Endothelium-Mediated Relaxation of Porcine Collateral-Dependent Arterioles Is Improved by Exercise Training

Kawanza L. Griffin, PhD; Christopher R. Woodman, PhD; Elmer M. Price, PhD; M. Harold Laughlin, PhD; Janet L. Parker, PhD

Background—Endothelium-dependent modulation of coronary tone is impaired in the collateral-dependent coronary microcirculation. We used a porcine model of chronic coronary occlusion and collateral development to evaluate the hypothesis that exercise training enhances endothelium-mediated relaxation and increases endothelial nitric oxide synthase (ecNOS) mRNA levels of collateral-dependent microvasculature.

Methods and Results—Adult female miniature swine were subjected to chronic, progressive ameroid occlusion of the proximal left circumflex coronary artery (LCx); after 2 months, animals were randomly exposed to 16-week exercise-training (EX group; treadmill running) or sedentary (SED group; cage confinement) protocols. After completion of EX or SED programs, coronary arterioles (≈100 μm in diameter) were isolated from collateral-dependent LCx (distal to occlusion) and nonoccluded left anterior descending coronary artery (LAD) regions of each heart. Arterioles were studied by in vitro videomicroscopy or frozen for ecNOS mRNA analysis (RT-PCR techniques). Relaxation to the endothelium-dependent vasodilator bradykinin was decreased (P<0.05) in arterioles isolated from collateral-dependent LCx versus nonoccluded LAD regions of SED animals. Bradykinin-mediated relaxation, however, was not different in LCx versus LAD arterioles isolated from EX animals. Nitroprusside-induced relaxation was unaffected by either chronic occlusion or exercise. Importantly, ecNOS mRNA expression was significantly decreased in arterioles isolated from LCx versus LAD regions of SED animals. After training, ecNOS mRNA expression was not different between LAD and LCx arterioles.

Conclusions—These data indicate that exercise training enhances bradykinin-mediated relaxation of collateral-dependent LCx arterioles isolated after chronic coronary occlusion, most likely because of effects on ecNOS mRNA expression and increased production of NO. (Circulation. 2001;104:1393-1398.)

Key Words: bradykinin ■ collateral circulation ■ occlusion ■ nitric oxide ■ microcirculation

Coronary collateral development secondary to myocardial ischemia is often adequate to support myocardial function at rest.1-5 Myocardial ischemia and inotropic dysfunction may occur, however, during periods of increased cardiac workload because of inadequate perfusion of the collateral-dependent myocardial region.1-3,6 Importantly, Roth et al7 demonstrated that the perfusion deficit and contractile dysfunction of collateral-dependent myocardium was improved by chronic (16-week) exercise training in pigs. Increased collateral development has been proposed to underlie training-induced improved perfusion of the diseased heart;2-3,6; however, synergistic effects of chronic exercise on improved microvascular vasomotor function of collateral-dependent vasculature may also be involved. Our studies addressed the possibility that in hearts exposed to chronic coronary artery occlusion, exercise training enhances microvascular endothelial function and may thereby contribute to optimization of perfusion to the myocardial region at risk.

Significant evidence indicates that in normal hearts, exercise training increases coronary endothelium-dependent relaxation7,8; these effects are associated with increases in nitric oxide (NO)7-10 production and endothelial nitric oxide synthase (ecNOS) gene expression.10,11 Conversely, canine and porcine hearts exposed to chronic coronary occlusion exhibit impaired endothelium-dependent vasorelaxation12-14 and enhancement of selected vasoconstrictor responses.12-15 Combined effects of coronary disease and exercise training on endothelial function are less clearly understood. In the present study, we evaluated exercise training-induced alterations of coronary endothelial function and ecNOS mRNA in porcine hearts...
arterioles isolated from hearts exposed to chronic atheromatous occlusion of the left circumflex coronary artery (LCx). We hypothesized that exercise training would improve impaired vasorelaxation in arterioles isolated from the collateral-dependent LCx region and that improved impaired relaxation would be associated with increased eNOS gene expression. Relaxation responses to the endothelium-dependent vasodilator bradykinin and the endothelium-independent vasodilator sodium nitroprusside were evaluated and compared in arterioles isolated from collateral-dependent LCX and nonoccluded left anterior descending coronary artery (LAD) myocardial regions of sedentary (SED group) and exercise-trained (EX group) animals.

Methods

Porcine Model of Chronic Coronary Occlusion and Collateral Development

Adult female Yucatan miniature swine (~25 to 45 kg; Charles River Laboratories, Windham, Maine) were randomly divided into normal nonoccluded and chronically coronary-occluded groups. Atherosclerosis-induced progressive coronary occlusion was performed as described previously. Briefly, animals were sedated with ketamine (20 mg/kg IM), midazolam (0.5 mg/kg IM), and glycopyrrolate (0.004 mg/kg IM) and then intubated and maintained on isoflurane for sterile surgery. A left thoracotomy was performed through the fifth intercostal space for cardiac exposure and pericardial incision. An atherosclerotic constrictor (2.5- to 3.5-mm ID) was placed around the proximal LCX. After surgery, animals received buprenorphine (0.01 mg/kg IV) as needed for pain. Antibiotics were given immediately before surgery (~30 000 U penicillin IM) and for 5 days after surgery (~480 mg/50 lb sulfathrometrimethoprim). Atherosclerosis occlusion is successful in >95% of animals; furthermore, because of coronary collateral development, this procedure results in minimal myocardial infarction. All experimental procedures were in accordance with the “Position of the American Heart Association on Research Animal Use” (November 11, 1984) and were approved by the Animal Care and Use Committee of the University of Missouri.

Exercise-Training Program

Coronary-occluded animals were allowed 2 months for recuperation and collateral development before random division into equal groups of either EX or SED animals. EX animals entered a progressive treadmill running program for 16 weeks, and SED animals remained sedentary (SED group). EX (n = 150) and SED (n = 150) pigs were housed individually, with animals of similar body weight grouped by sex and weight (mean body weight 25 to 45 kg; Charles River Laboratories, Windham, Maine) were randomly divided into normal nonoccluded and chronically coronary-occluded groups. Atherosclerosis-induced progressive coronary occlusion was performed as described previously. Briefly, animals were sedated with ketamine (20 mg/kg IM), midazolam (0.5 mg/kg IM), and glycopyrrolate (0.004 mg/kg IM) and then intubated and maintained on isoflurane for sterile surgery. A left thoracotomy was performed through the fifth intercostal space for cardiac exposure and pericardial incision. An atherosclerotic constrictor (2.5- to 3.5-mm ID) was placed around the proximal LCX. After surgery, animals received buprenorphine (0.01 mg/kg IV) as needed for pain. Antibiotics were given immediately before surgery (~30 000 U penicillin IM) and for 5 days after surgery (~480 mg/50 lb sulfathrometrimethoprim). Atherosclerosis occlusion is successful in >95% of animals; furthermore, because of coronary collateral development, this procedure results in minimal myocardial infarction. All experimental procedures were in accordance with the “Position of the American Heart Association on Research Animal Use” (November 11, 1984) and were approved by the Animal Care and Use Committee of the University of Missouri.

Solutions and Drugs

The Krebs solution contained (in mmol/L) NaCl 145, KCl 4.7, CaCl2 2.0, MgSO4 1.17, MOPS 3.0, NaHPO4 1.2, pyruvate 2, glucose 5, and EDTA 0.02, and 10 g/L (1%) albumin (pH 7.4 at 37°C). Bradykinin was purchased from Bachem; other drugs were purchased from Sigma Chemical Co. Drugs were prepared daily in distilled, deionized H2O.

Oxidative Enzyme Capacity

After removal of the heart, skeletal muscle samples were obtained from the middle of the triceps brachii and deltoid muscles, frozen in liquid N2, and stored at −70°C until processed. Citrate synthase activity was measured by spectrophotometry; increased activity is representative of successful training.

Data Analysis

Citrate synthase activities of skeletal muscle and heart weight/body weight ratios were compared by Student’s unpaired t test. Concentration-response curves were compared by 2-way ANOVA for repeated measures. Differences between individual points were ascertained with Fisher’s test for least significant difference. RT-PCR data from multiple arterioles were averaged, resulting in 1 data point.
Characteristics of Porcine Arterioles Isolated From Hearts Subjected to Chronic Coronary Occlusion and Collateral Development

<table>
<thead>
<tr>
<th>Group</th>
<th>Lumen Diameter, μm</th>
<th>Endothelin Concentration, μmol/L</th>
<th>Percent Preconstriction</th>
</tr>
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<tbody>
<tr>
<td>Sedentary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD (n=14)</td>
<td>105.55 ± 6.86</td>
<td>0.42 ± 0.19</td>
<td>63.05 ± 5.70</td>
</tr>
<tr>
<td>LCx (n=12)</td>
<td>99.17 ± 6.49</td>
<td>0.26 ± 0.11</td>
<td>58.82 ± 4.00</td>
</tr>
<tr>
<td>Exercise-trained</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD (n=12)</td>
<td>97.95 ± 6.18</td>
<td>0.30 ± 0.08</td>
<td>58.76 ± 5.32</td>
</tr>
<tr>
<td>LCx (n=10)</td>
<td>115.38 ± 5.89</td>
<td>0.75 ± 0.59</td>
<td>58.54 ± 5.89</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Numbers in parentheses indicate the total number of arterioles studied in each group.

point per animal. For all analyses, significance was defined as P<0.05.

Results

Efficacy of Training
Citrate synthase activity was significantly increased in samples obtained from the medial head of the triceps brachii muscle (P<0.05; EX 18.2±1.2, SED 15.1±0.6) and deltoid muscle (P<0.01; EX 19.4±2.0, SED 15.9±0.9) of EX animals. Other areas of the triceps brachii muscle also tended to have greater citrate synthase activity after training. The heart weight/body weight ratio of EX animals was also significantly higher than that of SED animals (P<0.05; EX 6.6±0.4 g/kg, SED 5.0±0.2 g/kg).

Vessel Characteristics
Arteriolar intraluminal diameters were not significantly different in LAD or LCx vessels from SED or EX animals (Table). For arterioles isolated from SED animals, endothelin preconstriction levels (expressed as percent reduction in diameter) averaged 63.1±5.7% and 58.8±4.0% in LAD and LCx, respectively (P>0.05). Endothelin concentrations needed to produce preconstriction were similar (LAD 0.4±0.2 nmol/L, LCx 0.3±0.1 nmol/L). For arterioles isolated from EX animals, preconstriction levels were similar between both myocardial regions (LAD 58.8±5.3%, LCx 58.6±5.9%), and the concentration of endothelin needed to produce this level of preconstriction was not significantly different.

Vasorelaxation Responses to Bradykinin
As shown in Figure 1A, in SED animals, relaxation to bradykinin was blunted in arterioles isolated from the collateral-dependent LCx region compared with the nonoccluded LAD region of the same hearts (P<0.05). We also compared LAD and LCx responses to bradykinin of arterioles isolated from normal (nonoccluded) pigs; in contrast to findings in coronary-occluded hearts, endothelin-induced relaxation was not significantly different between LCx and LAD arterioles isolated from normal hearts (data not shown). Importantly, after exercise training of coronary-occluded animals, there was no difference in bradykinin-stimulated relaxation in arterioles isolated from LCx and LAD myocardial regions (P>0.05; Figure 1B), suggesting that exercise training had improved relaxation of LCx arterioles. NO synthesis inhibition (NG-nitro-l-arginine, L-NMMA) inhibited bradykinin vasorelaxation responses of both LAD and LCx arterioles isolated from SED as well as EX animals (Figure 2, A and B) compared with initial bradykinin curves (Figure 1, A and B).

Responses to Sodium Nitroprusside
To examine the possibility that smooth muscle responsiveness to NO and/or downstream cGMP/protein kinase G (PKG)–dependent pathways are altered by chronic coronary occlusion or exercise training, we evaluated relaxation responses to the NO donor sodium nitroprusside. In contrast to our findings with bradykinin, nitroprusside-induced relaxation was similar in arterioles from the collateral-dependent LCx compared with the nonoccluded LAD region of SED (P>0.05; Figure 3) and EX (data not shown) animals.

Figure 1. Bradykinin relaxation responses of arterioles isolated from collateral-dependent (LCx) and nonoccluded (LAD) regions of hearts exposed to chronic LCx occlusion. A, In SED animals, bradykinin relaxation was significantly impaired (P<0.05) in LCx vs LAD arterioles. B, Bradykinin relaxation was not significantly different between LCx and LAD arterioles after 16-week exercise training. Data are mean±SEM. Number of animals for each protocol is shown in parentheses.

Figure 2. Bradykinin-induced relaxation of arterioles isolated from collateral-dependent (LCx) and nonoccluded (LAD) regions in presence of inhibition of NOS (L-NMMA). Bradykinin relaxation responses were shifted downward and to right in both LAD and LCx arterioles isolated from SED (A) and EX (B) animals compared with bradykinin relaxation in absence of inhibitor (Figure 1). Data are presented as in Figure 1.
Role of NOS: ecNOS mRNA Expression
Levels of ecNOS mRNA expression (normalized to GAPDH) were assessed to determine the role of altered NO regulation in our results. The ecNOS-to-GAPDH ratio was significantly lower in collateral-dependent LCx \((0.54 \pm 0.04)\) than in nonoccluded LAD arterioles \((0.70 \pm 0.07)\) isolated from SED animals (Figure 4A). Importantly, the ecNOS-to-GAPDH ratio of arterioles isolated from collateral-dependent LCx \((0.68 \pm 0.07)\) versus nonoccluded LAD \((0.74 \pm 0.06)\) arterioles was not significantly different after training (Figure 4B). Both LAD and LCx arteriolar ecNOS-to-GAPDH ratios exhibited a tendency to increase ecNOS mRNA content after EX; values of LCx arterioles after training were similar to those in LAD arterioles of SED animals. Arteriolar GAPDH mRNA expression was unaltered by coronary occlusion in both the SED and EX groups, as indicated in sample agarose gels (see insets, Figure 4) for the SED and EX groups. RT-PCR experiments were also performed in endothelial cells freshly dispersed from coronary arteries of occluded hearts; results indicate that occlusion had no effect on endothelial cell GAPDH mRNA (Figure 4C). Taken in concert with unaltered total arteriolar GAPDH mRNA (Figure 4A), unaltered endothelial GAPDH mRNA suggests that vascular smooth muscle GAPDH mRNA is also not altered by occlusion. These results indicate that GAPDH is unaffected by chronic coronary occlusion and also support previous findings that GAPDH mRNA expression is not altered by chronic exercise training.\(^{10,11}\)

Discussion
To the best of our knowledge, the present study is the first in vitro documentation that impaired endothelium-dependent relaxation of collateral-dependent arterioles is improved after exercise training. In addition, we report that enhanced bradykinin-mediated relaxation of collateral-dependent arterioles involves increased expression of ecNOS mRNA. Importantly, these findings provide insight into potential mechanisms underlying improved blood flow/ischemia of collateralized myocardium after exercise training in experimental animals,\(^2,3,6\) as well as the well-documented beneficial effects of exercise training in human patients with coronary artery disease.\(^{19–21}\)

Vasorelaxation to Sodium Nitroprusside
Arteriolar smooth muscle relaxation to the NO donor sodium nitroprusside was unaffected by either chronic coronary occlusion or exercise training. These results are in agreement with the previous report by Muller et al\(^8\) indicating that exercise training does not affect nitroprusside relaxation of coronary arterioles of normal animals and suggest that altered smooth muscle relaxation to NO and underlying cGMP/PKG pathways are also not involved in training effects on endothelium-dependent relaxation in collateral-dependent vasculature. Our finding that vasodilator...
responses to nitroprusside were not altered in coronary arterioles isolated from collateral-dependent myocardium of coronary-occluded hearts, however, contradicts the previous findings of Sellke et al.\textsuperscript{12,13} of enhanced relaxation to sodium nitroprusside and nitroglyceride after chronic occlusion. The reasons for these differences are unclear but may relate to temporal differences between occlusion models, because Sellke et al evaluated endothelium-dependent relaxation in arterioles isolated 4 to 7 weeks after occlusion, whereas our arterioles were isolated 6 months after ameroid occlusion. Potentially, time-dependent adaptations in guanylyl cyclase/cGMP levels and/or differences in basal NO release between these models may play a role; however, neither study directly measured levels of cGMP or NO.

Vasorelaxation to Bradykinin

Bradykinin-mediated relaxation was impaired in porcine coronary arterioles isolated from the collateral-dependent LCx region (Figure 1). Because arteriolar smooth muscle responses to NO (nitroprusside) were unaffected, these findings indicate impairment of endothelium-dependent relaxation after chronic coronary artery occlusion, supporting similar results in previous publications.\textsuperscript{12,13}

Muller et al.\textsuperscript{8} and Parker et al.\textsuperscript{7} reported that coronary resistance arteries and arterioles isolated from normal female Yucatan swine exhibited enhanced bradykinin-mediated relaxation after exercise training. These findings stimulated our hypothesis that exercise training would improve impaired relaxation to bradykinin in collateral-dependent microvessels. In the present study, we found enhanced bradykinin-mediated relaxation of collateral-dependent LCx arterioles compared with nonocluded LAD arterioles after exercise training. Thus, exercise training appears to exert beneficial effects on regulation of coronary microvascular tone distal to a chronic coronary stenosis/occlusion. Interestingly, relaxation in the nonocluded LAD region remained unchanged. These results are surprising, because Muller et al.\textsuperscript{8} and Parker et al.\textsuperscript{7} reported enhanced bradykinin-mediated relaxation in arterioles isolated from normal myocardium. It is unclear why training produces enhanced endothelial function in LAD arterioles of normal pigs but not in LAD arterioles from LCx-occluded hearts. Potentially, differences due to adaptive responses of the nonocluded artery after chronic occlusion may be involved, because basal flow in the nonocluded artery increases to provide collateral in addition to native coronary flow. These adaptations may differentially interact with the effects of training because of simultaneous and opposing alterations in resting flow and shear stress (stimuli for ecNOS regulation) in nonocluded versus collateral-dependent vasculature.

Endothelium-Mediated Relaxation Responses After Training: Role of NO

Previous studies in the normal dog\textsuperscript{10} have demonstrated increased NO production in both large coronary arteries and coronary microvessels and increased ecNOS gene expression in dispersed aortic endothelial cells after exercise training. Similarly, Woodman et al.\textsuperscript{11} reported increased ecNOS gene expression in coronary arterioles isolated from EX animals, supporting findings by Muller et al.\textsuperscript{8} that enhanced bradykinin-induced relaxation after exercise training was at least partially due to an increased production of NO. Thus, we hypothesize that the effects of exercise training on improved relaxation of collateral-dependent vasculature in our porcine model are due, at least in part, to increased production of NO. Indeed, NOS inhibition shifted bradykinin responses to the right in both nonocluded and collateral-dependent arterioles, similar to the findings of Muller et al.\textsuperscript{8} in normal pigs. To further test this hypothesis, we compared ecNOS mRNA expression in SED and EX animals. In SED animals, ecNOS mRNA expression was significantly decreased in arterioles from the collateral-dependent LCx region compared with the nonocluded LAD region (Figure 4). Importantly, ecNOS mRNA levels in collateral-dependent arterioles of EX animals were comparable to those from nonocluded LAD arterioles, suggesting a training-induced upregulation of ecNOS mRNA expression. To the best of our knowledge, this is the first report of the effects of exercise training on ecNOS gene expression in coronary arterioles isolated after chronic coronary occlusion. Another interpretation of these data, however, is that exercise training prevents the detrimental effects of occlusion from developing by maintaining ecNOS mRNA expression in the LCx region. Our results demonstrated similar ecNOS-to-GAPDH ratios in LAD arterioles isolated from SED and EX animals but decreased ecNOS-to-GAPDH ratios in LCx arterioles isolated 6 months after ameroid placement. In the present study, we did not measure ecNOS mRNA at earlier time points; Laham et al.\textsuperscript{22} however, reported similar ecNOS expression in LAD and LCx arterioles at 7 weeks after ameroid implantation in a similar model.

Limitations of the Study

Endothelin-mediated vasoconstriction is associated with the production of NO and prostacyclin; thus, it is possible that endothelin vasoconstriction may complicate NO-dependent relaxation to endothelium-dependent vasodilators such as bradykinin. Theoretically, decreased sensitivity to endothelin may result from the increased production of NO after exercise training. Thus, more endothelin would be necessary to overcome the NO-dependent vasodilation on the vascular smooth muscle. In the present study, however, preconstriction levels were well matched between LAD and LCx arterioles of SED and EX animals, and the concentration of endothelin needed to produce preconstriction (before exposure to bradykinin) was not significantly different. We also recognize that LAD versus LCx differences in relaxation to bradykinin were most apparent at higher concentrations of bradykinin (10\textsuperscript{-10} to 10\textsuperscript{-6} mol/L). We used bradykinin responses as a tool to assess endothelial function; direct correlations of these findings to in vivo bradykinin levels remain unknown. Finally, isolation of multiple coronary arteries and arterioles did not allow simultaneous histological assessment of infarction within the same heart; thus, individual contributions of infarction are unknown. Roth, White, and colleagues\textsuperscript{1–4} however, documented the presence of minimal (1% to 6%) infarction in collateral-dependent myocardium of similar porcine hearts. Furthermore, we initiated training at 8 weeks after surgery (when collateral development has plateaued), and random placement of animals into the SED and EX groups would not be expected to elicit these differences.

Conclusions and Clinical Implications

Collectively, these findings indicate that exercise training, potentially via increases in ecNOS gene expression and NO
production, enhances endothelium-mediated relaxation of the collateral-dependent coronary microcirculation. Current results also provide support to a previous report of beneficial effects of training in conduit-sized coronary arteries distal to occlusion.\(^3\) Furthermore, because NO exerts multiple beneficial vasodilator, antithrombotic, and antiadhesive properties, these findings have particular clinical relevance to known protective actions of exercise-based rehabilitation programs in human patients with coronary disease\(^{19–23}\) and an impact on recent reports of a beneficial role of physical activity in reducing the incidence of a second myocardial infarction in patients who survive a first infarction.\(^1\)

In vivo mechanisms whereby exercise training produces beneficial adaptations of coronary endothelium distal to a chronic coronary occlusion remain unclear. Sellke and colleagues\(^{24}\) documented that in vivo administration of exogenous growth factors increases collateral development and improves endothelium-dependent relaxation of collateral-dependent arteries. Similarly, exercise training enhances collateral development and regional myocardial perfusion in the porcine model of chronic coronary occlusion.\(^2,3,25,26\) Furthermore, exercise training has been shown to improve myocardial function in patients with ischemic cardiomyopathy\(^27\) and to stimulate blood flow to the myocardium via native and developing collaterals.\(^3\) Therefore, exercise training may act as a nonpharmacological therapeutic strategy\(^{20,21}\) to enhance collateral development and/or stimulate cellular pathways (ie, flow- and shear stress–dependent signaling mechanisms) involved in eNOS regulation and induce beneficial adaptations of collateral-dependent microvascular endothelium.

NO has been proposed to be a crucial molecular signal in the angiogenic response to certain growth factors.\(^{28,29}\) For instance, vascular endothelial growth factor stimulates proliferation of postcapillary endothelial cells via eNOS-dependent production of NO and cGMP accumulation.\(^28\) Endothelial NO/cGMP pathways in turn activate mitogen-activated protein kinase, evidence supportive of a “prosurvival” balancing role of NO between cell proliferation and differentiation.\(^29\) Exercise training–induced upregulation of eNOS mRNA may theoretically contribute to and/or amplify NO-dependent actions of endogenous angiogenic growth factors or exogenous therapeutic growth factors to enhance collateral development and improve perfusion to ischemic myocardium. Potential synergistic actions of exercise training and angiogenic growth factors remain to be determined.

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