

Chronic Exercise in Dogs Increases Coronary Vascular Nitric Oxide Production and Endothelial Cell Nitric Oxide Synthase Gene Expression

William C. Sessa, Kirkwood Pritchard, Nahid Seyedi, Jie Wang, Thomas H. Hintze

Abstract Recently, we have shown that chronic exercise increases endothelium-derived relaxing factor (EDRF)/nitric oxide (NO)-mediated epicardial coronary artery dilation in response to brief occlusion and acetylcholine. This finding suggests that exercise can provide a stimulus for the enhanced production of EDRF/NO, thus possibly contributing to the beneficial effects of exercise on the cardiovascular system. Therefore, the purpose of the present study was to examine whether chronic exercise could influence the production of NO (measured as the stable degradation product, nitrite) and endothelial cell NO synthase (ECNOS) gene expression in vessels from dogs after chronic exercise. To this end, dogs were exercised by running on a treadmill (9.5 km/h for 1 hour, twice daily) for 10 days, and nitrite production in large coronary vessels and microvessels and ECNOS gene expression in aortic endothelial extracts were assessed. Acetylcholine (10^{-7} to 10^{-5} mol/L) dose-dependently increased the release of nitrite (inhibited by nitro-L-arginine) from coronary arteries and microvessels in control and exercised dogs. Moreover, acetylcholine-stimulated nitrite production was markedly enhanced in large coronary arteries and microvessels prepared from hearts

of dogs after chronic exercise compared with hearts from control dogs. One potential mechanism that may contribute to the enhanced production of nitrite in vessels from exercised dogs may be the induction of the calcium-dependent ECNOS gene. Steady-state mRNA levels for ECNOS were significantly higher than mRNA levels for von Willebrand's factor (vWF, a specific endothelial cell marker) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a constitutively expressed gene) in exercised dogs. Densitometric analysis of ECNOS gene expression compared with vWF (to normalize for endothelial cell RNA isolation) or GAPDH expression revealed a twofold to threefold increase in ECNOS gene expression in exercised dogs relative to control dogs. Collectively, these data demonstrate that chronic exercise, presumably by increasing endothelial shear stress, increases EDRF/NO production and ECNOS gene expression and may contribute to the beneficial effects (ie, antihypertensive) of exercise on the cardiovascular system. (*Circ Res.* 1994;74:349-353.)

Key Words • exercise • endothelium • nitric oxide • gene expression

Endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO)^{1,2} or a closely related molecule,³ is released by the endothelium in response to local hormones, changes in blood flow velocity, or endothelial shear stress.⁴ However, the physiologically relevant stimulus for EDRF/NO release in vivo is not known and may reflect the summation of local hormones and blood flow. Evidence supporting the contribution of basally released EDRF/NO to the regulation of vascular tone is derived from experiments showing that specific inhibitors of NO synthase (NOS) elicit a prolonged pressor response and reduce regional blood flows in vivo and inhibit endothelium-dependent relaxations in vitro.⁵

The first demonstration that chronic alterations in local blood flow can exert an influence on EDRF/NO-mediated responses was shown by Miller and colleagues.^{6,7} They demonstrated that femoral arteries exposed to prolonged increases in flow (via opening an arteriovenous anastomosis) exhibited enhanced endothelium-dependent relaxations in vitro. In addition, increasing coronary blood flow by chronic cardiac pacing in dogs also enhances endothelium-dependent dilation.⁸ Conversely, in experimental models of congestive heart failure, where blood flow is compromised, endothelium-dependent relaxations in vitro and flow-mediated dilation in vivo are depressed.^{9,10} Collectively, these data suggest that chronic alterations in blood flow can regulate the expression of EDRF/NO-mediated endothelium-dependent dilation.

A physiological stimulus that increases intracoronary blood flow is acute or chronic exercise.¹¹ Acute exercise increases coronary blood flow and flow velocity, thereby causing dilation of epicardial coronary arteries. This is supported by experiments demonstrating that restriction of flow (by a critical stenosis) prevents exercise-induced epicardial vasodilation.¹² The flow velocity-mediated epicardial dilation is likely mediated by EDRF/NO, because inhibitors of NOS blunt this response.¹³ Recently, we have shown that chronic exercise

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increased EDRF/NO-mediated epicardial coronary artery dilation in response to brief occlusion and acetylcholine.¹³ This finding suggested that exercise could provide a stimulus for the enhanced production of EDRF/NO, thus possibly contributing to the beneficial effects of exercise on the cardiovascular system. Therefore, the purpose of the present study was to examine whether chronic exercise affected the production of NO (measured as the stable degradation product, nitrite) and endothelial cell NOS (ECNOS) gene expression in vessels from dogs after chronic exercise.

Materials and Methods

Surgical Preparation and Hemodynamic Measurements in Conscious Dogs

Before surgery, all dogs were screened to run on a treadmill (Talbot Carlson, Inc, Audubon, Iowa), and surgery was performed only on dogs that ran well at speeds of 10.9 km/h. Dogs (25 to 29 kg) of either sex were sedated with acepromazine (0.3 mg/kg IM, Ayerst) and anesthetized with pentobarbital sodium (25 mg/kg IV). Dogs were prepared for sterile surgery, and a thoracotomy was performed in the left fifth intercostal space. A Tygon catheter was placed in the descending thoracic aorta, a solid pressure gauge (Konigsberg Instruments, Inc, Pasadena, Calif) was placed into the apex of the left ventricle, and a flow-cuff transducer (3.5- to 5.0-mm diameter, Parks, Eugene, Ore) was implanted on the left circumflex coronary artery. Sonomicrometer crystals (7 MHz, 1×2 mm) were sutured on opposing surfaces of the same artery.

The catheter and wires were run subcutaneously to the intracapsular region. The chest was closed in layers, and the pneumothorax was reduced. Antibiotics were given after surgery as necessary. After 10 days, dogs (n=10) were trained to lie quietly on the laboratory table. A subset of control dogs (n=5) was then trained to exercise on a treadmill at 9.5 km/h for 1 hour in the morning and 1 hour in the afternoon for 10 consecutive days. The following hemodynamic variables were measured in dogs lying on the table or during exercise: aortic pressure, heart rate, left ventricular pressure and its first derivative (dP/dt), left circumflex coronary artery diameter, and blood flow. Late-diastolic coronary vascular resistance was calculated as an index of changes in the diameter of coronary resistance vessels according to the following equation: late-diastolic aortic pressure/diastolic coronary blood flow.¹⁴ All data were recorded on a 14-channel tape recorder and played back on a direct-writing oscillograph as previously described.¹⁴

The protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the guiding principles for the use and care of laboratory animals of the American Physiological Society and the National Institutes of Health.

Isolation of Coronary Microvessels and Large Coronary Arteries

Hearts were obtained immediately from pentobarbital-anesthetized dogs and kept in ice-cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, pH 7.4. Circumflex and left anterior descending coronary arteries were isolated, cleaned of periadventitial fat, and cut into 2- to 4-mm rings. Coronary microvessels from the left ventricle were prepared by the method of Gerritsen and Printz.¹⁵ Typically, microvessels (1.5 g) were obtained from the left ventricle of a single dog. After preparation, coronary vessels and microvessels were preincubated in PBS gassed with 95% O₂/5% CO₂ for 30 minutes at room temperature.

Measurement of Nitrite Release From Large Coronary Arteries and Microvessels

Coronary arterial rings (two or three rings) or microvessels (20 mg wet weight) prepared from hearts of control or

exercised dogs were incubated in PBS (500 μL) in the absence or in the presence of increasing concentrations of acetylcholine (10⁻⁷ to 10⁻⁵ mol/L) for 20 minutes at 37°C. In preliminary studies, the accumulation of nitrite in the supernatant was linear for 20 minutes (data not shown). After the 20-minute incubation period, the supernatant was removed and assayed for nitrite, the predominant breakdown product of NO in aqueous solution, via the Griess reaction. Supernatants (500 μL) were incubated with acidified sulfanilamide (0.1%, 450 μL) and *N*-(1-naphthyl)-ethylenediamine (0.2%, 50 μL) for 10 minutes at room temperature. Nitrite accumulation was monitored spectrophotometrically at 540 nm and compared with sodium nitrite standards. The lower limit of sensitivity of this assay is 0.5 nmol/mL.

Northern Blot Analysis

Aortas (n=5 each, from control and exercised dogs) were perfused with aerated sterile medium 199 plus Hanks' buffer (500 mL) *in situ*. The ascending and descending segments of thoracic aortas were removed, and endothelial cells were scraped with a scalpel blade (No. 10) into guanidine isothiocyanate as previously described.¹⁶ Total RNA (10 μg) was denatured by heating (65°C) in 50% (vol/vol) formamide and 4.4 mol/L formaldehyde, electrophoresed through a 1.2% agarose gel containing 2.2 mol/L formaldehyde, transferred to a nylon membrane, and hybridized to a ³²P-labeled cDNA probe by standard methods.¹⁷ Blots were hybridized overnight at 65°C with random-primed full-length bovine ECNOS cDNA (18, detects 4.4-kb mRNA, 2×10⁹ cpm/μg), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (detects 1.5-kb mRNA, 1.5×10⁹ cpm/μg), or human von Willebrand's factor (vWF) cDNA (detects 8-kb mRNA, 1.2×10⁹ cpm/μg). Blots were washed twice in 2×standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 15 minutes at room temperature and then twice in 0.4× SSC/0.1% SDS for 15 minutes at 65°C, rinsed in 0.1 mol/L phosphate buffer, pH 7.0, dried, and exposed to x-ray film in the presence of an intensifying screen for 4 days. Under these hybridization and wash conditions, the bovine ECNOS cDNA probe does not cross hybridize with rat neuronal,^{17,18} human hepatocyte, or murine macrophage NOS cDNAs or RNAs but does cross hybridize with human ECNOS cDNA and RNA (W.C. Sessa, unpublished data). Optical densities of hybridization signals on x-ray films were measured by densitometry for quantification of steady-state RNA levels.

Statistical Analysis

All data are expressed as mean±SEM from n dogs. The data were analyzed by an unpaired Student's *t* test, with *P*<.05 taken as statistically significant. Nitrite levels were initially evaluated using a one-way ANOVA. Differences were quantitated by converting the ratio of *F* values to a *t* distribution using Scheffé's test.

Results

Acute exercise (running at 9.5 km/h) of chronically instrumented, conscious dogs (n=5) significantly (*P*<.05) increased ventricular pressure (from 139±5 to 199±16 mm Hg), left ventricular dP/dt (from 3339±275 to 5552±455 mm Hg/s), mean arterial pressure (from 105±4 to 126±4 mm Hg), heart rate (from 99±10 to 201±9 beats per minute), and peak coronary blood flow (from 37±3 to 65±6 mL/min) and decreased late-diastolic coronary resistance (from 2.6±0.3 to 1.9±0.5 mm Hg·mL⁻¹·min). This level of exercise was repeated twice daily for 10 days. Exercise training for 10 days did not affect resting heart rate, coronary blood flow, or other cardiac parameters, suggesting that classic "cardiac conditioning" did not occur. In dogs, at least 4

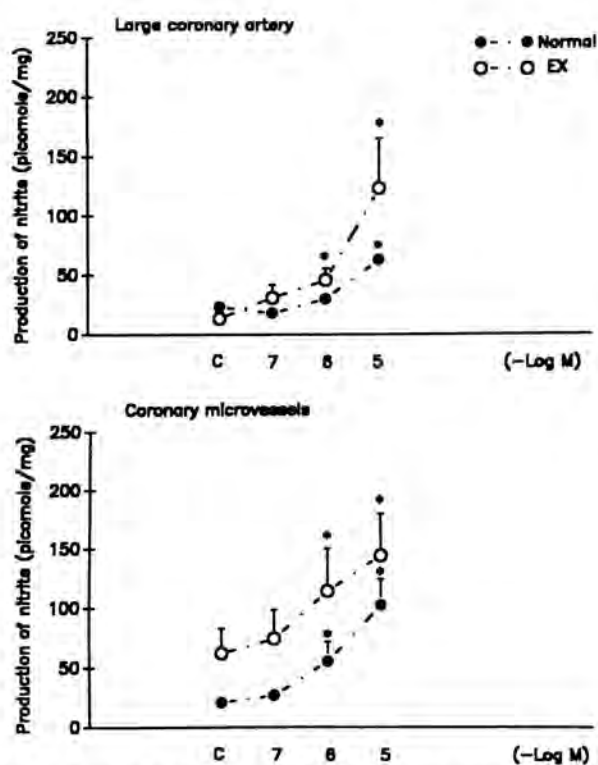


Fig 1. Graphs showing that acetylcholine-stimulated nitrite production is increased in large coronary arteries (top) and microvessels (bottom) isolated from exercised (EX) dogs (○) compared with nonexercised (normal) dogs (●). C represents control incubations in the absence of acetylcholine. Vessels from normal ($n=5$) or EX ($n=5$) dogs were incubated with acetylcholine (10^{-7} to 10^{-5} mol/L) for 20 minutes, and supernatants were processed for measurement of nitrite as described. * $P < .05$.

to 10 weeks of training is necessary to significantly increase epicardial coronary diameter, capillary density, and maximal coronary reserve and to reduce resting heart rate.¹⁹⁻²¹

Fig 1 depicts the production of nitrite in large coronary arteries and microvessels prepared from hearts of control and exercised dogs 1 day after the last training period. The basal production of nitrite was not significantly different in large coronary vessels or microvessels from control or exercised dogs ($n=5$). Acetylcholine (10^{-7} to 10^{-5} mol/L) dose-dependently increased the release of nitrite from coronary arteries and microvessels in both groups of dogs. Moreover, acetylcholine-stimulated nitrite production was enhanced in vessels prepared from hearts of exercised dogs compared with hearts from control dogs. The generation of nitrite was completely inhibited by preincubation of the vessels with nitro-L-arginine in an L-arginine-reversible manner, demonstrating that the nitrite was derived via the metabolism of L-arginine by ECNOS. Furthermore, all nitrate production was eliminated by boiling the tissue or by chelating both intracellular and extracellular calcium.

One potential mechanism that may contribute to the enhanced agonist-stimulated production of nitrite in vessels from exercised dogs may be the induction of the calcium-dependent ECNOS gene. In a separate group of dogs (aortas not used), we measured cardiac output before and during exercise. Exercise increased cardiac

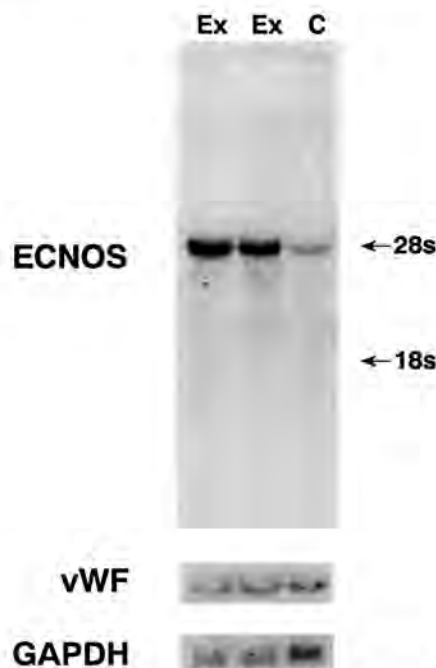


Fig 2. Northern blot showing that exercise induces endothelial cell nitric oxide synthase (ECNOS) gene expression in aortic extracts. Total RNA ($10 \mu\text{g}$) from two exercised (Ex) dogs and 1 control (C) dog was hybridized with full-length cDNAs for ECNOS, von Willebrand's factor (vWF), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and washed under high-stringency conditions as described.

output from 2.89 ± 0.50 to 6.20 ± 0.42 L/min ($n=5$), demonstrating that the proximal aorta was clearly being exposed to high-flow velocity. Because of difficulties in the isolation of adequate amounts and high-quality RNA from large coronary arteries and coronary microvessels, we isolated aortic endothelial cell RNA. Fig 2 shows Northern blot analysis of total RNA isolated from aortic endothelial cell scrapings from control or exercised dogs probed (under high-stringency conditions of hybridization and washing) with ^{32}P -labeled cDNAs for ECNOS, vWF, and GAPDH. Steady-state mRNA levels for ECNOS were significantly higher than mRNA levels for vWF (a specific endothelial cell marker) and GAPDH (a constitutively expressed gene) in exercised dogs. Densitometric analysis of ECNOS gene expression compared with vWF (to normalize for endothelial cell RNA isolation) or GAPDH expression revealed a twofold to threefold increase in ECNOS gene expression in exercised dogs relative to control dogs (Fig 3).

Discussion

The present study demonstrates that chronic exercise in dogs increases the release of L-arginine-derived nitrite from isolated large coronary arteries and microvessels and specifically increases ECNOS gene expression in aortic endothelial cells. These data support the observation that chronic exercise augments hormone- or flow velocity-induced endothelium-dependent dilation of epicardial coronary vessels in conscious dogs.¹³ More importantly, this is the first demonstration that the ECNOS gene can be regulated in vivo, thereby

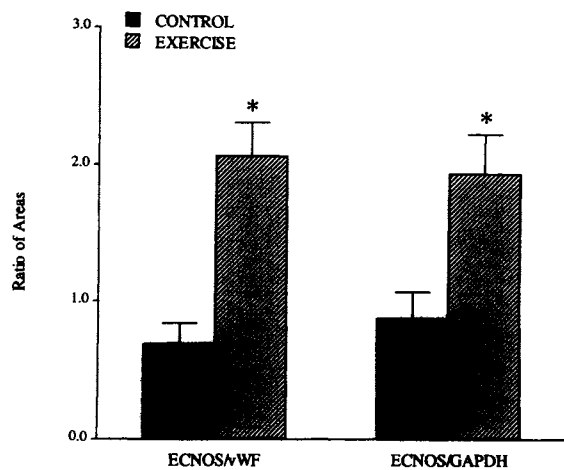


Fig 3. Bar graph showing quantification of endothelial cell nitric oxide synthase (ECNOS) gene expression relative to von Willebrand's factor (vWF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RNA extracts prepared from control (n=5) and exercised (n=5) dogs. Autoradiographs from blots hybridized with ECNOS, vWF, and GAPDH ³²P-labeled cDNA probes were quantified by densitometric analysis. *P<.05.

providing a potential molecular mechanism for increased epicardial dilation seen with chronic exercise.

Our previous work showing that chronic exercise for 7 days¹³ enhances endothelium-dependent epicardial coronary artery dilation, in response to acetylcholine or brief coronary occlusion, suggested that the production of EDRF/NO was increased. In addition, epicardial dilation in response to nitroglycerin was not enhanced with exercise, arguing against enhanced guanylate cyclase sensitivity. The present study demonstrates that coronary arteries and microvessels isolated from exercised dogs produce more nitrite when stimulated with the calcium-mobilizing agonist acetylcholine, confirming previous *in vivo* findings. The augmented production of nitrite was not agonist specific, because bradykinin and calcium ionophore A23187 produced similar responses (data not shown). This suggested that enhanced nitrite production was not due to upregulation or increased coupling of endothelial muscarinic receptors.

The equal production of nitrite in large coronary arteries and microvessels (and the increases seen in exercised dogs) suggests that these vessels have the same capacity to generate EDRF/NO. However, because vascular smooth muscle cells account for a larger portion of the vessel wall in large coronary vessels compared with microvessels, the amount of nitrite generated in large coronary vessels is probably an underestimation (when the data are expressed as nitrite per milligram of protein, Fig 1). Since exercise for 10 days does not influence vascular geometry, the enhanced release of nitrite from large coronary arteries and coronary microvessels from exercised dogs most likely reflects increased ECNOS activity.

Because exercise increases cardiac output and aortic flow velocity (see "Results"), we examined ECNOS gene expression in aortic endothelial cells isolated from the same exercised dogs exhibiting enhanced coronary vascular nitrite production. Steady-state ECNOS mRNA levels were consistently elevated in aortic extracts from exercised dogs (Figs 2 and 3). The induction

was specific, relative to the expression of vWF and GAPDH, demonstrating that inducibility of NOS genes need not be confined to cytokine-responsive isoforms (ie, from macrophages, hepatocytes, and vascular smooth muscle²²). Given the molecular differences between ECNOS and cytokine-inducible forms of NOS (50% amino acid identity¹⁷) and the lack of cross hybridization between the NOS cDNAs (brain, inducible, and endothelial), the increases in steady-state mRNA levels measured in aortic extracts from exercised dogs reflect upregulation of the ECNOS gene. The increased nitrite production evoked by the calcium-mobilizing agonist acetylcholine (Fig 1) and upregulation of the ECNOS gene (Figs 2 and 3) are consistent with exercise inducing the calcium-dependent endothelial NOS isoform responsible for the basal production of NO *in vivo*.¹³

The mechanisms by which chronic exercise increases aortic ECNOS gene expression and vascular nitrite release are not known. Exercise is an integrated physiological response that can increase circulating hormones or local autocooids (atrial natriuretic peptides, catecholamines, adenosine, and ATP) that may exert an influence on EDRF/NO production.²³ However, there is no direct evidence to support the involvement of these substances in the long-term regulation of NO biosynthesis. One potential mechanism consistent with our findings is that exercise increases myocardial oxygen consumption and reduces coronary vascular resistance, thus increasing coronary blood flow velocity.¹¹ The increased flow velocity will elicit endothelial shear stress, which may increase ECNOS gene expression. There are *in vitro* data showing that prolonged mechanical deformation of the endothelium by defined shear²⁴ or cyclic stretching²⁵ increases ECNOS, not inducible NOS, gene expression, protein, and activity. Moreover, the presence of myristoylated ECNOS in cellular membranes may permit the enzyme to respond to mechanical deformation of the endothelium.^{26,27} Whether shear-induced ECNOS gene expression is a direct result of shear *per se* or shear-induced mediator release (ie, ATP), activation of ion channels, or cytoskeletal rearrangements²⁸ in the above studies or in the present study is not known. In comparison with cytokine-induced transcriptional regulation of immunologically activated forms of NOS (in murine macrophages and human hepatocytes^{29,30}), the nature of exercise- or shear-induced upregulation of ECNOS gene expression is not known at the present time. Interestingly, the recent identification of a shear stress-responsive element in the platelet-derived growth factor promoter³¹ has also been found in the human ECNOS gene promoter,³² suggesting that shear can transcriptionally regulate ECNOS expression.

The finding that ECNOS mRNA is increased in aortic extracts from exercised dogs is in conflict with the current accepted nomenclature for NOS isozymes. Typically, ECNOS and neuronal NOS are categorized as calcium-dependent "constitutive" enzymes, whereas macrophage NOS, hepatocyte NOS, and smooth muscle NOS are calcium-independent cytokine-"inducible" enzymes. The results of the present study and other studies demonstrating that ECNOS mRNA and protein can be induced by mechanical forces^{24,25} or downregulated by cytokines (ie, tumor necrosis factor- α ^{24,33,34})

argue against the use of this nomenclature. We suggest the use of a provisional nomenclature based on molecular differences of the cloned NOS isoforms (ie, neuronal, cytokine-inducible, and endothelial NOS) and not terminology based on calcium sensitivity or subcellular localization. This is supported by the demonstrations that neuronal NOS and endothelial NOS are products of separate genes^{17,18,32,33} and that cytokine-induced forms (macrophage, hepatocyte, and vascular smooth muscle) of NOS are apparently derived from a single gene.^{30,35}

In summary, chronic exercise increases acetylcholine-stimulated nitrite production in coronary arteries and microvessels and ECNOS gene expression in aortic endothelial cell extracts. The functional significance of our results is not presently known. Perhaps, at rest, the increased production of EDRF/NO may be manifested by reduced platelet and leukocyte adherence to the vascular endothelium. Alternatively, during exercise (for 10 days), EDRF/NO may buffer sympathetic epicardial artery vasoconstriction. Future studies examining these possibilities and the time course, tissue distribution (ie, large vessels, skeletal muscle, and kidney), and reversibility of ECNOS induction after cessation of exercise may provide greater insights into the regulation and importance of NO during exercise training.

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