Exercise Training Enhances Vasodilation Responses to Vascular Endothelial Growth Factor in Porcine Coronary Arterioles Exposed to Chronic Coronary Occlusion

Jennifer A. Fogarty, PhD; Judy M. Muller-Delp, PhD; Michael D. Delp, PhD; Mildred L. Mattox, BS, M. Harold Laughlin, PhD; Janet L. Parker, PhD

Background—Chronic coronary occlusion (CCO) impairs endothelial function of distal collateral–dependent microvasculature; however, long-term exercise training (EX) seems to improve endothelial dysfunction. We hypothesized that EX enhances vasodilation responses to vascular endothelial growth factor (VEGF\textsubscript{165}), mediated via nitric oxide (NO), in arterioles exposed to CCO.

Methods and Results—The proximal left circumflex coronary artery (LCx) of female Yucatan miniswine was surgically instrumented with an ameroid occluder to induce CCO; 8 weeks after surgery, animals were randomized into 14-week sedentary (SED) or EX (treadmill; 5 d/wk) protocols. Coronary arterioles (~100 \(\mu\)m in diameter) were isolated from collateral-dependent (LCx) and nonoccluded (left anterior descending; LAD) perfused myocardium of SED and EX animals. Vasodilation was assessed by videomicroscopy and MacLab data acquisition. Responses to VEGF\textsubscript{165} were unaffected by EX in nonoccluded LAD arterioles; in contrast, EX markedly enhanced VEGF\textsubscript{165}-induced vasodilation of collateral-dependent LCx arterioles (\(P<0.05\); EX versus SED). Furthermore, VEGF\textsubscript{165}-induced vasodilation of EX LCx arterioles exceeded that of EX or SED LAD arterioles (\(P<0.05\)). Enhanced vasodilation of EX LCx arterioles was abolished by inhibition of NO synthase and tyrosine kinase activity. Combined inhibition of NO synthase and cyclooxygenase decreased VEGF\textsubscript{165}-induced vasodilation of all vessels.

Conclusions—EX enhances VEGF\textsubscript{165}-induced vasodilation in arterioles distal to CCO; EX effects seem to be mediated through increases in NO. (Circulation. 2004;109:664-670.)

Key Words: microcirculation ■ nitric oxide ■ occlusion ■ coronary disease ■ collateral circulation

Chronic coronary occlusion (CCO) often results in ischemia and dysfunction of distal myocardium during increased cardiac workload, partly because of limited development of collateral circulation.\textsuperscript{1–4} CCO creates a proangiogenic environment that is dependent on the presence of growth factors and appropriate receptors.\textsuperscript{1,5} Indeed, increased myocardial release of vascular endothelial growth factor (VEGF) has been demonstrated after coronary artery occlusion.\textsuperscript{6} Furthermore, a significant body of evidence indicates that impaired endothelial function and altered vaso- motor responsiveness of collateral-dependent vasculature contribute to abnormal regulation of coronary tone distal to CCO.\textsuperscript{7–10} For instance, impaired endothelium-dependent nitric oxide (NO)–mediated vasodilation persists in the microvasculature distal to the occlusion\textsuperscript{7,8,10} and is associated with CCO-induced reduction in endothelial cell NO synthase (ecNOS) mRNA in coronary arterioles.\textsuperscript{10} Because NO plays pivotal roles in key endothelial signaling pathways, altered NO-dependent functions may have important consequences on vasomotor and angiogenic responses in the diseased heart.

Interestingly, long-term exercise training (EX) has been shown to ameliorate endothelial dysfunction via undefined mechanisms.\textsuperscript{10–12} In normal pigs, ecNOS mRNA of coronary arterioles is upregulated by EX.\textsuperscript{13} Griffin et al\textsuperscript{10} recently reported that impaired microvascular responsiveness to bradykinin in porcine CCO is reversed by EX and that the occlusion-related reduction of ecNOS mRNA in arterioles is prevented or reversed by EX. These results indicate that EX-induced increases in production and/or stabilization of NO may contribute to improved coronary microvascular function in the CCO model.\textsuperscript{10}

In addition, EX increases VEGF protein expression in normally perfused muscle\textsuperscript{14} and increases VEGF mRNA in a model of femoral ligation.\textsuperscript{15} Exercise-induced increases in
VEGF mRNA and protein in muscle have also been demonstrated in heart failure patients. Importantly, because the effects of VEGF on vasodilation, angiogenesis, and collateral development are believed to involve NO production, we hypothesized that EX enhances VEGF-mediated vasodilation in collateral-dependent coronary arterioles and that enhanced VEGF responses are mediated by the effects of NOS and NO production.

Methods

Porcine Model of CCO and Collateral Development

CCO and collateral development were induced in adult female Yucatan swine (Sinclair Research Farm, Columbia, Mo) as previously described. Briefly, a left lateral thoracotomy was performed and an amnioretro caval (Research Instruments SW) placed around the proximal left circumflex coronary artery (LCX). This model has a >95% success rate and results in minimal infarction (<7% area at risk) because of gradual occlusion. In the present study, 75 pigs were instrumented; none died during surgery or in the immediate perioperative period, 12 died before the present study, 75 pigs were instrumented; none died during surgery or in the immediate perioperative period, 12 died before protocol assignment, and 1 died during exercise training. Protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee and conformed to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (DHHS Publication NIH 85-23, Office of Science and Health Guide for Care and Use of Laboratory Animals).

Training Procedures

After 8 weeks of postoperative recovery, pigs were randomly assigned to sedentary (SED; n = 29) or exercise training (EX; n = 33) groups. Animals were fed once daily and allowed water ad libitum. SED animals remained confined to pens. EX animals were exposed to a 14-week treadmill program used extensively by this laboratory and others.

Isolated Microvessel Preparation

After completion of the protocols, animals were anesthetized, the heart was removed, and transmural tissue samples were dissected from collateral-dependent myocardium and myocardium perfused by the unocccluded left anterior descending (LAD) coronary artery.

Subepicardial arteriolar branches (<150-μm ID; 0.6 to 1 mm axial length) were dissected, and cannulation was performed as described previously by Muller-Delp et al. Briefly, arterioles were cannulated in a Lucite chamber with micropipettes, secured with suture, transferred to an inverted microscope (Olympus IX70), and pressurized. Vessels were visualized and assessed via videocamera (Panasonic BP310), CCTV monitor (Panasonic), video micrometer (Microcirculation Research Institute, TAMUSHSC), and a data-acquisition system (Macintosh/MacLab). Vessels were warmed to 37°C and equilibrated for 60 minutes at a static intraluminal pressure of 40 mm Hg.

Training Efficacy/Oxidative Enzyme Capacity

Training efficacy was determined via comparison of skeletal muscle citrate synthase activities and heart-to-body weight ratios between SED and EX animals. Samples of deltoid and triceps brachii were immediately frozen in liquid N2 and stored at −70°C. Citrate synthase activity was determined from whole-muscle homogenate and spectrophotometry.

Experimental Protocols

Functional assessment to agonist (±inhibitors) was conducted at static pressure with no flow. Arterioles were assessed for generation of spontaneous tone sufficient for vasodilation studies, and if inadequate, endothelin-1 was administered abuminally. Once diameter had stabilized, concentration-response relationships to adenosine (10−10 to 10−4 mol/L) and VEGF165 (10−10 to 10−6 mol/L) (R&D Systems) were determined by cumulative additions.

Freshly prepared arterioles were used to evaluate responses to VEGF165 in the presence of selective inhibitors. Arterioles underwent a 20-minute preincubation with inhibitor and subsequent treatment with endothelin-1 to achieve adequate tone. N4-Monomethyl-L-arginine (L-NMMA; 10 μmol/L; Calbiochem) was used to inhibit NOS, and indomethacin (10 μmol/L) was used to block cyclooxygenase (COX)–mediated prostanooid release. Piceatannol (10 μmol/L) was used to inhibit tyrosine kinase activity. At the conclusion of the experiment, nitroprusside (100 μmol/L) was used to determine maximal vessel diameter.

Solutions and Drugs

Chemicals were obtained from Sigma, except as stated. Physiological saline solution (PSS) contained (in mmol/L) NaCl 145, KCl 4.7, CaCl2 2.0, MgSO4 1.17, NaH2PO4 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and MOPS buffer 3.0. All solutions were adjusted to pH 7.4. VEGF165 was prepared in PSS with albumin (USB/Amersham). L-NMMA and indomethacin were prepared in PSS. Piceatannol was dissolved in DMSO as a stock solution, and dilutions were in PSS.

Data Analysis

Citrate synthase activity of skeletal muscle and heart-to-body weight ratios were compared by Student’s unpaired t test. Vasodilator responses were expressed as percentage of maximal diameter. Concentration-response curves were compared by 2-way ANOVA for repeated measures with Fisher’s test for least significant difference (LSD). For all analyses, significance is defined as a value of P<0.05. Data are represented as mean±SEM. Animal numbers are in parentheses.

Results

Training Efficacy

Citrate synthase activity was significantly increased in skeletal muscle samples from EX versus SED animals (Table 1). The heart-to-body weight ratio of EX animals was significantly higher than that of SED animals (P<0.001; EX versus SED).

Vessel Characteristics

Lumen diameter, percent preconstriction, and concentration of endothelin-1 used were similar between LAD and LCX arterioles from EX and SED animals (Table 2).

<table>
<thead>
<tr>
<th>TABLE 1. Exercise Training Effects</th>
<th>Citrate Synthase Muscle Analysis, μmol · min⁻¹ · g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triceps Anterior Head</td>
<td>Triceps Medial Head</td>
</tr>
<tr>
<td>SED</td>
<td>7.19±0.61</td>
</tr>
<tr>
<td>EX</td>
<td>15.5±1.79</td>
</tr>
<tr>
<td>Significance, P</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

This table shows the exercise training effects on citrate synthase muscle analysis, with values expressed in μmol · min⁻¹ · g⁻¹ for different muscle groups. The data indicate significant differences in citrate synthase activity between sedentary (SED) and exercise (EX) groups, with EX animals showing higher values.
Vasodilation Response to VEGF<sub>165</sub>
Collateral-dependent LCx arterioles from EX animals displayed significantly enhanced VEGF<sub>165</sub>-induced vasodilation compared with LCx arterioles from SED animals and nonoccluded LAD arterioles from SED and EX animals (P<0.05; Figure 1). VEGF<sub>165</sub> produced similar concentration-dependent vasodilation of SED and EX LAD arterioles. SED LCx arterioles did not display an attenuated VEGF<sub>165</sub>-induced vasodilation, as demonstrated by comparison with SED LAD. Both occlusion and EX seem to be necessary to produce an enhanced vasodilation to VEGF<sub>165</sub>, as shown in Figure 1. On removal of VEGF, tone in these arterioles recovered to levels not different from initial tone (data not shown).

Role of NO in EX-Induced, Enhanced Response to VEGF<sub>165</sub>
NOS inhibition (L-NMMA; 10 μmol/L) decreased the maximal VEGF<sub>165</sub>-induced dilation but did not significantly reduce the overall vasodilation of EX LAD arterioles. In addition, LAD arterioles from EX and SED animals responded similarly to VEGF<sub>165</sub> in the presence of L-NMMA (Figure 2). L-NMMA significantly reduced vasodilation of EX LCx arterioles to VEGF<sub>165</sub>, indicating a potential role for enhanced NO production after EX, but did not significantly affect SED LCx arterioles (Figure 2). Importantly, L-NMMA abolished the enhanced vasodilation of EX LCx arterioles, as demonstrated by comparison of EX and SED LCx. Substantial VEGF<sub>165</sub>-induced vasodilation remained after NOS inhibition in all arterioles.

Role of Prostanoid Vasodilators in VEGF<sub>165</sub>-Induced Dilation
Combined inhibition of NOS (L-NMMA; 10 μmol/L) and COX (indomethacin; 10 μmol/L) did significantly inhibit VEGF<sub>165</sub>-induced vasodilation of EX LAD arterioles. However, dual blockade did not significantly inhibit response of SED LAD arterioles (Figure 3). Vasodilation of SED and EX LCx arterioles to VEGF<sub>165</sub> was significantly reduced by inhibition of NOS and COX; EX LCx arterioles were more profoundly affected (Figure 3). Combined inhibition did not abolish the vasodilation response to VEGF<sub>165</sub>.

Role of Tyrosine Kinase Activity in VEGF<sub>165</sub>-Induced Dilation
Vasodilation of SED LAD and LCX arterioles and that of EX LAD arterioles were similarly diminished by tyrosine kinase inhibition (piceatannol; 10 μmol/L) (Figure 4). VEGF<sub>165</sub>-induced vasodilation of SED LCx arterioles seemed to be unaffected by piceatannol (Figure 4). Importantly, vasodilation of EX LCx arterioles was significantly diminished by piceatannol; indeed, vasodilation was no longer enhanced (EX versus SED).

Vascular Smooth Muscle Responses
Sodium nitroprusside (100 μmol/L) was used to establish maximal diameter and vascular smooth muscle–dependent vasodilation. Responses were not different between vessels (not shown). In addition, adenosine produced responses that were not different in LAD and LCx arterioles from SED and EX animals (Figure 5) and were unaffected by L-NMMA (not shown).

Discussion
In the present study, we used a porcine model of CCO to document, for the first time, that long-term exercise training enhances vasodilation responses of collateral-dependent LCx arterioles to VEGF<sub>165</sub>. Furthermore, the enhanced response of LCx arterioles from EX animals seems to be attributable to
increased synthesis/release of NO. In addition, we report that NO, prostanoid vasodilators, and potentially other vasoactive substances are released by porcine coronary arterioles in response to VEGF165. Data from this study imply a beneficial role for chronic exercise training in CCO, with respect to enhanced endothelial function and coronary/myocardial responses to VEGF, as well as potential interactive effects of EX and CCO on optimization of collateralization and myocardial perfusion.

Vasodilation to VEGF165
VEGF plays a significant role in angiogenesis and collateral development in coronary and peripheral vascular occlusive diseases. In the present study, CCO combined with EX resulted in enhanced vasodilation of isolated coronary arterioles to VEGF165. After training, vasodilation of collateral-dependent LCx arterioles exceeded that of LCx arterioles from SED animals and nonoccluded LAD arterioles (SED and EX). We believe that enhanced vasodilation after training in arterioles subjected to CCO may imply an increased role of VEGF165 in ongoing collateral development in our model. Furthermore, previous studies have established that training increases release of VEGF and VEGF receptor mRNA expression in ischemic muscle. Taken together, these data imply a synergistic environment in collateral-dependent myocardium of EX animals because of increased VEGF and enhanced microvascular sensitivity to VEGF.

Sellke et al reported enhanced responses to VEGF in coronary arterioles of untrained animals exposed to chronic occlusion. Explanations for discrepancies with our findings in SED animals are unclear. However, Sellke et al performed studies 7 to 9 weeks after instrumentation, whereas our study assessed function 22 weeks after ameroid placement. Collateral development at 8 weeks in this model provides...
adequate blood flow in resting myocardium but insufficient flow during physiological stress. Possibly, at 7 to 9 weeks, collateral development may be more limited than at 22 weeks, inadequately perfusing “resting” myocardium and potentially causing variable ischemic episodes stimulating enhanced VEGF-induced responses under sedentary conditions.

VEGF<sub>165</sub>-Induced Vasodilation: Role of NOS and Cyclooxygenase

VEGF upregulates ecNOS expression and protein and induces production of NO and prostacyclin in endothelial cells, and increases circulating endothelial progenitor cells. Exercise training induces increases in ecNOS mRNA in normal porcine hearts, prevents reduction in ecNOS mRNA in porcine hearts exposed to CCO, and induces VEGF release from ischemic muscle. Thus, enhanced VEGF<sub>165</sub>-induced vasodilation after training may result from several underlying mechanisms, including VEGF receptor upregulation, increased ecNOS activity (via ecNOS mRNA/protein upregulation or enhanced Ca<sup>2+</sup> mobilization), and/or enhanced VEGF signal transduction in collateral-dependent arterioles. In addition, we speculate that VEGF-induced increases in circulating endothelial progenitor cells may contribute to beneficial reendothelialization of the microvasculature downstream from occlusion. These effects may culminate in improved endothelial function; increased endothelial production of NO, which mediates vasodilation; increased blood flow; and the angiogenic process. This explanation is substantiated by NOS inhibition abolishing EX-induced enhanced vasodilation of collateral-dependent arterioles to VEGF<sub>165</sub> (Figure 2). Interestingly, NOS inhibition did not significantly affect VEGF<sub>165</sub>-induced vasodilation of collateral-dependent arterioles from SED animals and minimally affected normally perfused arterioles from SED and EX animals. Therefore, NO may play a greater role in VEGF-mediated vasodilation after training compared with responses observed in SED animals. Also, although lack of NOS blockade by L-NMMA could theoretically contribute to our results in SED LCx arterioles, we believe that this possibility is unlikely. Hein and Kuo demonstrated that L-NMMA (10 μmol/L) significantly inhibits agonist-stimulated NO production in porcine coronary arterioles. Furthermore, 10 μmol/L L-NMMA does not affect basal diameter or basal NO release measured in isolated arterioles and is without nonspecific smooth muscle effects observed at higher concentrations. Nonetheless, our findings prompted experiments to further define the mechanisms by which VEGF<sub>165</sub> caused vasodilation in coronary arterioles from our model.

Figure 4. VEGF<sub>165</sub>-induced vasodilation of arterioles with and without tyrosine kinase inhibition (piceatannol; 10 μmol/L). EX LCx vasodilation was significantly reduced by piceatannol (*P<0.01; right). Indeed, piceatannol abolished training effect shown in Figure 1. SED and EX LAD concentration-response curves were minimally affected by tyrosine kinase inhibition; only last 3 doses of each concentration-response curve were significantly reduced (*P<0.05, unprotected Fisher’s LSD; control vs piceatannol).

Figure 5. Adenosine-induced vasodilation of coronary arterioles from SED and EX animals exposed to CCO. Responses of coronary arterioles to adenosine were unaffected by exercise training and/or CCO.
VEGF stimulates production of NO and prostacyclin, which increase vessel permeability, induce endothelial cell proliferation and migration, and cause vasodilation and hypotension. In agreement, we found that both NO- and COX-mediated vasodilators contribute to VEGF-induced vasodilation. Combined inhibition of NOS and cyclooxygenase significantly impaired vasodilation of collateral-dependent arterioles from EX and SED animals (Figure 3). However, the combined results of Figures 2 and 3 indicate that the relative contribution of NO to VEGF relaxation dominates in LCx arterioles from EX animals, whereas COX-mediated vasodilators play an increased role in LAD arterioles from SED and EX animals and LCx arterioles from SED animals. Interestingly, combined inhibition did not completely abolish the VEGF-induced vasodilation, which suggests that unidentified vasoactive substances may be released in response to VEGF and contribute to vasodilation. A prime candidate is endothelial hyperpolarizing factor, although methods to establish its role were not used.

**VEGF-induced Vasodilation: Role of Tyrosine Kinases**

VEGF is believed to confer its major effects via tyrosine kinase receptors. Therefore, inhibition of tyrosine kinase activity should abolish VEGF intracellular signaling and production of vasoactive substances. In our preparation, tyrosine kinase inhibition significantly reduced vasodilation of collateral-dependent arterioles from EX animals and abolished the effects of training on VEGF response (Figure 4). However, collateral-dependent arterioles from SED animals seemed to be unaffected by piceatannol. Reversal of the training effect strongly implies a role of tyrosine kinase activation in training-induced enhancement of VEGF-induced vasodilation and NO production. To the best of our knowledge, this is the first report that the effects of exercise training in experimental coronary disease involve alterations in VEGF signaling mechanisms such as tyrosine kinase pathways. Interestingly, tyrosine kinase inhibition did not completely abolish vasodilation to VEGF. This finding is in agreement with previous reports using porcine coronary arterioles and implies that VEGF may induce release of vasodilators through signaling cascades other than activation of tyrosine kinases.

**Vascular Smooth Muscle Responses**

Theoretically, training-induced enhanced vasodilation to VEGF could result from increased responsiveness of coronary microvascular smooth muscle. However, unlike vasodilation to VEGF, smooth muscle responses to nitroprusside (an NO donor) and adenosine (present study) in arterioles from EX and SED animals are unaffected by either CCO or training. These studies provide important controls for VEGF responses and indicate that arteriolar smooth muscle responses to NO and adenosine and downstream cGMP/cAMP mechanisms are unaltered and do not contribute to enhanced VEGF relaxation after training.

**Conclusions and Implications**

To the best of our knowledge, this study provides the first evidence that exercise training, in the setting of CCO, elicits enhanced microvascular vasodilation responses to VEGF. Training-induced effects on VEGF-induced vasodilation seem to involve increased synthesis/release of NO via tyrosine kinase–dependent pathways. These findings are in agreement with reports of enhanced endothelium-dependent, NO-mediated vasodilation to the agonist bradykinin in both normal and collateral-dependent coronary arterioles after training. In light of recent documentation that CCO-induced decreases in coronary arteriolar ecNOS mRNA levels are prevented/reversed by exercise training, these studies are supportive of the concept that exercise training results in an increased vasoregulatory role of NO in coronary microvascular function.

Increased NO production after training implies other beneficial effects of NO present in diseased myocardium, including reduction in platelet aggregation, aggregation, thrombogenicity, and vasospasm, as well as increased angiogenesis and optimized collaterals. We recognize the difficulties of extrapolating these in vitro findings to the intact heart under conditions of varying coronary flow. However, we speculate that these positive effects of exercise training on VEGF/NO responses may potentially contribute to improved perfusion of collateral-dependent myocardium and may be involved in improved myocardial function and enhanced coronary vasodilator reserve of collateral-dependent myocardium observed after long-term exercise in a similar porcine model of CCO. Thus, this study further substantiates the beneficial role of exercise training in improving endothelial function and blood flow to the myocardium distal to occlusion. Importantly, these findings also imply endothelium-related mechanisms underlying known beneficial effects of exercise in patients with coronary artery disease as well as reductions in morbidity and mortality associated with improved endothelial function.

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