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Exercise-induced activation of cardiac sympathetic nerve triggers cardioprotection via redox-sensitive activation of eNOS and upregulation of iNOS

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Exercise-induced activation of cardiac sympathetic nerve triggers cardioprotection via redox-sensitive activation of eNOS and upregulation of iNOS. Am J Physiol Heart Circ Physiol 292: H2051–H2059, 2007. First published January 26, 2007; doi:10.1152/ajpheart.01102.2006.—We investigated the mechanism of exercise-induced late cardioprotection against ischemia-reperfusion (I/R) injury. C57BL/6 mice received treadmill exercise (60 min/day) for 7 days at a work rate of 60–70% maximal oxygen uptake. Exercise transiently increased oxidative stress and activated endothelial isoform of nitric oxide synthase (eNOS) during exercise and increased expression of inducible isoform of NOS (iNOS) in the heart after 7 days of exercise. The mice were subjected to regional ischemia by 30 min of occlusion of the left coronary artery, followed by 2 h of reperfusion. Infarct size was significantly smaller in the exercised mice. Ablation of cardiac sympathetic nerve by topical application of phenol abolished oxidative stress, activation of eNOS, upregulation of iNOS, and cardioprotection mediated by exercise. Treatment with the antioxidant N-(2-mercaptopyrrolidinyl)-glycine during exercise also inhibited activation of eNOS, upregulation of iNOS, and cardioprotection. In eNOS−/− mice, exercise-induced oxidative stress was conserved, but upregulation of iNOS and cardioprotection was lost. Exercise did not confer cardioprotection when the iNOS selective inhibitor 1400W was administered just before coronary artery occlusion or when iNOS−/− mice were employed. These results suggest that exercise stimulates cardiac sympathetic nerves that provoke redox-sensitive activation of eNOS, leading to upregulation of iNOS, which acts as mediator of late cardioprotection against I/R injury.

oxidative stress; endothelial nitric oxide synthase; inducible nitric oxide synthase

ISCHEMIC HEART DISEASE is a leading cause of death in industrialized countries. Reduction of infarct size in the face of acute myocardial infarction is of prime importance in saving lives and in improving the prognosis of this devastating disease. Although several drugs such as β-blockers and nitrates are clinically available to mitigate angina and prevent the onset of acute myocardial infarction, a prophylactic use of cardioprotective drugs that are capable of reducing infarct size in a setting of experimental myocardial infarction is limited from the medical and the economical point of view. Thus alternative strategies aimed at alleviating acute myocardial infarction are required for a prophylactic purpose.

A growing body of evidence has demonstrated that brief periods of cardiac ischemia and reperfusion exert a protective effect against subsequent lethal periods of ischemia, a phenomenon termed as ischemic preconditioning (IPC). IPC was first discovered by Murry and associates (33) and has been extensively studied by many investigators in the last two decades. It is now evident that IPC has two distinct phases: an early phase, which lasts from a few minutes to 2–3 h, and a late phase, termed late preconditioning, which develops after 12 h, peaked between 24 and 48 h, and lasts for 72–96 h (1, 2, 40). Therefore, induction of late preconditioning is a promising approach to confer cardioprotection over a prolonged period of time.

Among the maneuvers that induce late preconditioning without pharmacological tools, regular exercise may represent the most convenient and effective means. It has been demonstrated that the incidence of myocardial infarction is reduced by heavy work in men (32, 38). Although improvement of endothelial function, thereby preventing atherosclerosis and coronary occlusion, and prevention of remodeling after myocardial infarction through the expression of oxidative metabolism-related genes have at least in part been attributed to this benefit (29), delaying acute ischemic injury after coronary occlusion by a late preconditioning effect may also be responsible for improving the prognosis of myocardial infarction in these subjects. Experiments in pigs (26) and dogs (17) showed that brief episodes of tachycardia that do not induce ischemia before a prolonged coronary occlusion decrease infarct size. Subsequent experiments in rats (39) and in dogs (18) showed that brief episodes of exercise induced early as well as late preconditioning protection in magnitudes larger than that obtained with ischemia- and tachycardia-induced preconditioning. However, the exact mechanism of late preconditioning against ischemia-reperfusion (I/R) injury induced by exercise remains unclear.

Exercise increases heart rate and contractile force mainly through cardiac sympathetic nerve (CSN) activation. Increased

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cardiac contractile activity activates endothelial nitric oxide (NO) synthase (eNOS) by imposing shear stress on vascular endothelial cells (25). Shear stress has been shown to increase the vascular generation of reactive oxygen species by an endothelium-dependent mechanism (28). Indeed, hydrogen peroxide can increase activity and expression of eNOS in endothelial cells (10, 19). Moreover, it was recently shown that hydrogen peroxide contributes to exercise-induced upregulation of eNOS (27). On the other hand, stimulation of CSN can activate eNOS by activating β-adrenergic receptors (9). NO presumably generated by eNOS has been demonstrated to be a trigger of late preconditioning (4). NO via the formation of reactive oxygen species activates redox-sensitive signaling modules that activate transcriptional factors with resultant upregulation of inducible NO synthase (iNOS), which plays an obligatory role in cardioprotection against I/R injury (3). Such circumstantial evidence prompted us to hypothesize that exercise induces a late preconditioning effect by primarily activating CSN, leading to redox-sensitive activation of eNOS that triggers upregulation of iNOS which acts as a mediator of cardioprotection against I/R injury.

**MATERIALS AND METHODS**

**Animals.** Male eNOS knockout (eNOS−/−) mice, iNOS knockout (iNOS−/−) mice, and wild-type littermates (genetic wild-type, based on C57BL/6 background) mice (10–12 wk of age and 24–28 g body wt) were obtained from Jackson Laboratory (Bar Harbor, ME). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996) and approved by the Kansas Medical University Institutional Animal Care and Use Committee. **Experimental protocol.** These mice were assigned to 14 groups according to the experimental protocol as shown in Fig. 1.

**Ablation of cardiac sympathetic nerve.** Ablation of CSN was performed as described previously (20). Briefly, mice were anesthetized with 2% isoflurane, intubated, and artificially ventilated. The chest was opened via the fifth intercostal space. The epicardial surface of the anterior wall of the left ventricle was painted with a solution of 10% phenol and 70% ethyl alcohol in the distribution of the left anterior descending coronary artery (LAD) to interrupt the cardiac sympathetic fibers innervating that region of the heart. The phenol solution was applied with a pointed handmade cotton swab. Every precaution was taken to prevent the spread of phenol to other parts of the heart and surrounding tissues. The sham-operated control animals received thoracotomy and pericardiotomy as described above without topical application of phenol.

**Measurements of norepinephrine content.** To confirm the efficacy of CSN ablation, anterior wall of the left ventricle was taken 7 days after topical application of phenol. The tissue samples were homogenized with a Polytron in 10% TCA. After centrifugation of the myocardial homogenate (10,000 g, 30 min), the supernatant was neutralized and analyzed for norepinephrine by high-performance liquid chromatography (1a).

**Exercise training protocol.** Exercise was performed 7 days after topical application of phenol as described previously (15). The exercised animals performed 7 days of consecutive treadmill exercise (60 min/day) at an estimated work rate of 60–70% maximal oxygen uptake. Mild electrical shocks were used sparingly to motivate animals to run. Control animals did not perform treadmill exercise but were placed on a nonmoving treadmill for 60 min/day for 7 days. The antioxidant, N-(2-mercaptopyrrolinyl)-glycine (MPG) was obtained from Sigma (Tokyo, Japan), dissolved in PBS, and injected intraperitoneally at a dose of 100 mg/kg and final volume of 0.1 ml 30 min before the animal was placed on a treadmill.

**Treatment with 1400W.** The iNOS selective inhibitor 1400W was purchased from Alexis (San Diego, CA), dissolved with dimethyl sulfoxide (DMSO), diluted with PBS, and injected intraperitoneally at a dose of 10 mg/kg and final volume of 0.1 ml 30 min before coronary artery occlusion. The rest of the groups received the same amount and concentration of DMSO as a vehicle.

**Myocardial I/R protocol.** Mice were anesthetized with a mixture of ketamine-xylazine (80 and 10 mg/kg, respectively, diluted in PBS) by an intraperitoneal route. Mice were intubated, and artificial respiration was maintained at a fraction of inspired oxygen of 0.8 by using 100 strokes/min and a 2- to 3-ml stroke volume delivered through a loose connection from the rodent ventilator.

A left thoracotomy was performed at the fifth intercostal space, and the pericardium was opened to expose the heart. The LAD was ligated 1 to 2 mm from its origin by a 7-0 silk suture with an atraumatic needle, and the ends of this ligature were passed through a small vinyl tube to form a snare. After the completion of the surgical procedure, the heart was returned to its normal position in the thorax. The
thoracic cavity was covered with saline-soaked gauze to prevent the heart from drying. The animals were then allowed to stabilize for 15 min before LAD ligation. Myocardial ischemia was induced by one-stage occlusion of the LAD by pressing the polyethylene tubing against the ventricular wall and then fixing it in place by clamping the vinyl tube with a hemostat. The animals then underwent 30 min of ischemia, confirmed visually in situ by the appearance of regional epicardial cyanosis and ST-segment elevation. The myocardium was ischemia, confirmed visually in situ by the appearance of regional ischemia cyanotic segment.

Measurements of myocardial norepinephrine content. Control mice with sham-operated surgery and mice treated with topical application of phenol were euthanized 7 days later, and myocardial norepinephrine content was measured as described in the text. Each bar graph represents means ± SE of 5 experiments. *P < 0.05 compared with control.

Myocardial ischemia was induced by 5 min before LAD ligation. Myocardial ischemia was induced by 5 min before LAD ligation. Myocardial ischemia was induced by injecting into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned along the long axis in 1-mm-thick sections, which were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five 1-mm-thick myocardial slices was weighed and digitally photographed to determine infarct size as a percentage of risk region, and the areas of infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using an image-analyzing software (Win Roof, Mitani, Fukui, Japan) and a computer-assisted planimeter.

Measurements of myocardial GSH and GSSG. Myocardial GSH and GSSG were measured using a Bioxytech GSH/GSSG-412 colorimetric assay kit from Oxis Research (Portland, OR). At an indicated time, mice were anesthetized by an overdose of pentobarbital sodium, and the heart was rapidly excised and snap frozen in liquid nitrogen. Frozen myocardial tissue samples were homogenized (g/10 ml) in 5% metaphosphoric acid. The procedure was followed as per manufacturer’s instructions, and the levels were quantitated as micromolar GSH or GSSG based on standard supplied along with the kit.

Western blot analysis. Frozen myocardial tissue samples were taken as in GSH/GSSG assay, ground with a mortar and pestle, and subsequently placed into a tissue grinder containing lysis buffer containing 30 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail Complete (Roche Diagnostics, Mannheim, Germany). The protein concentration was determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The lysate samples were separated by a 7.5% SDS-PAGE, and the separated proteins were transferred to a polyvinylidene-difluoride membrane with a transfer buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol. The membranes were blocked with 5% skimmed milk and incubated in primary antibodies specific for eNOS, phospho-eNOS (Ser1177), and rabbit polyclonal anti-nitrotyrosine (NT) (Cell Signaling Technology, Beverly, MA) or iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and they were subsequently incubated with a peroxidase-conjugated secondary antibodies and developed using an enhanced chemiluminescence detectionsystem (Amersham Biosciences, Tokyo, Japan) according to the manufacturer’s instructions. The immunolabeling was quantified with a densitometric analysis using Win Roof (Mitani). Equal loading of the samples and consistency in the data analysis were ensured by normalization of each immunoblot signal to the corresponding Coomassie blue stain signal as described previously (31).

NOS activity assay. iNOS activity assay was performed as described (37). In brief, the frozen heart tissues were homogenized in 4 vol of buffer containing 10 mM HEPES (pH 7.2), 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor cocktail. The homogenate was centrifuged, and aliquots of the supernatant were incubated for 60 min at 37°C with l) assay cocktail containing 50 mM L-valine, 1 mM DTT, 0.1 mM NADPH, 0.1 mM tetrahydrobiopterin, 1 mM L-citrulline, 18 μM l-arginine, 2 μM L-[14C]arginine, 1 mM MgCl2, and 0.2 mM CaCl2 in 50 mM KH2PO4 (pH 7.2); 2) cocktail plus 1 mM EGTA; or 3) cocktail plus 1 mM EGTA plus 1 mM ml-

![Fig. 2. Myocardial norepinephrine content. Control mice with sham-operated surgery and mice treated with topical application of phenol were euthanized 7 days later, and myocardial norepinephrine content was measured as described in the text. Each bar graph represents means ± SE of 5 experiments. *P < 0.05 compared with control.](http://ajpheart.physiology.org/)
N\textsuperscript{\textcircled{-}}-nitro-L-arginine methyl ester to determine the total and Ca\textsuperscript{2+}-
independent NOS (iNOS) activity. NOS activity was quantified by measuring L-[\textsuperscript{14}C]citrulline with a liquid scintillation counter following the removal of untreated L-[\textsuperscript{14}C]arginine with 50W-X8 Dowex resin (Muromachi Technos, Tokyo, Japan).

Statistical analysis. All numerical data are expressed as means ± SE. Statistical analysis of data was performed with one-way ANOVA, followed by the Bonferroni post hoc test.

RESULTS

Effect of topical application of phenol on norepinephrine content. Norepinephrine was completely depleted from the anterior wall of left ventricle 7 days after topical application of phenol (Fig. 2), indicating that CSN was successfully ablated by topical application of phenol. However, we were not able to detect a significant change in norepinephrine content in the control heart during and after exercise (not shown).

Effect of topical application of phenol and MPG on exercise-induced reduction of GSH/GSSG and formation of NT. We then examined whether activation of CSN exists upstream of oxidative stress during exercise. To this end, we measured glutathione redox state (GSH/GSSG) during exercise, because changes in the GSH/GSSH accurately reflect global changes in all of the intracellular antioxidant redox systems (23). GSH/GSSG was temporarily reduced by exercise (Fig. 3A). However, it returned to the baseline level 24 h after exercise. An exercise-induced decrease in GSH/GSSG was blocked by topical application of phenol or by treatment with MPG during exercise.

We also measured 3-NT formation during exercise. Formation of 3-NT detected as a 30-kDa band was determined as a marker of oxidative-nitrosative stress (24). 3-NT in the heart was increased during exercise training but was dissipated 24 h after exercise (Fig. 3B). Exercised-induced increase in 3-NT formation was blocked by topical application of phenol or by treatment with MPG during exercise.

Effect of topical application of phenol and MPG on activation of eNOS. Phosphorylation of eNOS on Ser1177 was thought to be an indicator of Ca\textsuperscript{2+}-independent activation of eNOS (16). Phosphorylation of eNOS on Ser1177 was significantly increased during exercise and was attenuated 24 h after exercise (Fig. 4A). Exercise-induced phosphorylation of eNOS was blocked by topical application of phenol or by treatment with MPG during exercise. Expression of eNOS was modestly but significantly increased 7 days after daily exercise (Fig. 4B).
and remained elevated 24 h after the last exercise session. Topical application of phenol or treatment with MPG during exercise significantly inhibited expression of eNOS. Activation of eNOS was evaluated by calculating the relative ratio of phosphorylation of eNOS to total eNOS (Fig. 4C). The ratio of phosphorylation of eNOS to total eNOS was significantly increased during exercise but returned to the baseline 24 h after exercise. Topical application of phenol or treatment with MPG during exercise significantly inhibited the increase in this ratio. These results indicate that exercise produces oxidative stress by activating CSN which temporarily activates eNOS and subsequently increases expression of eNOS 7 days after exercise training.

**Effect of topical application of phenol and MPG on expression and activity of iNOS and infarct size.** We then studied whether CSN promotes upregulation of iNOS and triggers cardioprotection after exercise. A significant increase in iNOS expression (Fig. 5A) and activity (Fig. 5B) was observed 7 days after daily exercise. Expression and activity of iNOS remained elevated 24 h after the last exercise session. Topical application of phenol or treatment with MPG during exercise inhibited the upregulation of iNOS.

Coronary artery occlusion was performed 24 h after the last exercise session. Infarct size measured 2 h after reperfusion following 30 min of coronary artery occlusion was significantly smaller in exercised animals (Fig. 5C). Topical application of phenol or treatment with MPG during exercise had no effect on infarct size in mice without exercise but blocked the infarct size-limiting effect of exercise.

**Effect of exercise on GSH/GSSG and formation of NT in eNOS−/− and iNOS−/− mice.** To investigate whether exercise-induced oxidative stress is indeed an upstream event of eNOS activation and iNOS expression, we measured GSH/GSSG and 3-NT in eNOS−/− and iNOS−/− mice. The reduced GSH/GSSG level induced by exercise was not affected in both eNOS−/− and iNOS−/− mice (Fig. 6A), suggesting that exercise-induced oxidative stress exists upstream of eNOS activation and iNOS expression. However, the increase in 3-NT formation induced by exercise was inhibited in eNOS−/− mice but not in iNOS−/− mice (Fig. 6B), suggesting that eNOS-mediated NO was necessary for 3-NT formation during exercise.

**Effect of exercise on expression and activity of iNOS and infarct size in eNOS−/− mice.** We then investigated the role of activation of eNOS in the upregulation of iNOS and cardioprotection induced by exercise. Exercise did not increase the expression and activity of iNOS in eNOS−/− mice 24 h after the last exercise session (Fig. 7, A and B), suggesting that eNOS plays a crucial role in the upregulation of iNOS induced by daily exercise.

In contrast to the wild-type mice, daily exercise did not reduce infarct size in eNOS−/− mice (Fig. 7C). Effect of 1400W and iNOS gene ablation on expression and activity of iNOS and exercise-induced limitation of infarct size. We next investigated the role of iNOS in cardioprotection conferred by 7 days of exercise. Although treatment with 1400W had no effect on enhanced expression of iNOS induced by exercise (Fig. 8A), it abolished the activation of iNOS (Fig. 8B). Both the enhanced expression and activity of iNOS 24 h after the last exercise session were not observed in iNOS−/− mice (Fig. 8, A and B). Treatment with 1400W just before coronary artery occlusion or the ablation of iNOS gene had no effect on infarct size in nonexercised mice but abolished the infarct size-limiting effect of daily exercise (Fig. 8C), suggesting that an upregulation of iNOS plays a crucial role in mediating cardioprotection after daily exercise.

**DISCUSSION**

We investigated the mechanism of cardioprotection conferred by daily exercise. The results suggest that exercise produces a late preconditioning effect primarily through the activation of CSN. Activation of the CSN provokes oxidative stress and transient activation of eNOS leading to upregulation of iNOS, which acts as a mediator of cardioprotection against I/R injury. The salient findings to support this hypothesis are summarized as follows: 1) exercise training at an estimated work rate of 60–70% maximal oxygen uptake reduced a
GSH/GSSG level and increased 3-NT formation associated with increased eNOS activity in the heart; 2) iNOS expression and activity were increased 24 h after the last exercise session; 3) upregulation of iNOS was associated with reduced infarct size after 30 min of coronary artery occlusion followed by 2 h of reperfusion; 4) topical application of phenol depleted nor-epinephrine in the heart and blocked reduction of GSH/GSSG, 3-NT formation, and activation of eNOS during exercise that inhibited the upregulation of iNOS 24 h after the last exercise session and the reduction of infarct size; 5) treatment with MPG during exercise also blocked the reduction of GSH/GSSG, 3-NT formation, and activation of eNOS that inhibited the upregulation of iNOS 24 h after the last exercise session and the reduction of infarct size; 6) reduction of GSH/GSSG during exercise was not altered in eNOS−/− mice, but the upregulation of iNOS 24 h after the last exercise session and the reduction of infarct size were not observed in these mice; 7) treatment with 1400W before coronary artery occlusion abolished iNOS activation and the reduction of infarct size mediated by daily exercise training; and 8) upregulation of iNOS 24 h after the last exercise session and the reduction of infarct size were not observed in iNOS−/− mice.

The present study employed 7 days of consecutive exercise aimed at producing a significant cardioprotective effect. In a preliminary study, we adopted 1 day of exercise. Although activation of eNOS was observed during this exercise protocol, similar to that observed after 7 days of consecutive exercise, there was no significant upregulation of iNOS 24 h after exercise and no infarct size-limiting effect (Y. Akita and H. Otani, unpublished observation). We attributed the inability of 1 day of exercise to completely mimic the 7 days of consecutive exercise to the lack of increased expression of eNOS. In the present study, we demonstrated that eNOS expression was modestly but significantly increased after 7 days of consecutive exercise, whereas 1 day of exercise failed to induce an upregulation of iNOS (not shown). The increased expression of eNOS resulted in a net increase in phosphorylation of eNOS on Ser1177 despite a similar relative increase in phosphorylation of eNOS during exercise between 1 day of exercise and 7 days of consecutive exercise. Such enhanced eNOS activation during exercise by itself may not be crucial in mediating cardioprotection 24 h after the last exercise session but is capable of promoting a signal transduction pathway for expression of the iNOS gene during exercise, leading to the upregulation of
iNOS and iNOS-dependent cardioprotection against I/R injury. This assumption raises an important clinical implication that daily regular exercise is necessary to provoke a powerful late preconditioning effect by increasing the expression of eNOS. It has been shown that upregulation of eNOS by regular exercise takes 10 days to several months (21). We have chosen only 7 days of consecutive exercise, because prolonged periods of regular exercise may induce innervation of CSN that had been ablated by topical application of phenol. Nevertheless, our exercise protocol induced a modest but significant upregulation of eNOS. The duration of exercise training to induce an expression of eNOS in the heart may differ because of species differences, sedentary conditions before exercise, and intensity of exercise training.

The present study suggests that oxidative stress is an upstream event of eNOS activation induced by CSN stimulation during exercise, because exercise-induced reduction of GSH/GSSG was observed in eNOS−/− mice. However, the molecular link between CSN activation and oxidative stress and between oxidative stress and activation of eNOS remains unclear. There are several lines of evidence suggesting that the molecular mechanism of exercise-induced activation of eNOS is closely related to the changes in frequency and magnitude of physical forces in the vasculature, in particular, in fluid shear stress. We could not measure hemodynamic changes during exercise in the present study simply because of a methodological reason. However, it is reasonable to speculate that exercise-induced activation of CSN increases cardiac contractility and heart rate, which in turn increases blood flow and vascular shear stress. Shear stress has been shown to increase the vascular generation of reactive oxygen species by an endothelium-dependent mechanism (28). Reactive oxygen species trigger activation of signal transduction, leading to phosphorylation of eNOS. It has been demonstrated that shear stress-induced eNOS activation does not depend on a rise of intracellular Ca2+ but is directly dependent on phosphorylation of eNOS on Ser1177 mediated by the serine/threonine protein kinase Akt (16). However, activation of eNOS through an Akt-phosphorylation-dependent pathway appears to be dependent on the degree of oxidative stress. Mild oxidative stress activates the Akt/eNOS pathway, whereas severe oxidative stress inhibits phosphorylation of eNOS by depleting cell surface thiols, suggesting that eNOS activation is exquisitely sensitive to regulation by redox signaling (34). Indeed, phosphorylation of eNOS on Ser1177 and coronary vascular diameter after reperfusion were closely related to the severity of

**Fig. 8.** A: Western blot analysis for iNOS. B: iNOS activity. C: infarct size. Experimental groups are shown in Fig. 1. Each bar graph represents means ± SE of 5 experiments. *P < 0.05 compared with Wild Ex (−); †P < 0.05 compared with Wild Ex (+).
ischemia and the degree of oxidative stress (22). It should be
noted, however, that activation of Akt is not a sole mechanism
for phosphorylation of eNOS. In addition to Akt, phosphoryla-
tion of eNOS is also mediated by AMP-activated protein
kinase, an enzyme that is activated by vigorous exercise and
ischemic stress, in the presence of Ca\(^{2+}\) -calmodulin (11) as
well as by a coordinated interaction between Akt and cAMP-
dependent protein kinase, i.e., protein kinase A (8). Protein
kinase A activation in endothelial cells may be mediated by
increased norepinephrine release associated with CSN activa-
tion through activation of β-adrenergic receptors and a result-
ant production of cAMP. Therefore, the exact mechanism of
exercise-induced phosphorylation of eNOS remains to be in-
vestigated in our model.

Expression of eNOS was also found to be dependent on CSN
activation and oxidative stress, because increased expression of
eNOS 7 days after exercise was blocked by topical application
of phenol or by treatment with MPG during exercise. The regula-
tion of eNOS expression is highly complex. A variety of
factors, such as lysophosphatidylcholine, cGMP analogs,
lipoproteins, inhibitors of protein kinase C, and different cyto-
kines, is known to alter eNOS expression (30). However, a
growing body of evidence suggests that shear stress plays a
 crucial role in increasing the expression of eNOS induced by
exercise. For example, laminar shear stress was found to
 provoke a robust upregulation of eNOS mRNA and protein in
cultured endothelial cells (36). Moreover, it has been dem-
onstrated that shear stress increases expression of eNOS mRNA
in a vascular region that is exposed to high shear stress in vivo
(12). Thus an increased intensity of physiological shear stress,
as expected by activation of CSN during exercise, might
increase vascular eNOS expression through oxidative stress in
line with the role of shear stress in generating reactive oxygen
species (28) and the role of reactive oxygen species in exercise-
duced upregulation of eNOS (19).

The present study indicates that not only exercise-induced
oxidative stress but also activation of eNOS is required for an
upregulation of iNOS and cardioprotection. This is because an
upregulation of iNOS and a reduction of infarct size were not
observed in eNOS 
−/− mice despite a generation of oxidative
stress as demonstrated by a reduction of GSH/GSSG during
exercise in these mice. It has been demonstrated that eNOS-
diated formation of NO and reactive oxygen species triggers
late preconditioning (13, 14). NO generated by eNOS via the
formation of reactive oxygen species activates protein kinase
C-ε, which activates transcriptional factors with a resultant
upregulation of iNOS that plays an obligatory role in cardio-
protection against I/R injury (3, 14, 35). It has been previously
shown that the delayed cardioprotective effects of exercise in
dogs are sensitive to aminoguanidine, an inhibitor of iNOS,
suggesting that exercise-induced late preconditioning is medi-
ated by iNOS. The results of the present study demonstrating
that activation of iNOS and cardioprotection induced by daily
exercise were abolished by preischemic treatment with a highly
selective iNOS inhibitor, 1400W, or in iNOS knockout mice
point to the same conclusion that iNOS is a mediator of late
preconditioning induced by exercise. The role of iNOS as a
mediator of exercise-induced late preconditioning is consistent
with the hypothesis that iNOS is a common mediator of late
preconditioning induced by ischemic challenges or by pharma-

cological agents (5, 6, 41).

In conclusion, the present study demonstrated for the first
time that exercise stimulates CSN which provokes redox-
sensitive activation of eNOS, leading to an upregulation of
iNOS that acts as a mediator of late cardioprotection against
I/R injury.

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