57Blk/6 mice but had no effect on eNOS protein levels in c-Src$^{+/-}$ mice. In contrast to exercise, treatment of mice with mevastatin, which stimulates expression of eNOS posttranscriptionally, increased eNOS protein in both strains. Training also increased aortic extracellular superoxide dismutase protein expression, which is regulated by nitric oxide, in C57Blk/6 mice but not in c-Src$^{+/-}$mice. These data indicate that c-Src has an important role in modulating vascular adaptations to exercise training, in particular increasing eNOS and extracellular superoxide dismutase protein expression.

extracellular superoxide dismutase; radiotelemetry; Western analysis; mice

A MAJOR VASCULAR ADAPTATION to exercise training is increased expression of the endothelial cell nitric oxide (NO) synthase (eNOS). This has been demonstrated in dogs, mice, rats, and pigs (11, 20, 21, 23, 28). Furthermore, chronic exercise training in humans enhances endothelium-dependent vasodilatation and increases plasma levels of nitrate and nitrite, the oxidation products of NO (14, 16). This phenomenon seems to have therapeutic implications, in that exercise training increases endothelium-dependent vasodilatation in humans with heart failure (5, 6). Because endothelial production of NO has numerous antiatherosclerotic properties, including inhibition of adhesion molecule expression, prevention of platelet aggregation (26, 27), and inhibition of vascular smooth muscle proliferation (18), increased eNOS expression in response to exercise may explain in part the beneficial effects of exercise in preventing cardiovascular disease.

One mechanism whereby exercise could affect eNOS expression is by increasing levels of endothelial cell shear stress. During exercise, the increase in cardiac output likely augments shear stress in vessels where blood flow is increased. As an example, it has recently been shown that shear stress in the abdominal aorta is doubled during exercise (25). The increase in eNOS expression in response to exercise training seems to differ between artery sizes and may reflect different levels of shear stress encountered by the endothelium in different-sized vessels during exercise training (13).

Shear stress exerts beneficial effects on the endothelium beyond increasing eNOS expression. Laminar shear stress has been shown to stimulate expression of the endothelial antioxidant enzymes Cu/Zn superoxide dismutase (SOD) (7) and glutathione peroxidase (24). Prior studies from our laboratory have shown that exercise training increases expression of the extracellular SOD (ecSOD) and that this occurs concurrently with an increase in eNOS expression. These studies demonstrated that the increase in ecSOD expression during exercise training was dependent on increased NO, because exercise training in eNOS-deficient mice had no effect on ecSOD expression (4).

Recently, we examined the signaling pathways responsible for increased eNOS expression in response to shear stress in cultured endothelial cells. These studies show that shear stress increases eNOS mRNA levels through two pathways: one controlling transcription of the message and the other regulating its stability. Both of these mechanisms require c-Src activation (2), because inhibition of c-Src by pharmacological mechanisms or by adenoviral overexpression of a dominant-negative c-Src mutant completely prevents upregulation of eNOS in response to shear stress. The increase in transcription is entirely dependent on activation of a classical signaling pathway involving Ras, Raf, mitogen-activated protein kinase kinase 1/2 (MEK1/2), and extracellular-related kinase 1/2 (ERK1/2). The increase in eNOS mRNA stability, although dependent on c-Src, was not affected by inhibition of this Ras/Raf/MEK/ERK pathway.

Address for reprint requests and other correspondence: D. G. Harrison, Div. of Cardiology, Emory University, 1639 Pierce Dr., WMB 319, Atlanta, GA 30322 (E-mail: dharr02@emory.edu).

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Although the studies discussed above strongly suggest that c-Src modulates eNOS expression in response to shear stress, it remains unclear whether c-Src plays a role in the increase in eNOS caused by exercise training. In the present study, we examined the effect of exercise training on eNOS protein levels in the heart and aorta of wild-type mice and mice heterozygous for the c-Src gene. Our findings clearly indicate that c-Src plays a role in modulating eNOS and ecSOD protein expression during exercise training and, therefore, illustrate an important role of this tyrosine kinase in cardiovascular adaptations not only to shear stress but also to this important physiological stimulus.

MATERIALS AND METHODS

Animals. Recently, c-Src knockout mice have become available for study (22). The homozygous knockouts of this strain have impaired long bone development, signs of osteoporosis, incomplete eruption of teeth, and reduced size (22). These features make exercise training impractical. Heterozygous littermates (c-Src$^{+/-}$), however, display none of these features and appear to develop normally. Furthermore, these mice have reduced levels of c-Src protein (22) and are ideally suited to examine the effects of c-Src on exercise training. c-Src$^{+/-}$ and C57Blk/6 (wild-type) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The c-Src$^{+/-}$ mice were backcrossed 10 generations to the C57BL/6J strain. Genotypes of the c-Src$^{+/-}$ offspring were determined from tail cuts by polymerase chain reaction as described previously (22). Studies were performed on 12- to 18-wk-old male mice.

Determination of c-Src, eNOS, and ecSOD protein levels. After 3 wk of exercise training, mice were euthanized 18 h after their last bout of exercise training by CO_2 inhalation. Their aortas and hearts were isolated, dissected free of adherent tissue, and homogenized in a buffer containing Tris (50 mM), EDTA (0.1 mM), EGTA (0.1 mM), 1% Triton X-100, and protease inhibitors. Western analysis was performed using a monoclonal antibody (BD Transduction Laboratories, San Diego, CA) as described elsewhere (4). Levels of ecSOD protein were determined using a previously generated polyclonal antibody (3). Levels of c-Src were determined using a polyclonal antibody specific for c-Src (Santa Cruz Biotechnology, Santa Cruz, CA).

Exercise protocol. Exercise training was performed on a motorized treadmill (Columbus Instruments, Columbus, OH) for 3 wk at 15 m/min for 5 days/wk as previously described (4, 11).

Determination of blood pressure and heart rate. In selected wild-type and c-Src$^{+/-}$ mice, blood pressure and heart rate were monitored at baseline and during exercise using telemetry (Data Sciences International, St. Paul, MN). Mice were anesthetized with an intraperitoneal injection of ketamine-xylazine. After isolation of the left common carotid artery, the catheter connected to the transducer was introduced into the carotid and advanced until the tip was just inside the thoracic aorta. The transmitter was positioned along the right flank, close to the hindlimb. Mice were allowed to recover for 2 wk, and measurements of heart rate and blood pressure before and during exercise were obtained daily for 1 wk.

Treatment with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Mevastatin (Sigma, St. Louis, MO) was converted to its open acid form as previously described (10). The resulting solution was brought up to a final concentration of 2.5 mg/ml with sterile phosphate-buffered saline, and 0.1 ml was injected subcutaneously as previously described (12).

Statistical analysis. Values are means ± SE. Data were compared between groups of animals by *t*-test when one comparison was performed or by ANOVA for multiple comparisons. When significance was indicated by ANOVA, the Tukey-Kramer post hoc test was used to specify between group differences.

RESULTS

Characterization of c-Src$^{+/-}$ mice. It was previously reported that c-Src$^{+/-}$ mice may have reduced levels of c-Src protein as determined by autophosphorylation kinase assay (22). To determine the amounts of c-Src protein in c-Src$^{+/-}$ mice, hearts were homogenized and subjected to Western analysis. As shown in Fig. 1, c-Src$^{+/-}$ mice had significantly less c-Src protein than wild-type mice.

To determine whether both strains had similar hemodynamic responses to exercise, heart rate and blood pressure were monitored at rest and during peak exercise using telemetry. Wild-type and c-Src$^{+/-}$ mice had similar baseline heart rate and blood pressure. At peak exercise, heart rate and blood pressure increased to a similar extent in both strains (Fig. 2). These data show that wild-type and c-Src$^{+/-}$ mice exercised to the same extent, increasing their cardiac output to the same degree.

Determination of eNOS levels in aortic and cardiac tissue. To examine the effect of exercise training on aortic and cardiac eNOS expression, Western analysis was performed on aortic and cardiac homogenates. Aortic eNOS protein levels increased significantly in response to 3 wk of exercise training in wild-type mice (Fig. 3). A similar increase in eNOS protein was observed in the hearts of wild-type mice (Fig. 4). These changes in eNOS expression in the heart or aorta were not observed in c-Src$^{+/-}$ mice (Figs. 3 and 4). These

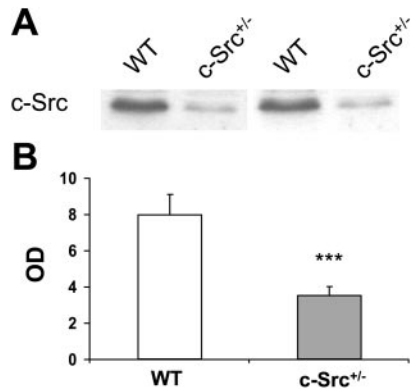

Fig. 1. Western analysis demonstrating c-Src protein expression in hearts of wild-type (WT) and c-Src$^{+/-}$ mice. Mouse hearts were homogenized, and 50 μg of protein were loaded onto a 12.5% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane, and membranes were probed with an antibody for c-Src. *A*: blots for c-Src from 2 mice from each group. *B*: grouped densitometric data expressed as optical density (OD) units. Values are means ± SE ($n = 9$ for each group). ***$P < 0.001$ vs. WT (*t*-test).

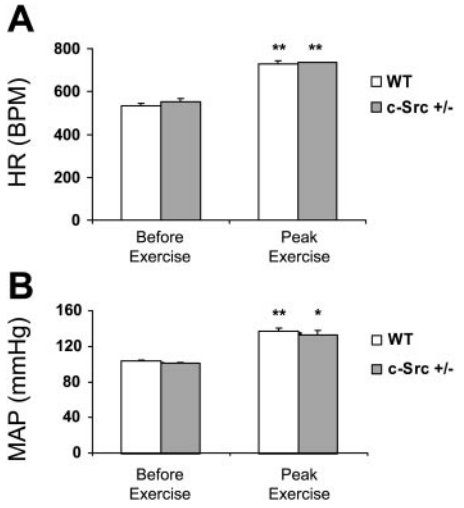

Fig. 2. Heart rate (HR; *A*) and mean arterial pressure (MAP; *B*) in wild-type and c-Src$^{+/-}$ mice before and during exercise. Mice were instrumented with intrarterial catheters attached to radiotransmitters, and hemodynamics were monitored by telemetry beginning 2 wk after surgery. Measurements were obtained at baseline and during exercise once daily for 1 wk. Values are means ± SE ($n = 3$ for each group). Significantly different from before exercise: *$P < 0.05$; **$P < 0.01$ (*t*-test).

data implicate c-Src activation in the increases in eNOS levels in response to exercise training.

Mevastatin has been shown to potently increase eNOS levels in the vasculature by posttranscriptional mechanisms, specifically through a Rho-dependent mechanism involving inhibition of geranylgeranyl pyrophosphate (12). To determine the effect of this Src-independent stimulus on eNOS expression, wild-type and c-Src$^{+/-}$ mice were treated with mevastatin (10 mg·kg^{-1}·day^{-1} sc) for 2 wk. In contrast to exercise training, 2 wk of mevastatin treatment significantly increased eNOS protein expression in wild-type and c-Src$^{+/-}$ mice to the same extent (Fig. 5). These data

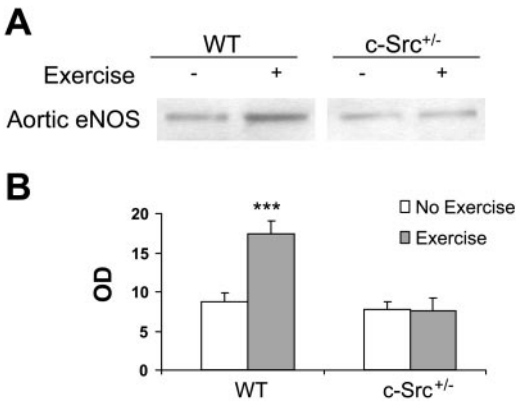

Fig. 3. Western analysis illustrating effect of exercise on aortic endothelial nitric oxide synthase (eNOS) expression in wild-type and c-Src$^{+/-}$ mice. Aortas were homogenized, and 15 μg of protein were separated on a 10% SDS-polyacrylamide gel. Membranes were probed with an antibody for eNOS. *A*: representative blot for eNOS. *B*: grouped densitometric data. Values are means ± SE ($n = 9$ for each group). ***$P < 0.001$ vs. nonexercised (Tukey-Kramer test after 1-way ANOVA).

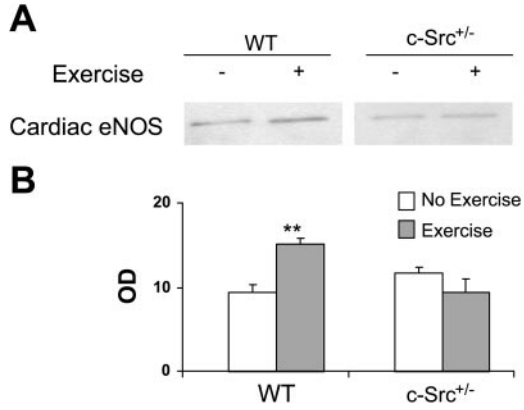

Fig. 4. Western analysis demonstrating effect of exercise on cardiac eNOS expression in wild-type and c-Src$^{+/-}$ mice. Protein (50 μg) was separated on a 10% SDS-polyacrylamide gel, and membranes were probed with an antibody for eNOS. *A*: representative blot for eNOS. *B*: grouped densitometric data. Values are means ± SE ($n = 9$ for each group). **$P < 0.01$ vs. nonexercised (Tukey-Kramer test after 1-way ANOVA).

demonstrate that a stimulus different from exercise training that is Src independent can increase eNOS levels in c-Src$^{+/-}$ mice.

Determination of ecSOD levels in wild-type and c-Src$^{+/-}$ mice. Our laboratory has previously shown that increases in ecSOD levels in response to exercise training are dependent on increased NO from eNOS. Thus the increased ecSOD provides insight into the biological activity of the increase in eNOS expression that occurs during exercise training. Exercise training significantly increased ecSOD protein levels in wild-type mice but had no effect in c-Src$^{+/-}$ mice (Fig. 6). These data demonstrate a role for c-Src activity in exercise-induced ecSOD protein upregulation.

DISCUSSION

We previously defined a role for c-Src in modulating eNOS mRNA transcription and stability in response to

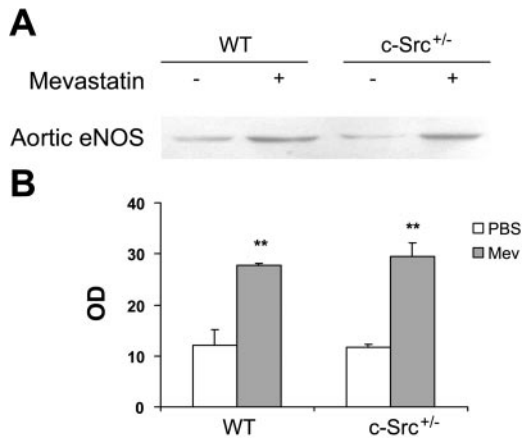

Fig. 5. Western analysis showing effect of chronic mevastatin treatment on aortic eNOS expression in wild-type and c-Src$^{+/-}$ mice. Aortic homogenates (25 μg) were loaded onto a 10% SDS-polyacrylamide gel, and membranes were probed with an antibody for eNOS. *A*: representative blot for eNOS. *B*: grouped densitometric data. Values are means ± SE ($n = 4$ for each group). **$P < 0.01$ vs. nonexercised (Tukey-Kramer test after 1-way ANOVA).

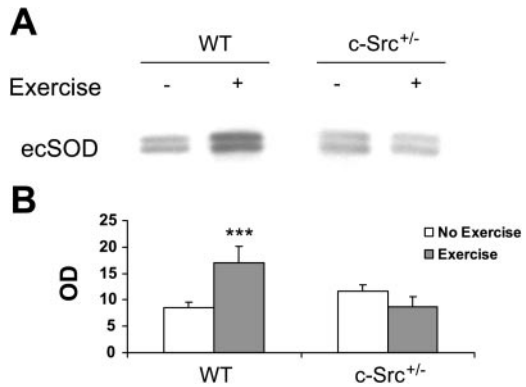

Fig. 6. Western analysis demonstrating effect of exercise training on extracellular superoxide dismutase (ecSOD) levels in wild-type and c-Src$^{+/-}$ mice. Aortic homogenates (15 μg) were loaded onto a 12.5% SDS-polyacrylamide gel, and membranes were probed with an antibody for ecSOD. *A*: representative blot for ecSOD. *B*: grouped densitometric data. Values are means ± SE ($n = 9$ for each group). ***$P <$ 0.001 vs. nonexercised (Tukey-Kramer test after ANOVA).

laminar shear stress. This was shown to occur through two separate pathways: one leading to activation of Ras/Raf/MEK/ERK and resulting in increased transcription of eNOS; and a second, unidentified pathway also signaled by c-Src and leading to prolonged message stabilization. However, there has been limited knowledge of the mechanisms by which exercise training leads to upregulation of eNOS expression. Here we show a critical in vivo role for full c-Src activation/expression in modulating eNOS expression in response to exercise training. This upregulation of eNOS expression has biological significance, inasmuch as it eventually leads to increased expression of ecSOD.

The availability of c-Src knockout mice has allowed us to study the effects of c-Src in exercise-induced vascular responses. Mice completely deficient in c-Src (c-Src$^{-/-}$) are very small, have impaired tooth and bone development, and do not survive past weaning. Therefore, these mice cannot undergo exercise training. The heterozygous mice (c-Src$^{+/-}$), on the other hand, are phenotypically normal. It had been shown by autophosphorylation kinase activity that c-Src$^{+/-}$ mice have reduced c-Src activity (22). We found that these mice had less than one-half the amount of aortic c-Src protein as estimated by Western analysis, in keeping with these prior studies.

To ensure that exercise training had similar effects on hemodynamics in wild-type and c-Src$^{+/-}$ mice, we used telemetry to monitor heart rate and blood pressure under baseline conditions and during exercise. Our data show an increase in heart rate and blood pressure to the same extent during exercise in wild-type and c-Src$^{+/-}$ mice. Furthermore, there was no difference in either of these values during resting conditions. These results show that both strains of mice exercised to the same extent and that the differences in eNOS and ecSOD expression observed in response to exercise training were not due to differences in levels of exercise training in the two groups of mice.

Interestingly, we observed a complete absence of an increase in eNOS and ecSOD expression in mice heterozygous for c-Src. It might have been expected that these animals would have a response to exercise training that was intermediate between that of untrained and exercise-trained wild-type mice. Our data are most compatible with the concept that there must be a threshold effect of c-Src protein level needed for initiation of this transcriptional cascade.

To ensure that deletion of one c-Src gene did not nonspecifically affect the ability of these mice to increase eNOS to any stimulus, we also treated a group of mice with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor mevastatin. Statins have been shown to increase eNOS expression via posttranscriptional mechanisms that depend on activation of the small G protein Rho (12). Our findings indicate that the c-Src$^{+/-}$ mice increased eNOS levels to an extent similar to that in wild-type mice when treated with mevastatin. Thus eNOS expression was not nonspecifically affected in these animals.

Our laboratory has previously shown that NO regulates vascular smooth muscle expression of ecSOD via a pathway involving activation of guanylate cyclase, formation of cGMP, and activation of protein kinase G and p38 MAP kinase. In this prior study, we found that exercise training increased expression of eNOS and ecSOD and that the increase in ecSOD was dependent on the increase in eNOS, inasmuch as it did not occur in eNOS$^{-/-}$ mice (4). In keeping with this prior study, we found that exercise training had no effect on eNOS expression in c-Src$^{+/-}$ mice, in which there is also no increase in eNOS expression. These findings provide evidence that the differences in eNOS expression as a result of exercise training between wild-type and c-Src$^{+/-}$ mice have physiological significance, because a well-defined molecular target of long-term NO upregulation, the ecSOD, was very differently regulated in these animals. Our findings also indicate that c-Src activation importantly modulates the ability of the antioxidant status of the vessel, inasmuch as ecSOD is an important scavenger of extracellular superoxide.

In addition to enhanced shear stress, other stimuli that are altered by exercise might affect eNOS expression. Several studies have suggested that exercise imposes a transient oxidant stress, reflected by increased circulating levels of lipid peroxides and even hydrogen peroxide (8, 9). Indeed, we have shown that hydrogen peroxide is a potent stimulus for eNOS expression in cultured endothelial cells; however, this pathway is not dependent on c-Src (1). There is recent evidence to support the concept that increased eNOS protein expression in response to exercise is dependent on increased flow. Indeed, Miyauchi et al. (17) showed that exercise training in rats increases eNOS expression in the lungs, where blood flow increases during exercise. In contrast, these authors found that exercise training decreases eNOS expression in the kidneys, where flow decreases during exercise (17). Thus the changes in blood flow with exercise seem to parallel changes in eNOS expression.

It is of interest that Src tyrosine kinases were first identified as tumor-promoting enzymes expressed by

the Rous sarcoma virus, and mammalian homologs were first referred to as oncogene kinases (15, 19). It is now abundantly clear that c-Src plays a crucial role in normal cell signaling and, in particular, that endothelial cells respond to mechanical forces via stimulation of c-Src, leading to activation of a number of downstream signaling events. In the present study, we showed a critical role of c-Src in allowing vascular adaptation to the very physiological stimulus of exercise training. Thus, in contrast to a pathological function of c-Src, our data show that this enzyme plays a central role in the beneficial effects of exercise training on vascular function.

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