Exercise training enhances multiple mechanisms of relaxation in coronary arteries from ischemic hearts

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Deer RR, Heaps CL. Exercise training enhances multiple mechanisms of relaxation in coronary arteries from ischemic hearts. Am J Physiol Heart Circ Physiol 305: H1321–H1331, 2013. —Exercise training of coronary artery disease patients is of considerable interest, since it has been shown to improve vascular function and, thereby, enhance blood flow into compromised myocardial regions. However, the mechanisms underlying exercise-induced improvements in vascular function have not been fully elucidated. We tested the hypothesis that exercise training increases the contribution of multiple mediators to endothelium-dependent relaxation of coronary arteries in the underlying setting of chronic coronary artery occlusion. To induce gradual occlusion, an anterior wall constrictor was placed around the proximal left circumflex coronary artery in Yucatan miniature swine. At 8 wk postoperatively, pigs were randomly assigned to sedentary or exercise (treadmill, 5 days/wk) regimens for 14 wk. Exercise training significantly enhanced the contribution of nitric oxide, prostanoids, and large-conductance Ca\(^{2+}\)-dependent K\(^+\) (BK\(_{Ca}\)) channels to endothelium-dependent, bradykinin-mediated relaxation in nonoccluded and collateral-dependent arteries. Combined nitric oxide synthase, prostanoïd, and BK\(_{Ca}\) channel inhibition ablated the enhanced relaxation associated with exercise training. Exercise training significantly increased nitric oxide levels in response to bradykinin in endothelial cells isolated from nonoccluded and collateral-dependent arteries. Bradykinin treatment significantly increased PG\(_{I2}\) levels in all artery treatment groups and tended to be further enhanced after nitric oxide synthase inhibition in exercise-trained pigs. No differences were found in whole cell BK\(_{Ca}\) channel currents, BK\(_{Ca}\) channel protein levels, or arterial cyclic nucleotide levels. Although redundant, upregulation of parallel vasodilator pathways appears to contribute to enhanced endothelium-dependent relaxation, potentially providing a more refined control of blood flow after exercise training.

THE ENDOTHELIUM PLAYS AN IMPORTANT role in mediating vascular tone. In response to mechanical stimuli (shear stress and pulsatile pressure) or vasoactive agonists (bradykinin and ace-tylcholine), endothelium-dependent relaxation occurs through the release of mediators such as nitric oxide and PG\(_{I2}\), which affect the adjacent vascular smooth muscle in a paracrine manner. In addition, a third endothelium-derived factor [endo- thelium-derived hyperpolarizing factor (EDHF)] relaxes vascular smooth muscle by causing hyperpolarization of the underlying smooth muscle cells. While a single EDHF has not been elucidated, numerous candidate mechanisms or pathways have been proposed. Large-conductance Ca\(^{2+}\)-dependent K\(^+\) (BK\(_{Ca}\)) channels are a common downstream effector for several of these potential EDHFs (2, 27), as well as for nitric oxide (29) and PG\(_{I2}\) (7). BK\(_{Ca}\) channels are prominent in coronary vascular smooth muscle cells, and, thus, small changes in open probability have significant effects on membrane potential and vasomotor tone (6, 25).

Endothelial dysfunction is characterized by impaired vasodilation, enhanced vasoconstriction, cell proliferation, platelet activation, vascular permeability, and inflammation and aggregation of platelets. In humans, all major cardiovascular risk factors, including hypercholesterolemia, hypertension, diabe-tes, and smoking, have been associated with endothelial dys-function and impaired nitric oxide bioavailability (24). A primary feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to vasoactive agonists (11). However, other vasodilatory pathways have been shown to be augmented in conditions in which nitric oxide availability is reduced (3, 14, 30, 36). This redundancy in vasodilator signaling pathways allows for compensation if one mechanism is impaired.

The effect of exercise on the vascular health of patients with coronary artery disease is of considerable interest. Moderate exercise training has been shown to markedly improve myocardial perfusion and cardiac contractile function in compromised myocardium of diseased patients (17, 18). Previous studies have shown that exercise training enhances endothelial nitric oxide synthase (eNOS) mRNA expression in coronary arteries (41) and eNOS protein levels in coronary arterioles (26) of control animals. Exercise training also has been shown to increase nitric oxide generation, eNOS (15, 16, 44), and phosphorylated (Ser\(^{1177}\)) eNOS gene expression in animal models of disease (20, 49), as well as phosphorylated (Ser\(^{1177}\)) eNOS gene expression in human coronary artery disease patients (17).

Despite evidence that exercise training restores nitric oxide-dependent relaxation in the coronary circulation, little is known about adaptations in other endothelium-dependent signaling pathways that may function to compensate for reduced nitric oxide bioavailability. In the current study, we tested the hypothesis that exercise training increases the contribution of multiple mediators to endothelium-mediated relaxation of coronary arteries in the underlying setting of chronic coronary artery occlusion. Mechanistic adaptations in response to chronic coronary artery occlusion, as well as subsequent exercise training, were investi-gated.

METHODS

Experimental animals and surgical procedures. All animal protocols were carried out in accordance with “US Government Principles
for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training,” as detailed in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Texas A & M University in accordance with Association for the Assessment and Accreditation of Laboratory Animal Care procedures. As described in detail previously (22), adult female Yucatan miniature swine (Sinclair Research Center, Avon, Mongo) were surgically instrumented with an aortoconstrictor around the proximal left circumflex (LCX) coronary artery. Animals were preanesthetized with glycopyrrolate (0.004 mg/kg im), midazolam (0.5 mg/kg im), and ketamine (20 mg/kg im). Surgical anesthesia was induced with 3% isoflurane. Animals were intubated, and anesthesia was maintained with 2–3% isoflurane-balance O2 throughout aseptic surgery. During the surgery, pigs received the following drugs as necessary: pancuronium or vecuronium bromide (0.1 mg/kg; neuromuscular blockers) and lidocaine (1 mg/kg iv; antiarrhythmic). Immediately following surgery, pigs received ketoprofen (Ketofen, 3.0 mg/kg iv; nonsteroidal anti-inflammatory drug). Prior to surgery and during surgical recovery, animals received buprenorphine hydrochloride (0.1 mg/kg iv; analgesic) or butorphanol tartrate (0.5 mg/kg iv; analgesic) every 3–6 h, as needed for pain relief. An antibiotic (ceftriaxone sodium [Naxcel], 4 mg/kg im) was administered 24 h before surgery, immediately prior to surgery, and for 2 days following surgery.

Exercise training. At 8 wk postoperatively, pigs were randomly assigned to sedentary (n = 39) or exercise-training (n = 38) protocols, in which pigs underwent a progressive treadmill exercise-training program 5 days/wk for 14 wk or remained confined to their pens. By week 12 of the progressive exercise program, animals were running 85 min/day, 5 days/wk, which was maintained throughout the remainder of the training regimen, as described in detail previously (22). Efficacy of the exercise-training regimen was verified by comparison of skeletal muscle citrate synthase (oxidative enzyme) levels (42) and heart-to-body weight ratio at the conclusion of the study.

Preparation of coronary arteries. At the completion of the 14-wk exercise-training or sedentary protocols, pigs were anesthetized using xylazine (Rompun, 2.25 mg/kg im), ketamine (35 mg/kg im), and thiopental sodium (Pentothal, 30 mg/kg iv) and then given heparin (1,000 U/kg iv). Pigs were intubated and ventilated with room air, and a left lateral thoracotomy was performed in the fourth intercostal space. The heart was removed and placed in iced Krebs bicarbonate buffer (0–4°C) and weighed. Under a dissection microscope, the left anterior descending (LAD; nonoccluded) and LCX (collateral-dependent) coronary arteries were cut into rings (axial length = 5 mm) that were size-matched to have similar internal diameters, as indicated below for 20 min and then preconstricted with PGF2α (30 μM) until steady-state contraction was achieved. Concentration-response relationships to bradykinin (10⁻¹¹–10⁻⁶ M) or nitroprusside (10⁻⁹–10⁻⁴ M) were determined by cumulative addition in half-log increments directly into the tissue bath. Inhibitors include I) the nitric oxide synthase (NOS) inhibitor nitro-l-arginine methyl ester (l-NAME, 300 μM), 2) the prostaglandin inhibitor indomethacin (Indo, 5 μM), and 3) the BKCa channel blocker IBTX (100 nM).

Endothelial cell dissociation. Endothelial cells were enzymatically dissociated from segments of collateral-dependent LCX and nonoccluded LAD coronary arteries (~1.0 mm luminal diameter). Artery segments were cut longitudinally and pinned lumber-side-up in low-Ca²⁺ (0.1 mM) physiological buffer containing 294 U/ml collagenase, 5 U/ml elastase, 2 mg/ml BSA, 1 mg/ml soybean trypsin inhibitor, and 0.4 mg/ml DNase I in a 37°C water bath for 20 min. The enzyme solution was replaced with enzyme-free low-Ca²⁺ solution, and isolated cells were obtained by repeated direction of a stream of low-Ca²⁺ solution over the artery via a fire-polished Pasteur pipette. Enzymatically dissociated cells were collected and transferred to a 15-ml conical tube. The cells were centrifuged (Sorvall RT7 Plus swinging-bucket rotor RTH-75B; 800 rpm) for 3 min, the supernatant was removed, and the pellet was resuspended in enzyme-free low-Ca²⁺ solution.

Detection of bradykinin-stimulated nitric oxide levels in isolated endothelial cells. Bradykinin-stimulated nitric oxide levels were measured from freshly isolated endothelial cells in real-time using the fluorescence indicator 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA; Molecular Probes). Cells were incubated with DAF-FM DA (2.5 μM) for 10 min in the dark at room temperature and centrifuged (800 rpm) for 3 min, the supernatant was removed, and the pellet was resuspended in enzyme-free low-Ca²⁺ solution. Cells were pipetted into a superfusion chamber and observed using a epifluorescence microscopy system, which permitted evaluation of DAF fluorescence from multiple user-selected endothelial cells simultaneously throughout the experimental protocol (NIS-Elements AR 3.0). Endothelial cells were morphologically distinguishable from other cell types in the dispersion, as characterized previously (46). Cells were excited with a 175-W xenon arc lamp with a 475-nm interference filter for excitation wavelength (Lambda DG-4, Sutter Instruments). Fluorescence emission was captured at 530 nm every 15 s and reflected to an interline transfer, progressive-scan, cooled charge-coupled device video camera (CoolSNAP HQ, Photometrics) with a dichroic mirror. The microscope was equipped with a ×40 oil immersion objective with a numerical aperture of 1.3. After a 3-min baseline period, bradykinin (10⁻⁶, 10⁻⁷, or 10⁻⁸ M) was superfused at 5-min intervals. To determine delta peak fluorescence, baseline fluorescence was subtracted from the peak fluorescence response to each bradykinin concentration for each cell.

Measurement of PGF2α levels. Nonoccluded and collateral-dependent arteries were cut into rings (~1 mm axial length) and placed in cold Krebs buffer (4°C) on ice for 30 min. Supernatant was removed and fresh Krebs buffer (in the presence or absence of Indo or l-NAME) was added and slowly warmed in a 37°C water bath. Supernatant was once again removed and replaced with 175 μl of Krebs buffer containing the following agonists/antagonists: 1) control, 2) Indo (5 μM), 3) bradykinin (10⁻⁶ M), 4) Indo (5 μM) + bradykinin (10⁻⁸ M), or 5) l-NAME (300 μM) + bradykinin (10⁻⁶ M). After 10 min of incubation at 37°C, rings were removed from the solution. Arterial rings and supernatant were snap-frozen in separate tubes. Supernatant samples were prepared in accordance with the protocol provided with the 6-keto-PGFα enzyme immunoassay kit (catalog no. 515211, Cayman Chemical) and diluted 1:20 immediately prior to addition to the 96-well plate. The bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce) was used to determine total protein concentration of arterial rings.

Smooth muscle cell dissociation. All electrophysiology experiments were performed using freshly dispersed arterial smooth muscle
cells from nonoccluded and collateral-dependent arteries. Coronary arteries were pinned lumen-side-up in low-Ca\(^{2+}\) (0.1 mM) physiological buffer containing 1.4 mg/ml papain, 0.4 mg/ml DTT, and 0.4 mg/ml BSA. Cells were enzymatically dissociated by incubation in a 37°C water bath for 30–45 min. The enzyme solution was replaced with enzyme-free low-Ca\(^{2+}\) solution, and the arteries were dispersed with gentle trituration by micropipette for isolation of single smooth muscle cells. Smooth muscle cells were morphologically distinguishable from other cell types, as described previously (46). Isolated cells were maintained in low-Ca\(^{2+}\) solution at 4°C until use (0–6 h).

**Voltage clamp.** \(K^+\) channel currents were determined using standard whole cell voltage-clamp technique, as routinely done in our laboratory (19, 23, 47). All proposed experiments were performed using freshly dispersed smooth muscle cells on the day the animals were euthanized. Cells were initially superfused with low-Ca\(^{2+}\) physiological saline solution (PSS) containing (in mM) 138 NaCl, 5 KCl, 0.1 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 20 HEPEs, pH 7.4, during gigaseal formation. Heat-polished glass pipettes (2–5 M\(\Omega\)) were filled with a solution containing (in mM) 120 KCl, 10 NaCl, 1 MgCl\(_2\), 10 EGTA, and 10 HEPEs, pH 7.1, with KOH. Ionic currents were amplified by an Axopatch 200B patch-clamp amplifier (Axon Instruments). Cells were continuously perfused under gravity flow at room temperature (22–25°C). IBTX-sensitive currents were obtained by subtraction of currents in the presence of IBTX from control currents in smooth muscle cells isolated from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained animals.

**Immunoblots.** Arterial rings (~3 mm long, ~1.0 mm diameter) were isolated from the collateral-dependent LCX and nonoccluded LAD coronary arteries, quick-frozen, and stored at −80°C for later immunoblot analysis, as described in detail previously (12). Arterial lysate (20 μg total protein) was subjected to SDS-PAGE (4–20% gradient gel), transferred to a polyvinylidene difluoride membrane, and probed overnight with primary antibody. Primary antibodies included BK\(_{Ca}\) channel α-subunit (catalog no. APC-021, Alomone Labs; 1:200 dilution) and smooth muscle α-actin (catalog no. ab21027, Abcam; 1:1,000 dilution).

**Cyclic nucleotide measures.** Arterial rings (~5 mm long, ~1.0 mm diameter) were isolated from the collateral-dependent LCX and nonoccluded LAD and pretreated with the nonselective phosphodiesterase inhibitor IBMX (100 μM) for 5 min at 37°C in PSS. Subsequently, bradykinin (30 nM) or an equivalent volume of PSS (control rings) was added for an additional 10 min at 37°C. Rings were removed from PSS, quick-frozen in liquid N\(_2\), and stored at −80°C for later analysis of cAMP and cGMP by enzyme immunoassay according to instructions from the manufacturer (catalog nos. 581001 and 581021, Cayman Chemical).

**Statistics.** Animal body weight, heart-to-body weight, IC\(_{50}\), and citrate synthase activity were compared between sedentary and exercise-trained pigs by Student’s t-test. Dimensional characteristics of coronary arteries, fluorescence (DAF-FM DA), PG\(_I_2\), immunoblot, and cyclic nucleotide data were compared using two-way ANOVA. Bradykinin-mediated relaxation and whole cell \(K^+\) currents were evaluated by repeated-measures two-way ANOVA and the Greenhouse-Geisser adjustment to control for type 1 error due to unequal group sizes (28). If a main effect was identified by ANOVA, Bonferroni’s tests were used to detect individual differences. \(P \leq 0.05\) was considered significant. When more than one coronary arterial ring from the nonoccluded or collateral-dependent region of a given animal was used in identical protocols, the responses from those rings were averaged before data were analyzed.

## RESULTS

**Efficacy of the exercise-training program.** Effectiveness of the 14-wk exercise-training regimen was demonstrated by significant increases in skeletal muscle oxidative enzyme activity and an increased heart-to-body weight ratio in exercise-trained compared with sedentary animals. Citrate synthase activity increased significantly (\(P < 0.02\) for all comparisons) in the deltoid muscle (45.6 ± 1.4 vs. 36.9 ± 1.3 μmol·min\(^{-1}\)·g\(^{-1}\)) and lateral (38.7 ± 1.1 vs. 32.9 ± 1.0 μmol·min\(^{-1}\)·g\(^{-1}\)), medial (43.0 ± 1.8 vs. 36.3 ± 1.5 μmol·min\(^{-1}\)·g\(^{-1}\)), and long (34.7 ± 1.6 vs. 29.7 ± 1.2 μmol·min\(^{-1}\)·g\(^{-1}\)) heads of the triceps brachii muscle in exercise-trained (\(n = 38\)) compared with sedentary (\(n = 39\)) pigs. Although body weight did not differ between sedentary and exercise-trained animals at the time of death (34.42 ± 0.77 vs. 33.80 ± 0.75 kg), heart-to-body weight ratio was significantly greater in exercise-trained than sedentary pigs (4.47 ± 0.10 vs. 5.27 ± 0.10, \(P < 0.001\)).

**Coronary artery dimensions and characteristics.** Analyses of dimensional characteristics of arterial rings used for isometric tension studies revealed statistically smaller mean luminal diameter of collateral-dependent LCX than nonoccluded LAD coronary artery rings of sedentary pigs (Table 1). Additional artery dimensions, including outer diameter, wall thickness, and axial length, were not significantly different between groups. Furthermore, the resting tension of coronary ring segments at \(L_{\text{max}}\) was not significantly different between the four artery treatment groups.

**Contribution of nitric oxide, PG\(_I_2\), and BK\(_{Ca}\) channel to resting tension.** Incubation of coronary arterial rings with the NOS inhibitor L-NAME resulted in increases in resting tension that were not statistically different between treatment groups (Fig. 1A). Combined NOS and prostanoid (L-NAME + Indo) inhibition significantly increased resting tension of collateral-dependent arteries of exercise-trained pigs compared with all other treatment groups (Fig. 1B). Importantly, comparison of Fig. 1, A and B, revealed that L-NAME + Indo significantly attenuated the increase in resting tension generated by L-NAME alone in nonoccluded arteries of sedentary and exercise-trained pigs and tended (\(P = 0.09\)) to lessen the increase in collateral-dependent arteries of sedentary pigs. These data suggest that constricting, rather than dilating, prostanoids contribute to resting tone in three of the treatment groups, while

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Outer Diameter, mm</th>
<th>Lumen Diameter, mm</th>
<th>Wall Thickness, mm</th>
<th>Axial Length, mm</th>
<th>RT at (L_{\text{max}}), g</th>
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<tbody>
<tr>
<td>Sedentary</td>
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<tr>
<td>Nonoccl</td>
<td>59</td>
<td>1.52 ± 0.05</td>
<td>1.07 ± 0.04</td>
<td>0.21 ± 0.01</td>
<td>3.29 ± 0.03</td>
<td>1.34 ± 0.10</td>
</tr>
<tr>
<td>Coll-dep</td>
<td>55</td>
<td>1.37 ± 0.05</td>
<td>0.92 ± 0.04*</td>
<td>0.21 ± 0.01</td>
<td>3.23 ± 0.04</td>
<td>1.28 ± 0.11</td>
</tr>
<tr>
<td>Exercise-trained</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nonoccl</td>
<td>54</td>
<td>1.49 ± 0.04</td>
<td>1.04 ± 0.03</td>
<td>0.21 ± 0.01</td>
<td>3.25 ± 0.03</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>Coll-dep</td>
<td>54</td>
<td>1.37 ± 0.05</td>
<td>0.94 ± 0.04</td>
<td>0.20 ± 0.01</td>
<td>3.26 ± 0.04</td>
<td>1.29 ± 0.11</td>
</tr>
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</table>

Values are means ± SE; \(n\), number of arteries. RT at \(L_{\text{max}}\), resting tension at optimal length, where maximal active tension to KCl-induced depolarization is developed; Nonoccl, nonoccluded; Coll-dep, collateral-dependent. \(* P < 0.05\) vs. sedentary Nonoccl.
prostanoids do not appear to contribute to basal tension in collateral-dependent arteries of exercise-trained pigs. Addition of IBTX had a negligible effect on resting tension, suggesting that BKCa channels have little role in basal tone.

Effects of exercise training and chronic occlusion on bradykinin-mediated relaxation. We examined the effect of exercise training and chronic coronary artery occlusion on bradykinin-mediated relaxation (Fig. 2, Table 2). Comparison of control curves in Fig. 2, A–D, revealed no significant difference in relaxation from nonoccluded and collateral-dependent coronary arteries of sedentary or exercise-trained animals. Concurrent studies were performed to determine the contribution of nitric oxide, prostanoids, and BKCa channels to bradykinin-mediated relaxation. NOS inhibition significantly attenuated relaxation in all groups compared with the control curve; however, relaxation responses remained significantly more persistent in arteries from exercise-trained than sedentary pigs. Similarly, sensitivity (IC50, Table 2) of the arterial rings to L-NAME and indomethacin (Indo, 5 μM; B) and L-NAME + Indo + IBTX (100 nM; C). Values are means ± SE; n, number of animals. *P < 0.05 vs. all other treatments within A, B, or C.

Fig. 1. Effect of chronic occlusion and exercise training on the contribution of nitric oxide, prostanoids, and large-conductance Ca2+/H11001-dependent K+/H11001 (BKCa) channel to resting tension. A–C: increase in baseline tension in nonoccluded (nonoccl) and collateral-dependent (coll-dep) arteries of sedentary (SED) and exercise-trained (EX) pigs in response to administration of nitro-l-arginine methyl ester (L-NAME, 300 μM; A), L-NAME + indomethacin (Indo, 5 μM; B), and L-NAME + Indo + IBTX (100 nM; C). Values are means ± SE; n, number of animals. *P < 0.05 vs. all other treatments within A, B, or C.

Fig. 2. Effect of chronic occlusion and exercise training on bradykinin-mediated relaxation. A–D: relaxation responses of rings from nonoccluded and collateral-dependent arteries of sedentary (A and B) and exercise-trained (C and D) pigs in response to increasing concentrations of bradykinin in the absence and presence of inhibitors. Values are means ± SE; n = 8–18 pigs for each condition. *P < 0.05 vs. all other treatment groups within A, B, C, or D. †P < 0.05 vs. L-NAME within A, B, C, or D. ‡P < 0.05 vs. respective SED curve across A–D.

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bradykinin was significantly reduced following pretreatment with L-NAME, although the reduction of sensitivity was less pronounced in the arteries of exercise-trained pigs. Combined inhibition of NOS and prostanoids partially reversed the enhanced relaxation observed in exercise-trained pigs after NOS inhibition alone; however, significantly increased relaxation persisted in nonoccluded and collateral-dependent arteries of exercise-trained animals. Sensitivity (Table 2) of the arterial rings to bradykinin was not further reduced following pretreatment with L-NAME + Indo compared with L-NAME alone in all groups. Finally, combined blockade with NOS, prostanoids, and BKCa channels ablated the enhanced exercise-training-mediated relaxation in nonoccluded and collateral-dependent arteries, such that relaxation responses were similar across treatment groups when all three mediators were inhibited. Sensitivity (Table 2) of the arterial rings to bradykinin was not further reduced following pretreatment with L-NAME + Indo + IBTX compared with L-NAME alone or L-NAME + Indo. In additional studies, pretreatment with Indo or IBTX alone did not significantly alter the bradykinin-mediated relaxation response in any of the artery treatment groups (Table 2).

Smooth muscle responsiveness to nitroprusside. We also evaluated the response of coronary arterial rings to the endothelium-independent nitric oxide donor nitroprusside. Concentration-dependent relaxation responses to nitroprusside were not significantly different in arteries from the collateral-dependent or nonoccluded arteries of sedentary or exercise-trained pigs (IC50 = 9.72 ± 0.18, 9.72 ± 0.10, 9.72 ± 0.16, and 9.72 ± 0.16 M, respectively). These data suggest that smooth muscle responsiveness to nitric oxide was not altered by exercise training or chronic occlusion.

Measurement of PGI2. We evaluated basal and bradykinin-stimulated changes in intracellular PGI2 levels in arterial rings of the collateral-dependent LCX and nonoccluded LAD coronary arteries (Fig. 4). PGI2 levels were assessed by measurement of the stable end product of PGI2 metabolism 6-keto-PGF1α. Basal PGI2 levels were not significantly altered by chronic occlusion or exercise training. There was no difference in PGI2 release with bradykinin (10−9 M) stimulation (data not shown); however, bradykinin (10−6 M) caused a significant increase in PGI2 levels in all artery treatment groups. To examine the specificity of the assay, we pretreated a subset of arterial rings with the prostanoid inhibitor Indo (5 μM). Pretreatment with Indo confirmed the specificity of the assay and caused a significant decrease in basal and bradykinin-stimulated PGI2 levels (Fig. 4). To assess the possibility of cross talk between nitric oxide and PGI2, we examined PGI2 levels in the presence of NOS inhibition, anticipating that nitric oxide may hinder PGI2 production. However, pretreatment with L-NAME did not significantly enhance bradykinin-stimulated PGI2 in any of the artery treatment groups.

Table 2. IC50 values for bradykinin-mediated relaxation in the absence and presence of inhibition of select signaling pathways

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n = 15)</th>
<th>L-NAME (n = 8–10)</th>
<th>L-NAME + Indo (n = 16–18)</th>
<th>L-NAME + Indo + IBTX (n = 13–15)</th>
<th>Indo (n = 6)</th>
<th>IBTX (n = 6)</th>
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<tr>
<td>Sedentary</td>
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<tr>
<td>Nonoccl</td>
<td>−8.73 ± 0.09</td>
<td>−7.92 ± 0.14*</td>
<td>−7.72 ± 0.10*</td>
<td>−7.83 ± 0.16*</td>
<td>−8.70 ± 0.22</td>
<td>−8.70 ± 0.27</td>
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<tr>
<td>Coll-dep</td>
<td>−8.75 ± 0.11</td>
<td>−7.67 ± 0.30*</td>
<td>−7.62 ± 0.11*</td>
<td>−8.10 ± 0.27*</td>
<td>−8.78 ± 0.33</td>
<td>−8.67 ± 0.38</td>
</tr>
<tr>
<td>Exercise-trained</td>
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</tr>
<tr>
<td>Nonoccl</td>
<td>−8.73 ± 0.06</td>
<td>−7.87 ± 0.16*</td>
<td>−7.96 ± 0.11*</td>
<td>−7.95 ± 0.17*</td>
<td>−8.50 ± 0.20</td>
<td>−8.57 ± 0.21</td>
</tr>
<tr>
<td>Coll-dep</td>
<td>−8.73 ± 0.08</td>
<td>−8.07 ± 0.18*</td>
<td>−7.94 ± 0.13*</td>
<td>−7.97 ± 0.10*</td>
<td>−8.43 ± 0.15</td>
<td>−8.36 ± 0.33</td>
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Values (log M) are means ± SE; n, number of animals. L-NAME, N-nitro-L-arginine methyl ester; Indo, indomethacin. *P < 0.05 vs. corresponding IC50 from control (no inhibitors).
Whole cell $K^+$ channel current. We also determined the effects of chronic occlusion and exercise training on coronary smooth muscle $K^+$ channel currents (Fig. 5). Whole cell currents were elicited by 500-ms step depolarizations to test potentials ranging from $-70$ to $+100$ mV from a holding potential of $-80$ mV. Representative traces for currents of cells from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained pigs are shown in Fig. 5A. For current-voltage relationships in Fig. 5, B and C, we plotted the mean value of outward current for the last 100 ms of each test potential normalized to cell membrane capacitance (pA/pF). Cell capacitance was not significantly different between smooth muscle cells from nonoccluded and collateral-dependent arteries of sedentary (11.1 ± 0.8 and 11.7 ± 0.9 pF, respectively) and exercise-trained (12.0 ± 0.8 and 12.2 ± 0.8 pF, respectively) pigs. Comparison of the current-voltage relationships indicated that neither chronic coronary occlusion nor exercise training altered whole cell $K^+$ currents in arterial smooth muscle cells.

IBTX-sensitive $K^+$ channel current. We further explored the effects of chronic occlusion and exercise training on IBTX-sensitive (BK$_{Ca}$) channel currents (Fig. 6). IBTX-sensitive currents were obtained by subtraction of currents in the presence of IBTX from control currents (difference currents) (Fig. 6A). Findings from these studies revealed that BK$_{Ca}$ channel currents were not significantly altered by occlusion or exercise training in coronary arterial smooth muscle of our porcine model (Fig. 6, B and C).

BK$_{Ca}$ channel protein. Determination of protein by immunoblot revealed that neither occlusion nor exercise training significantly altered BK$_{Ca}$ channel protein levels in coronary arteries (Fig. 7). The control protein, smooth muscle $\alpha$-actin, also was not significantly altered by occlusion or exercise training in these arteries.

Cyclic nucleotide levels. We examined levels of cGMP and cAMP by enzyme immunoassay in nonoccluded and collateral-dependent arterial rings of sedentary and exercise-trained pigs. Bradykinin treatment of rings did not alter cyclic nucleotide levels compared with control conditions (PSS); thus control and bradykinin-treated rings for each pig were averaged prior to final data analysis. Findings from these studies revealed that neither cGMP nor cAMP levels were altered by chronic occlusion or exercise training (Fig. 8).

**DISCUSSION**

These studies provide the first evidence that exercise training increases the contribution of multiple, redundant mediators to endothelium-dependent relaxation of coronary arteries in the underlying setting of chronic occlusion that is not observed in the sedentary pig. This discovery has plausible clinical relevance, in that it suggests that, after exercise training, the diseased heart may be less susceptible to an ischemic episode as a result of these redundant and potentially compensatory pathways of vascular relaxation. Our data reveal that bradykinin-stimulated nitric oxide levels were significantly increased after exercise training in endothelial cells of nonoccluded and collateral-dependent arteries. Furthermore, PGI$_2$ levels were slightly, although not significantly, increased in arteries of exercise-trained pigs compared with respective arteries of sedentary pigs. Interestingly, despite increases in signaling molecules with exercise training, bradykinin-mediated, endothelium-dependent relaxation was not enhanced in arteries from exercise-trained pigs. On the other hand, bradykinin-mediated relaxation was more persistent in arteries from exercise-trained pigs after inhibition of select endothelium-dependent signaling molecules, suggesting redundancy in signaling pathways of vascular relaxation.

Specifically, while NOS inhibition significantly reduced bradykinin-mediated relaxation in all artery treatment groups, the effect was much more marked in arteries of sedentary than exercise-trained animals. This finding might be interpreted to indicate that the contribution of NOS to endothelium-mediated relaxation is significantly greater in sedentary than exercise-trained pigs or that other pathways of relaxation better compensate for the loss of nitric oxide in the exercise-trained...
animals. While combined inhibition of nitric oxide and prostanoids partially reversed the difference in bradykinin-mediated relaxation in the presence of NOS inhibition alone, the significantly enhanced relaxation continued in arteries from exercise-trained pigs. Finally, combined inhibition of nitric oxide, prostanoids, and BKCa channels eliminated the persistent relaxation observed in arteries from exercise-trained pigs. Indeed, prostanoids and BKCa channels contributed to bradykinin-mediated relaxation only after the inhibition of NOS in exercise-trained pigs, providing further evidence of a compensatory role for these pathways after loss of nitric oxide. Taken together, these data suggest that nitric oxide, dilating prostanoids, and BKCa channels eliminated the persistent relaxation observed in arteries from exercise-trained pigs. Indeed, prostanoids and BKCa channels contributed to bradykinin-mediated relaxation only after the inhibition of NOS in exercise-trained pigs, providing further evidence of a compensatory role for these pathways after loss of nitric oxide. Taken together, these data suggest that nitric oxide, dilating prostanoids, and BKCa channel contribution are increased after exercise training but that some limitation exists regarding the contribution of these molecules to the overall relaxation response, such as negative modulation between the mediators or their pathways. Indeed, numerous reports have revealed interaction of various signaling pathways that can negatively or positively regulate one another (3, 33, 34, 38). We anticipated that PGI2 levels in the presence of NOS inhibition may be increased above those observed in the absence of NOS inhibition, which would suggest that nitric oxide negatively modulated PGI2 production, as previously reported (4, 34, 38). However, while PGI2 levels in the presence of NOS inhibition in exercise-trained pigs were slightly increased above PGI2 levels in the absence of NOS inhibition, they did not reach statistical significance ($P_{\text{NS}}$). Previous studies reported cross talk between nitric oxide and PGI2 in the coronary circulation. In canine coronary arteries, prostanoid inhibition had no effect on bradykinin-mediated relaxation until combined with NOS inhibition (38), and bradykinin-stimulated PGI2 release was significantly enhanced by chronic NOS blockade (4).

Another critical aspect of our study was quantification of nitric oxide and PGI2 levels in response to bradykinin stimulation. Our findings demonstrate that bradykinin-stimulated nitric oxide levels were significantly increased in endothelial cells from exercise-trained compared with sedentary pigs. Taken together with our functional data in the presence of NOS inhibition, these data support better compensation of other pathways of relaxation after inhibition of nitric oxide production, rather than a lesser contribution of nitric oxide to relaxation, in exercise-trained pigs. These results agree with previous studies from our laboratory that reported significant exercise-training-induced adaptations in mechanisms that control nitric oxide production in nonoccluded and collateral-dependent arteries.

Fig. 5. Effect of chronic occlusion and exercise training on whole cell K+ channel current ($I_{K}$) in coronary artery smooth muscle cells. Currents were elicited by 500-ms step depolarizations to test potentials (TP) ranging from −70 to +100 mV in 10-mV increments from a holding potential (HP) of −80 mV. A: representative traces for currents from cells from nonoccluded and collateral-dependent arteries of sedentary and exercise-trained pigs. B and C: current-voltage relationships obtained by plotting the mean outward current at the end of each test potential normalized to cell membrane capacitance. Values are means ± SE; $n$, number of animals, followed by number of cells. There were no significant differences.
dent coronary arteries, resulting in enhanced nitric oxide production (49). Other studies have shown increased eNOS mRNA and protein levels after exercise training in control animals (26, 41) and in numerous animal models of disease (15, 16, 20, 44). Additionally, exercise training of patients with stable coronary artery disease was also shown to improve in vivo endothelium-dependent dilation and mean peak blood flow velocity concomitant with increased phosphorylation (Ser1177) of eNOS and total eNOS protein and mRNA expression in the left internal mammary artery of these patients (17). Although statistical significance was not attained, PGI2 levels were slightly enhanced in nonoccluded and collateral-dependent arteries of exercise-trained pigs compared with their respective arteries in sedentary animals. These findings coincide with our functional data that showed a significantly greater effect of combined NOS and prostanoid inhibition than NOS inhibition alone in the exercise-trained, but not sedentary, pigs. Additionally, exercise training tended to increase bradykinin-stimulated PGI2 levels after nitric oxide inhibition in nonoccluded and collateral-dependent arteries. Taken together, our data suggest that exercise training enhances nitric oxide and PGI2 contributions to vasorelaxation and that nitric oxide may have inhibitory actions on PGI2 production. While the effects of exercise training on nitric oxide production have been widely studied, exercise-training-induced adaptations of PGI2-dependent responses are not well understood. There is debate on the importance of prostanoids in the regulation of coronary blood flow in humans. Studies of patients with coronary artery disease have shown that Indo diminishes vasodilation and reduces resting coronary blood flow (13, 35), suggesting that PGI2 contributes to blood flow in disease states under resting conditions. In contrast, in healthy patients, prostanoids were not essential to basal coronary flow or to modulation of coronary flow during exercise (9). Taken together, these findings suggest that blunted NOS activity often associated with human disease may be compensated by an increased contribution of prostanoids. In agreement, our data clearly suggest that, following NOS inhibition, PGI2 significantly contributes to bradykinin-mediated relaxation in coronary arteries of exercise-trained pigs in the underlying setting of chronic coronary occlusion.
artery occlusion. However, the role of PGI2 in the absence of NOS inhibition was not examined in our studies.

In a canine model of permanent occlusion of the LAD artery, resting myocardial blood flow in the control and collateral-dependent zones was not altered by cyclooxygenase blockade (1). However, during exercise, Indo produced an increase in transcollarateral resistance that was associated with a decrease in subendocardial flow in the collateral-dependent zone (1), suggesting a role for PGI2 to maintain blood flow to compromised myocardial regions during stress. Furthermore, in patients not affected by coronary artery disease, inhibition of coronary prostanoids did not alter coronary hemodynamics at rest; however, sympathetic stimulation (cold pressor test) induced significantly increased coronary blood flow and decreased coronary vascular resistance, which were associated with enhanced levels of PGI2 and PGE2 (32). Subsequent prostanoid inhibition abolished these changes in coronary hemodynamics (32).

In this study there was no effect of chronic occlusion or exercise training on K\textsuperscript{+} channel currents. These findings agree with previous studies that showed no effect of exercise training on whole cell K\textsuperscript{+} currents of smooth muscle cells from coronary arteries of control (5, 19) and hypercholesterolemic (19) pigs. Furthermore, there was no effect of occlusion or exercise training on BKCa channel currents in our studies. In contrast, the effect of IBTX in functional data suggests that BKCa channels do contribute to the enhanced relaxation after exercise. Taken together, these data suggest that a bradykinin-sensitive cellular signaling pathway that mediates smooth muscle relaxation via BKCa channels may be upregulated by exercise training and contribute to enhanced relaxation, rather than an increase in BKCa channel number. We postulate that this pathway may be a putative EDHF, since the contribution of BKCa channels persisted in the presence of NOS and prostanoid inhibition. EDHF and PGI2 have been suggested to act as backup vasodilators; hence, their role in dilation may be more relevant after the nitric oxide pathway is compromised (3, 33, 34). Indeed, the role of compensatory mechanisms has been shown to be very important in numerous vascular diseases associated with decreases in nitric oxide production. In coronary artery disease, compensation occurs via increased K\textsuperscript{+} channel activity and increased release of hyperpolarizing factors (30), while in hypercholesterolemia, compensatory increases in Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels is evident (31). EDHF has also been shown to compensate for diminished nitric oxide-dependent dilation in numerous disease states, including type 2 diabetes (36), hypertension (14), and hyperparathyroidism (45).

We recently reported on responses of the coronary microcirculation in this porcine model of chronic occlusion and exercise training. In these experiments, bradykinin-mediated dilation was significantly attenuated in collateral-dependent arterioles of sedentary pigs, an impairment that was reversed with exercise training (47). These data reveal that one or more of the endothelium-dependent dilation pathways is impaired in the collateral-dependent microcirculation and are in contrast to the current findings in the macrocirculation, in that overall relaxation was not altered by chronic occlusion or exercise training in conduit arteries. Furthermore, we evaluated the role of nitric oxide in the microcirculation and found that while nitric oxide contributed significantly to bradykinin-mediated dilation in all arteriole treatment groups, it was not the underlying mechanism for enhanced dilation of collateral-dependent arterioles after exercise training (48). Thus, on the basis of our
collective data, it is apparent that nitric oxide is a major contributor to endothelium-dependent dilation/relaxation in the micro- and macrocirculations of our pig model. However, while nitric oxide concentrations are increased by exercise training in nonoccluded and collateral-dependent arteries, overall endothelium-dependent relaxation was not enhanced in these arteries. We have not made direct measures of nitric oxide in the microcirculation. Our previous data in the microcirculation also reveal that blockade of BKCa channels alone significantly attenuated bradykinin-mediated dilation in all arteriole treatment groups (47). Indeed, the reversal of impaired dilation in collateral-dependent arterioles with exercise training was attributable to BKCa channels (47). This is in contrast to our findings in the conduit arteries, in which blockade of BKCa channels alone did not significantly alter relaxation. However, BKCa channels do contribute significantly in a compensatory manner in the macrocirculation after exercise training when other pathways of dilation are inhibited. Taken together, while many of the same pathways contribute to dilation/relaxation in the micro- and macrocirculations, the level of their involvement (primary or secondary) appears to vary depending on the in vivo role of the vessel. This heterogeneity of responses across vessel sizes has been well documented previously.

Clinical significance and conclusions. Exercise training has been shown to improve vascular function and contribute to enhanced myocardial perfusion and cardiac contractile function in numerous vascular disease states (17, 18). Despite remarkable evidence for the therapeutic benefits of physical activity, the mechanisms by which regular exercise improves vascular function in the setting of coronary artery disease are not fully understood. Furthermore, while conduit arteries typically contribute little to total coronary vascular resistance, the presence of disease has been reported to enhance the contribution of epicardial arteries to total resistance and, therefore, blood flow regulation (13a). Thus the exercise-training-induced adaptations in epicardial coronary arteries in the underlying setting of coronary artery disease may give rise to enhanced blood flow into the collateral-dependent region. The current study provides new evidence that exercise training enhances the contribution of numerous vasodilators, including nitric oxide, PGI2, and BKCa channels, in the underlying setting of coronary artery disease. The apparent redundancy in these signaling pathways is thought to ensure vascular relaxation and, thereby, provide sufficient blood flow to the myocardium during cardiac stress. These exciting findings provide novel insights into the effects of exercise training on coronary vascular function in the underlying setting of coronary artery disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.R.D. and C.L.H. are responsible for conception and design of the research; R.R.D. performed the experiments; R.R.D. and C.L.H. analyzed the data; R.R.D. and C.L.H. interpreted the results of the experiments; R.R.D. prepared the figures; R.R.D. drafted the manuscript; R.R.D. and C.L.H. edited and revised the manuscript; R.R.D. and C.L.H. approved the final version of the manuscript.

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