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Exercise training-induced adaptations in mediators of sustained endothelium-dependent coronary artery relaxation in a porcine model of ischemic heart disease

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Abstract

Objective—Test the hypothesis that exercise training enhances sustained relaxation to persistent endothelium-dependent vasodilator exposure via increased nitric oxide contribution in small coronary arteries of control and ischemic hearts.

Methods—Yucatan swine were designated to a control group or a group in which an ameroid constrictor was placed around the proximal LCX. Subsequently, pigs from both groups were assigned to exercise (5 days/week; 16 weeks) or sedentary regimens. Coronary arteries (~100–350 μ m) were isolated from control pigs and from both nonoccluded and collateral-dependent regions of chronically-occluded hearts.

Results—In arteries from control pigs, training significantly enhanced relaxation responses to increasing concentrations of bradykinin (10^{-10} to 10^{-7} M) and sustained relaxation to a single bradykinin concentration (30 nM), which were abolished by NOS inhibition. Training also significantly prolonged bradykinin-mediated relaxation in collateral-dependent arteries of occluded pigs, which was associated with more persistent increases in endothelial cellular Ca^{2+} levels, and reversed with NOS inhibition. Protein levels for eNOS and p-eNOS-(Ser1179), but not caveolin-1, Hsp90, or Akt, were significantly increased with occlusion, independent of training state.

Conclusions—Exercise training enhances sustained relaxation to endothelium-dependent agonist stimulation in small arteries of control and ischemic hearts by enhanced nitric oxide contribution and endothelial Ca^{2+} responses.

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Disclosures

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Keywords

coronary occlusion; collateral; isometric tension; bradykinin; ischemic heart disease; calcium

INTRODUCTION

It has become apparent in recent years that exercise training/physical activity improves the functional integrity of the vascular endothelium. Numerous reports have documented that exercise training may provide protective benefits through alterations in coronary vascular reactivity, primarily through improved endothelium-dependent vasodilation in health and disease (13–15, 17–19, 28, 38–40, 43). These studies have contributed greatly to our understanding of exercise-induced adaptations of the coronary endothelium and the mediators that contribute to these adaptations. However, these studies have primarily examined the effect of exercise training on large coronary arteries ($>500\text{ }\mu\text{m}$) or microvascular coronary arterioles ($\sim 100\text{ }\mu\text{m}$). In contrast, little is known regarding effects of exercise training on intermediate-sized coronary arteries ($\sim 150\text{--}400\text{ }\mu\text{m}$), even though these vessels have been shown to contribute significantly to total coronary vascular resistance and its regulation during metabolic stress. Chilian and colleagues have reported that under basal conditions, roughly 45–50% of total coronary resistance is proximal to arteries $\sim 100\text{ }\mu\text{m}$ in diameter, while 20–25% of this resistance is proximal to arteries $\sim 200\text{ }\mu\text{m}$ in diameter (3, 4). Furthermore, during metabolic vasodilation of coronary arterioles ($<100\text{ }\mu\text{m}$), an even greater fraction of the remaining coronary resistance resides in the small coronary arteries, as these arteries appear less responsive to metabolic vasodilators (22). This phenomenon is likely of greatest significance under conditions of myocardial ischemia when small arterioles have undergone metabolic vasodilation. The ability of small coronary arteries in the ischemic myocardial region to respond to endogenous vasodilators may ultimately control regional blood flow and the subsequent fate of the ischemic myocardium (6).

Furthermore, prior studies of coronary vascular reactivity have focused on assessment of rapid arterial responses to step increases in agonist concentrations rather than vascular responses to sustained agonist exposure; a condition which may be more relevant by closely mimicking the *in vivo* myocardial milieu. Therefore, the purpose of the current study was to determine the effects of chronic exercise training on the time course of endothelium-dependent relaxation of small coronary arteries (range $\sim 100\text{--}350\text{ }\mu\text{m}$) and the underlying mechanisms which initiate and maintain relaxation during sustained exposure to an endothelium-dependent agonist (bradykinin). We hypothesized that exercise training would enhance the role of nitric oxide in sustained endothelium-dependent relaxation in small coronary arteries from both control and ameroid-occluded pigs and thereby, contribute to protracted bradykinin-mediated relaxation responses after exercise training. We further hypothesized that bradykinin-stimulated increases in endothelial intracellular Ca^{2+} levels would be more sustained after exercise training and thus, provide a primary mechanism for the prolonged relaxation response via Ca^{2+} -dependent activation of endothelial nitric oxide synthase.

MATERIALS AND METHODS

Experimental Animals

All animal protocols were in accordance with The American Physiological Society's "Guiding Principles in the Care and Use of Animals" and approved by the Institutional Animal Care and Use Committee at Texas A&M University in accordance with AALAC procedures. Furthermore, all methods conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHHS Publication NIH 85-23, Office of Science and Health Reports, Bethesda, MD). Yucatan miniature swine were designated to a control group (n=40) or a group in which animals were surgically instrumented with an ameroid constrictor (n=28) around the proximal LCX artery. Subsequently, pigs from both groups were randomly assigned to exercise training (5 days/week) or sedentary (pen-confined) protocols for 16 weeks.

Surgical instrumentation

Female Yucatan miniature swine were surgically instrumented with ameroid constrictors around the LCX artery as described in detail previously (14, 20). Animals were preanesthetized with glycopyrrolate (0.004 mg/kg, im) and midazolam (0.5 mg/kg, im). Anesthesia was induced with ketamine (20 mg/kg, im) and maintained with 2–3% isoflurane, and supplemental O₂ throughout aseptic surgery. During the surgery, pigs received the following drugs as necessary: pancuronium or vecuronium bromide (0.1 mg·kg⁻¹; neuromuscular blockers) and lidocaine (1 mg·kg⁻¹, i.v.; antiarrhythmic). Immediately following surgery, pigs received ketofen (3.0 mg·kg⁻¹, i.v.; NSAID). Prior to surgery and during surgical recovery, animals received either buprenorphine hydrochloride (0.1 mg·kg⁻¹, i.v.; analgesic) or butorphanol tartrate (0.5 mg·kg⁻¹, i.v.; analgesic) every 3–6 hr, as needed for pain relief. Antibiotics (Naxcel, 4 mg·kg⁻¹, i.m.) were administered 24 hours before surgery, immediately prior to surgery and for two days following surgery. Pigs recovered from surgery for eight weeks before experimental exercise training and sedentary protocols were initiated.

Exercise training

Exercise trained pigs underwent a progressive treadmill exercise training program, 5 days/week for 16 weeks, as described previously (15, 20). During the first week of the experimental protocol, a typical exercise training session included treadmill running at 3 mph for 20–25 min (endurance) and at 5 mph for 5–10 min (sprint). All exercise training sessions were preceded by a 5 min warm-up at 2.5 mph and followed by a 5 min warm-down at 2 mph. Speed and duration of the exercise training sessions were progressively increased so that during the last four weeks of training, animals ran at 4–5.5 mph for 60 min and at 6 mph for 5–15 min. Grade of the treadmill was maintained at 0% throughout the experimental protocol. The progressive nature of the exercise training was dependent upon the tolerance of each animal and therefore, ranges of running speed and duration presented represent differing abilities of the animals. Pigs were given positive reinforcement for exercise by feeding them after each training bout. Sedentary animals remained pen-confined throughout the 16-week duration. Effectiveness of the exercise training program was

determined by comparing heart-to-body weight ratio and skeletal muscle citrate synthase activity as previously described (14, 20).

Preparation of coronary arteries

Following completion of the 16-week exercise training or sedentary protocols, pigs were anesthetized with ketamine (30 mg/kg) and sodium pentothal (35 mg/kg). Hearts were removed, placed in Krebs bicarbonate buffer (0–4 °C) and weighed. Visual inspection of the ameroid occluder during dissection of the LCX artery validated 100% occlusion in all animals that had undergone surgery for this study. With the aid of a dissection microscope, size-matched arteries (~160 µm inner diameter, unpressurized) were isolated from the LCX-supplied myocardium of control animals and both the nonoccluded LAD artery region and collateral-dependent LCX region (distal to occlusion) of ameroid-occluded pigs. Arteries selected for study were isolated from the midmyocardium and were typically second or third order branches arising from the main artery (LAD or LCX). Arterial segments were trimmed of fat and connective tissue, cut into rings and measured with a calibrated Filar micrometer eyepiece (Hitschfel Instruments, St. Louis, MO) in a relaxed, unstretched state.

Arterial rings were mounted in specialized isometric microvessel myographs (Living Systems Instrumentation) as used previously (19, 31, 34). Initially, arteries were stretched using the micrometer to yield a baseline tension of ~0.1–0.2 mN/mm. Vessels were superfused continuously with aerated (95% O₂, 5% CO₂) Krebs bicarbonate buffer, warmed to 37 °C and allowed to equilibrate for 30 min. Following the initial equilibration, the resting tension-internal circumference relationship was determined with progressive stretches, and measurement of passive tension and internal circumference (L) of the artery at each level of stretch. L was calculated by using the following formula:

$L = 2f + 4d/2 + 2(\pi/2)$, where d is wire diameter and f is the distance between the wires as measured by the micrometer. Coronary rings were set at L₉₅, where $L_{95} = 0.95 * L_{100}$ and L₁₀₀ is the internal circumference the artery would have under a transmural pressure of 100 mmHg (23, 31). Arterial rings were allowed to equilibrate for 15 min at L₉₅ prior to subsequent evaluation of pharmacological responsiveness.

Experimental Protocols

Concentration-response relationships to bradykinin (10⁻¹⁰ to 10⁻⁷ M) and nitroprusside (10⁻¹⁰ to 10⁻⁴ M) were determined by cumulative addition of these agents directly to the superfusate. Additional arteries were used to determine the relaxation response to a sustained exposure to a single concentration of bradykinin (30 nM) for 20 minutes. All arteries were precontracted with endothelin-1 (2 nM) or KCl (35 mM) prior to bradykinin and nitroprusside exposure. In select experiments, arteries were incubated with the cyclooxygenase inhibitor, indomethacin (5 µM), to block the production of prostanoids or combined inhibition with indomethacin and the nitric oxide synthase inhibitor, L-NAME (100 µM), beginning 20 min prior to bradykinin exposure.

Endothelial cell dissociation

Small coronary arteries were placed in low-Ca²⁺ (0.1 mM) physiological buffer containing 294 U/ml collagenase, 5 U/ml elastase, 2 mg/ml bovine serum albumin, 1 mg/ml soybean

trypsin inhibitor, and 0.4 mg/ml DNase I. Cells were enzymatically dissociated by incubation in a 37 °C water bath for 45 min. The enzyme solution was then replaced with enzyme-free low-Ca²⁺ solution and the arteries dispersed with gentle trituration by micropipette.

Endothelial intracellular free Ca²⁺ measurement

Freshly dissociated endothelial cells were incubated with the fluorescent Ca²⁺ indicator fura-2/AM (2.5 μM) at 37 °C for 20 min. Cells were washed at 37 °C for 20 min in sterile modified Eagle's minimal essential storage media to cleave extracellular fura-2/AM. Isolated cells were maintained in low-Ca²⁺ solution at 4 °C until use (0–4 h). Fura-2 loaded cells were placed in a superfusion chamber and observed using an epifluorescence microscopy system, which permitted simultaneous evaluation of fura-2 fluorescence from multiple user-selected endothelial cells throughout an experimental protocol, as described in detail previously (45). Cells were continuously superfused under gravity flow with PSS. Addition of bradykinin (30 nM) was made to PSS for determination of bradykinin-stimulated intracellular Ca²⁺ responses.

Immunoblots

Arterial rings (~100–350 μm diameter; ~6–10 mm total length) were dissected from both the nonoccluded and collateral-dependent myocardial regions, quick-frozen in liquid N₂ and stored at –80°C for later immunoblot analysis, as described in detail previously (12). Arterial lysate (10 μg total protein) was subjected to SDS–PAGE (10 % gel), transferred to PVDF membrane, and probed with primary antibody. Primary antibodies included eNOS (no. 610297, 1:500), phosphorylated eNOS (pSer1179, no. 612393, 1:250), caveolin-1 (no. 610407, 1:1000), and protein kinase B (Akt; no. 610861, 1:1000) all from BD Biosciences, Hsp90 (Abcam no. ab13495, 1:1000), and β-actin (Novus Biologicals no. 600-501, 1:10,000).

Solutions and Drugs

Krebs bicarbonate buffer used for isometric tension studies contained (in mM): 131.5 NaCl, 5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 11.2 glucose, 13.5 NaHCO₃ and 0.025 EDTA. Physiological saline solution used for fura-2 studies contained (in mM): 138 NaCl, 5 KCl, 0.1 CaCl₂, 1 MgCl₂, 10 glucose, 20 HEPES, pH 7.4. Drugs were obtained from Sigma Chemical unless otherwise noted. Endothelin-1 was purchased from Peninsula Laboratories and bradykinin from Bachem.

Data Analysis

Maximal relaxation in these vessels was obtained in the presence of zero Ca²⁺ to account for variable levels of basal tone. Relaxation responses to bradykinin and nitroprusside are presented as the percentage decrease in tension at each agonist concentration relative to the agonist (endothelin-1 or KCl)-stimulated preconstriction $[(CT_{zero} - CT) / (DT_{zero})] * 100$, where CT_{zero} is total contractile tension (agonist-stimulated plus basal tone), CT is the steady state contractile tension that remains after each bradykinin concentration, and DT_{zero} is the developed tension produced by the preconstricting agonists (endothelin-1 or KCl).

Developed tension (T) was calculated as the mN of force generated (F) per axial vessel length (g; in mm), where $T = F/2g$ (29). Concentration-response relationships were analyzed using two-way analysis of variance with repeated measures followed by Bonferroni correction for multiple comparisons when a main effect was identified. IC₅₀ values were defined as the concentration of agonist that produced 50% of the maximal relaxation. Body weight, heart-to-body weight ratio and citrate synthase values were analyzed using unpaired t-tests. Dimensional characteristics of coronary arteriolar rings, fura-2 Ca²⁺ responses, and immunoblot results were compared using one-way analysis of variance. When more than one coronary artery from the same myocardial region of a given animal was used in identical protocols, the responses from these rings were averaged before data analyses were conducted. For all analyses, a *P* value ≤ 0.05 was considered significant. Data are presented as mean $\pm$ S.E.M., and *n* values in parentheses reflect the number of animals studied.

RESULTS

Efficacy of the exercise training program

Effectiveness of the 16-week exercise training regimen was demonstrated by significant increases in skeletal muscle oxidative enzyme capacity and heart-to-body weight ratios in exercise trained compared with sedentary animals in both control and ameroid-occluded pig groups. In control animals, citrate synthase activity increased significantly in the deltoid muscle (24.6 ± 1.8 vs. $19.5 \pm 0.8 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) and the long head of the triceps brachii muscle (19.3 ± 2.2 vs. $12.9 \pm 0.9 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) in exercise trained (*n*=19) compared with sedentary (*n*=21) pigs, respectively. Although body weight did not differ between sedentary and exercise trained animals at the time of sacrifice (33.0 ± 1.4 vs. 32.0 ± 1.4 kg, respectively), heart-to-body weight ratio was significantly greater from exercise trained compared with sedentary pigs (5.81 ± 0.16 vs. $4.93 \pm 0.16 \text{ g}\cdot\text{kg}^{-1}$, respectively). Similarly, in ameroid-occluded pigs, citrate synthase activity increased significantly in the deltoid muscle (19.3 ± 1.6 vs. $14.7 \pm 0.7 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) and the long head of the triceps brachii muscle (16.0 ± 1.2 vs. $12.9 \pm 0.7 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) in exercise trained (*n*=14) compared with sedentary (*n*=14) pigs, respectively. Similar to control pigs, body weight did not differ between sedentary (*n*=18) and exercise trained (*n*=16) ameroid-occluded animals at the time of sacrifice (37.8 ± 1.5 vs. 34.3 ± 1.6 kg, respectively), however, heart-to-body weight ratio was significantly greater in exercise trained compared with sedentary pigs (6.1 ± 0.2 vs. $5.0 \pm 0.2 \text{ g}\cdot\text{kg}^{-1}$, respectively). While comparison of the effectiveness of the exercise training regimen between control and occluded pigs may be tempting, these pig have made across pig groups (control vs. occluded) in the current manuscript. We propose that differences in citrate synthase data between these two groups of pigs are not representative of differences in training adaptations but rather the result of differences in tissue handling and inter-assay variability. For comparison across groups (control vs. occluded pigs), we propose that evaluation of the heart-to-body weight may be more appropriate.

Coronary artery dimensions and characteristics

Characteristics of coronary arteries isolated from both control and ameroid-occluded pigs are presented in Table 1. No significant differences in dimensional characteristics or basal tension of rings following stretch to L₉₅ was observed between arteries of sedentary and

exercise trained animals from the control group. Similarly, no significant differences in dimensional characteristics or basal tension was observed between nonoccluded and collateral-dependent arterial rings from either sedentary or exercise trained animals.

Relaxation responses to bradykinin – control animals

We assessed endothelium-dependent relaxation responses to increasing concentrations of bradykinin (10^{-10} to 10^{-7} M) in small coronary arteries from control pigs that had been precontracted with endothelin-1 or KCl. The peak relaxation response at each bradykinin concentration was used to generate concentration-response curves. As shown in Figure 1A, relaxation responses to bradykinin in the presence of the prostanoid inhibitor, indomethacin, were significantly enhanced in exercise trained compared with sedentary animals. Furthermore, as illustrated in Figure 1A *inset*, IC_{50} was attained at a significantly lower bradykinin concentration for arteries from exercise trained compared with sedentary pigs (0.74 ± 0.12 vs. 3.17 ± 1.09 nM, respectively).

Pretreatment of coronary arteries with the NOS inhibitor, L-NAME, in addition to indomethacin, reduced the maximal bradykinin-induced relaxation response in rings from both sedentary and exercise trained pigs (Figure 1B). Importantly, the exercise training-enhanced relaxation to bradykinin was abolished by the addition of L-NAME, suggesting that bradykinin-mediated nitric oxide production is likely increased after exercise training in small coronary arteries of control animals. Furthermore, in the presence of indomethacin and L-NAME, IC_{50} values (Figure 1B *inset*) were not significantly different between exercise trained and sedentary animals (1.10 ± 0.45 vs. 1.22 ± 0.44 nM, respectively). We also compared bradykinin-mediated relaxation following KCl precontraction in the presence of indomethacin and L-NAME (Figure 1C). Findings from these studies revealed that increased extracellular K^+ (35 mM) further attenuated bradykinin-mediated relaxation, providing evidence that an EDHF also contributes to bradykinin-mediated relaxation. Since the bradykinin concentration-response curves were similarly attenuated after addition of extracellular K^+ , our data suggest that an EDHF contributes to the same extent under these conditions in arteries of sedentary and exercise trained pigs. Additionally, in the presence of indomethacin, L-NAME and KCl precontraction, IC_{50} values (Figure 1C *inset*) were not significantly different between exercise trained and sedentary animals (7.88 ± 2.64 vs. 15.22 ± 7.07 nM, respectively). Comparison of the level of precontraction between arteries of sedentary and exercise-trained pigs revealed no significant differences within any of the panels of Figure 1 (panel: A. SED = 2.28 ± 0.23 , EX = 2.37 ± 0.36 ; B. SED = 3.49 ± 0.26 , EX = 3.49 ± 0.39 ; C. SED = 3.85 ± 0.59 , EX = 4.79 ± 1.08). On the contrary, comparison between panels A and B in Figure 1 showed that pretreatment with INDO + L-NAME produced significantly more precontraction than INDO alone in sedentary pigs with a propensity ($P=0.06$) for the same effect in exercise-trained pigs. Comparison of panels B and C revealed no significant differences in the level of precontraction with ET-1 vs. KCl (in the presence of inhibitors) for either sedentary or exercise-trained pigs.

We next examined the time-dependent relaxation response to a single bradykinin concentration (30 nM) in arteries from control pigs. As revealed in Figure 2A, the peak relaxation response to bradykinin was not significantly increased in arteries from exercise

trained compared with sedentary pigs (94.2 ± 3.6 vs. 65.6 ± 9.7 %) when the curves were compared using two-way ANOVA with repeated measures. In contrast, the sustained response to bradykinin was significantly increased after exercise training (Figure 2A). These experiments were also intended to evaluate the time course over which underlying mediators contribute to peak and sustained bradykinin-mediated relaxation. Pretreatment of arterial rings with indomethacin did not reduce the peak or sustained response to bradykinin suggesting that prostanoids do not contribute to bradykinin-mediated relaxation in small coronary arteries of sedentary or exercise trained pigs (Figure 2B). In contrast, pretreatment with L-NAME in addition to indomethacin reduced both the peak and sustained responses to bradykinin in arteries from both sedentary and exercise trained pigs (Figure 2C). Importantly, nitric oxide synthase inhibition also corrected the significant difference between arterial rings of sedentary and exercise trained animals suggesting that nitric oxide mediates the exercise training-enhanced bradykinin-mediated sustained response. Interestingly, a large component of the initial relaxation phase (< 5 min) to bradykinin was insensitive to nitric oxide synthase and prostanoid inhibition (Figure 2C). Comparison of the level of preconstriction between arteries of sedentary and exercise-trained pigs indicated no significant differences within any of the panels of Figure 2 (panel: A. SED = 3.47 ± 0.52 , EX = 3.54 ± 0.34 ; B. SED = 3.93 ± 0.39 , EX = 3.33 ± 0.37 ; C. SED = 5.11 ± 0.86 , EX = 4.47 ± 0.29). Comparison between panels B and C in Figure 2 demonstrated that pretreatment with INDO + L-NAME produced significantly more preconstriction than INDO alone in exercise-trained pigs with a tendency ($P=0.07$) for the same effect between panels A and C. Although the level of preconstriction in arteries of sedentary pigs increased as arteries were treated with more inhibitors, there were no statistically significant differences between panels.

Relaxation responses to bradykinin – ameroid-occluded animals

We also assessed the time-dependent relaxation response to a single bradykinin concentration (30 nM) in arteries from ameroid-occluded pigs. These studies were completed in arteries isolated from both the nonoccluded and collateral-dependent myocardial regions. Continuous exposure to bradykinin, in the presence of indomethacin, produced similar relaxation responses in nonoccluded arteries from both sedentary and exercise trained ameroid-occluded pigs (Figure 3A). Additionally, inhibition of nitric oxide synthase (L-NAME) completely blocked sustained bradykinin-mediated relaxation, but only partially blocked initial relaxation in nonoccluded arteries of both sedentary and exercise trained pigs.

In contrast to arteries isolated from the nonoccluded region, bradykinin-induced relaxation was significantly prolonged in the collateral-dependent arteries of exercise trained compared to sedentary animals in the presence of indomethacin (Figure 3B). Furthermore, inhibition of nitric oxide synthase markedly attenuated the exercise training-enhanced relaxation to bradykinin, so that the exercise training effect was eliminated, suggesting that nitric oxide mediated the exercise training-enhanced bradykinin response (Figure 3B). Initial relaxation to bradykinin in the collateral-dependent arteries was only partially reduced by combined nitric oxide synthase and prostanoid inhibition suggesting another mediator, such as an EDHF, also may have contributed to the initial, peak response to bradykinin in small

coronary arteries. Comparison of the level of precontraction between nonoccluded arteries of sedentary and exercise-trained pigs within panel A of Figure 3 revealed that pretreatment with INDO + L-NAME produced significantly more precontraction than INDO alone in sedentary pigs and INDO + L-NAME in exercise-trained pigs (panel A. SED = 3.19 ± 0.26 , EX = 2.91 ± 0.40 ; SED + L-NAME = 5.00 ± 0.32 ; EX + L-NAME = 3.67 ± 0.22). In panel B of Figure 3, pretreatment with INDO + L-NAME produced significantly more precontraction in sedentary than exercise-trained pigs (panel B. SED = 4.89 ± 0.72 , EX = 5.78 ± 1.20 ; SED + L-NAME = 6.10 ± 0.64 , EX + L-NAME = 4.47 ± 0.42).

Relaxation responses to nitroprusside

To examine the effects of exercise training on coronary smooth muscle responsiveness to nitric oxide in both control and ameroid-occluded pigs, we evaluated the response of arterial rings to the endothelium-independent nitric oxide donor, nitroprusside. Concentration-dependent relaxation responses to nitroprusside were not significantly different in coronary arteries from sedentary and exercise trained pigs of the control group (Figure 4A). Similarly, nitroprusside-mediated relaxation was not different in arteries isolated from control and collateral-dependent regions of sedentary or exercise trained pigs of the ameroid-occluded group (Figure 4B).

Bradykinin-stimulated intracellular Ca^{2+} levels in arterial endothelial cells

We evaluated cytosolic free Ca^{2+} levels (fura-2) under sustained bradykinin-stimulated conditions in freshly isolated endothelial cells from small arteries of both the collateral-dependent and nonoccluded myocardial regions of sedentary and exercise trained pigs. Figure 5A illustrates our experimental protocol, as well as demonstrating a representative endothelial cell Ca^{2+} response to prolonged bradykinin exposure. The peak cytosolic Ca^{2+} response to bradykinin stimulation was calculated by subtraction of basal Ca^{2+} levels from the peak Ca^{2+} response to bradykinin to generate the peak fura-2 ratio (Figure 5B). Chronic occlusion and/or exercise training did not significantly alter the peak Ca^{2+} response to bradykinin in endothelial cells isolated from both control and collateral-dependent arteries (Figure 5B). Based on our hypothesis that bradykinin-stimulated Ca^{2+} response would be more persistent in cells from the collateral-dependent artery of exercise trained pigs, we also assessed the plateau in the Ca^{2+} response at the end of the 20-min bradykinin exposure in the same cells. The plateau Ca^{2+} response was calculated by subtraction of the basal Ca^{2+} levels from the average of the final 30 sec of the 20-min bradykinin exposure. The plateau Ca^{2+} response was significantly increased in endothelial cells from collateral-dependent arteries of exercise trained compared with sedentary pigs (Figure 5C). Neither occlusion nor exercise training alone significantly altered the plateau in the Ca^{2+} response.

Protein levels of eNOS and associated regulatory molecules

Determination of protein levels by immunoblot revealed that chronic occlusion significantly increased eNOS and phosphorylated eNOS (pSer1179) protein content in collateral-dependent arteries of both sedentary and exercise trained pigs (Figure 6). Protein content of eNOS or phosphorylated eNOS was not altered significantly by exercise training alone in arteries of the nonoccluded regions. We further examined proteins that interact with and

regulate eNOS signaling to determine if these were altered by occlusion and/or exercise training. Protein levels for caveolin-1, Akt, and Hsp90 were not altered by occlusion or exercise training (Figure 6).

DISCUSSION

Few studies have examined the sustained dilation response of vasculature to agonist-stimulation and the mechanisms that underlie both initial and persistent vasodilation (35). Furthermore, evaluation of the effects of exercise training or chronic occlusion on vasoreactivity of arteries of this size (~100–350 μm) is very limited (19, 31, 32, 36). Thus, several novel and important findings have emerged from these studies. Our results indicate that exercise training resulted in more persistent bradykinin-mediated relaxation in small coronary arteries of control animals, as well as arteries from the collateral-dependent myocardial region of ameroid-occluded animals compared with sedentary pigs. Additional studies revealed that in collateral-dependent arteries, the prolonged bradykinin-mediated relaxation was associated with a more sustained increase in endothelial intracellular Ca^{2+} (fura-2) after exercise training. Interestingly, exercise training did not alter relaxation responses to sustained bradykinin exposure in arteries isolated from the nonoccluded myocardial region in animals that underwent ameroid placement. While inhibition of cyclooxygenase had negligible effect, nitric oxide synthase inhibition reversed the exercise-enhanced bradykinin-mediated relaxation in both the concentration-response curves (Figure 1B) and during sustained bradykinin exposure (Figure 2C) in arteries from control pigs. Similarly, combined inhibition of nitric oxide synthase and cyclooxygenase in collateral-dependent arteries reversed the exercise training-enhanced relaxation response to bradykinin that was observed in the presence of cyclooxygenase inhibition alone (Figure 3). Relaxation responses to the nitric oxide donor, nitroprusside, were not altered by exercise training in coronary arteries of control or ameroid-occluded pigs. Chronic occlusion also did not alter responsiveness to nitroprusside. Furthermore, eNOS protein levels were increased by chronic occlusion in both sedentary and exercise trained pigs, whereas, exercise training alone did not significantly alter eNOS protein levels.

Previous studies have revealed that exercise training enhances endothelium-dependent relaxation in large coronary arteries (>500 μm) (14, 17, 18, 38–40, 43) and small coronary arterioles (~100 μm) (13, 15, 28) isolated from both control and diseased hearts. Many of these studies also provide evidence that the enhanced endothelium-mediated relaxation is attributable to increased nitric oxide bioavailability. However, these studies have focused primarily upon rapid responses to step increases in agonist concentrations. We postulated that during sustained endothelium-dependent relaxation, time-dependent underlying roles of vasodilator mediators would differentially contribute to overall endothelium-dependent responses. Thus, we evaluated vascular responses to sustained agonist exposure to mimic persistent vasodilation of the coronary circulation throughout an exercise training bout. Importantly, the current study is the first to report a time course response to an endothelium-dependent vasodilator (bradykinin) in coronary arteries from either control or diseased animals.

During exercise, the ability of small coronary arteries and arterioles to dilate in response to increased myocardial oxygen demand is revealed by the markedly close relationship between myocardial oxygen consumption and coronary blood flow. This increase in blood flow during prolonged exercise is essential for preserving an adequate supply of oxygen to the myocardium. While numerous mediators are thought to contribute to the coronary vasodilation associated with increased cardiac work, specific mechanisms have not been clearly established. Interestingly, there is also evidence that mechanisms underlying exercise-induced coronary vasodilation in diseased hearts may differ from that observed in control hearts (7). The diminishing responses of both bradykinin-induced relaxation (Figure 3) and associated fura-2 responses (Figure 5) suggest that bradykinin does not mimic the elusive endogenous mediator or more than one mediator may be necessary for the persistent increase in coronary blood flow throughout exercise. Indeed, previous reports (8, 9) have revealed variable relaxation and fura-2 responses to sustained (~4 min) exposure to various mediators (acetylcholine, adenosine, flow). Finally, it is noteworthy that studies in the human coronary circulation support a role for endogenous bradykinin release based on findings that antagonism of the bradykinin B₂ receptor led to a significant increase in coronary vascular resistance and decrease in coronary blood flow under both basal and flow-stimulated conditions (16). However, the specific contribution of bradykinin to coronary vasodilation during exercise has not been examined.

Bradykinin-induced relaxation in control pigs was characterized by an immediate dilation followed by a secondary sustained relaxation. Pretreatment with the cyclooxygenase inhibitor, indomethacin, did not significantly alter the response to bradykinin in sedentary or exercise trained animals suggesting minimal role for prostanoids as mediators of bradykinin-induced relaxation in the small coronary arteries examined in these studies. These findings are supported by previous studies which have reported no effect of cyclooxygenase inhibition on blood flow into the collateral-dependent region under resting conditions in conscious dogs, although the effect was significant during exercise (1). Previous studies have also demonstrated negligible contribution of prostanoids to bradykinin-mediated vasodilation in coronary arterioles from control pigs (28). Furthermore, sustained bradykinin-mediated relaxation remained significantly increased in exercise trained compared with sedentary pigs in the presence of indomethacin implicating an alternative mediator in the training-enhanced bradykinin relaxation. Indeed, we found that nitric oxide synthase inhibition, in the presence of cyclooxygenase blockade (indomethacin), markedly inhibited the sustained relaxation response to bradykinin, but only minimally inhibited the maximal (peak) response. Importantly, the difference in relaxation responses in arteries isolated from sedentary and exercise trained animals was abolished by nitric oxide synthase inhibition. The residual peak and sustained responses to bradykinin in the presence of cyclooxygenase and nitric oxide synthase inhibitors indicates a potential role for another mediator, such as endothelium-derived hyperpolarizing factor. Furthermore, these data also implicate differential time-dependent roles of endothelial-derived vasoactive mediators (nitric oxide, prostanoids, and hyperpolarizing factor) in mediating the initial and sustained phases of relaxation to bradykinin in both sedentary and exercise trained animals. Our cursory evaluation of bradykinin-mediated relaxation in the presence of KCl-induced precontraction in Figure 1 suggests that while EDHF appears to contribute to relaxation, the

role of EDHF may not be augmented by exercise training in this artery size, although future studies are warranted to systematically explore a potential role for EDHF in exercise training-induced adaptations.

In the ameroid-occluded swine, arteries from the nonoccluded myocardial region from sedentary and exercise trained pigs responded similarly to protracted exposure to bradykinin. We have previously observed negligible effects of exercise training in arteries isolated from the nonoccluded region of ameroid-occluded hearts (12, 15, 19, 21), which suggests that chronic occlusion may produce global coronary adaptations that differ from that observed in control hearts. In contrast, the collateral-dependent arteries of exercise trained swine displayed significantly prolonged bradykinin-mediated relaxation compared with collateral-dependent arteries from sedentary animals. It is interesting that nitric oxide synthase inhibition essentially abolished the protracted response in the nonoccluded arteries of sedentary and exercise trained animals, similar to that observed in the arteries of sedentary control pigs. These data suggest that the sustained response in the nonoccluded arteries and the arteries of control sedentary animals is nearly completely dependent on nitric oxide bioavailability. In contrast, the bradykinin-mediated relaxation response was more persistent in the collateral-dependent compared with nonoccluded arteries in the presence of nitric oxide synthase inhibition, suggesting the potential for an alternative mediator of endothelium-dependent relaxation after chronic coronary occlusion.

Our finding that the plateau in the endothelial cell Ca^{2+} response at the end of the 20-min bradykinin exposure was significantly increased in collateral-dependent arteries of exercise trained pigs suggests that the sustained intracellular Ca^{2+} levels may be a primary mediator for the prolonged bradykinin-mediated relaxation in these arteries. Indeed, intracellular Ca^{2+} levels are a critical determinant of agonist-mediated eNOS activation since the binding of Ca^{2+} -dependent calmodulin is required for the enzyme to achieve maximal catalytic activity and nitric oxide production (33). Interestingly, eNOS and p-eNOS (S1179) protein levels were significantly enhanced in small coronary arteries from the collateral-dependent region compared with the nonoccluded region, independent of exercise status. These findings are consistent with our previous reports that eNOS and p-eNOS protein levels were enhanced in both epicardial artery segments and coronary arterioles by chronic occlusion, but not by exercise training (44, 45). These data reveal that increased eNOS protein alone was not sufficient to produce prolonged bradykinin-mediated relaxation in the collateral-dependent arteries of sedentary pigs. Taken together, these data suggest that the enhanced bradykinin-mediated increase in endothelial cell Ca^{2+} levels likely contributes to greater eNOS activity and prolonged nitric oxide-dependent bradykinin-induced relaxation in collateral-dependent coronary arteries from exercise trained pigs, whereas increased eNOS protein levels alone does not appear sufficient in the collateral-dependent arteries of sedentary pigs. On the other hand, there may be an issue of limited nitric oxide availability in the collateral-dependent arteries of sedentary pigs, despite the increased eNOS protein levels. It is noteworthy that exercise training of control animals typically has been linked with increased eNOS mRNA and protein levels in coronary arteries and arterioles (24, 37, 41), yet our data do not reveal an increase in eNOS protein levels in arteries of the nonoccluded myocardial region. These data suggest that the global response to exercise training may differ in ischemic compared with control hearts. We speculate that exercise training-induced adaptations in eNOS protein

levels in the collateral-dependent region that are absent in the nonoccluded region may function as a protective mechanism to increase blood flow into the compromised myocardium without the diversion of blood into the nonoccluded region, thus potentially reducing the debilitating effects of coronary steal responses downstream of occlusion.

We have previously reported that treatment of small coronary arteries with the nitric oxide synthase inhibitor, L-NAME, increased resting tension significantly more in the collateral-dependent arteries of exercise trained animals than arteries from the nonoccluded region of sedentary or exercise trained pigs and arteries of the collateral-dependent region of sedentary animals (19). These studies implicate an increased basal coronary tone in the collateral-dependent myocardial region of trained animals, which is inhibited by enhanced endogenous levels of nitric oxide, and revealed by NOS inhibition. Taken together with our current findings, these data suggest that nitric oxide production is significantly increased in the collateral-dependent arteries of exercise trained animals under both basal and agonist-stimulated conditions.

Despite reversal of the protracted bradykinin-mediated relaxation in the collateral-dependent arteries of exercise trained pigs with combined inhibition of cyclooxygenase and nitric oxide synthase, a tendency for more sustained relaxation persisted in the collateral-dependent arteries of exercise trained compared with sedentary animals. These data suggest that endothelium-derived hyperpolarizing factor may also be enhanced in the collateral-dependent arteries of exercise trained pigs. Previous studies have revealed enhanced bradykinin-mediated relaxation in arteries of exercise trained animals that is only partially corrected by nitric oxide synthase inhibition (14) and appears dependent on endothelium-derived hyperpolarizing factor. Indeed, increases in shear stress and distention, such as during exercise are reported to increase nitric oxide production (10, 42), as well as increase release of endothelium-derived hyperpolarizing factor (2, 11, 25–27). Thus, the role of various hyperpolarizing factors is an important goal for future studies.

Our finding that the relaxation response to the nitric oxide donor, nitroprusside, was similar in arteries of all treatment groups indicates that the smooth muscle responsiveness to nitric oxide and the downstream protein kinase G-dependent pathway are not altered by occlusion or exercise training in arterioles. These findings are in agreement with previous findings in small arterioles (15, 28) and large arteries (14, 30) of both normal and diseased hearts (15). Thus, these data provide evidence that the exercise training-enhanced bradykinin-mediated relaxation in arteries from control animals, as well as in the collateral-dependent region of ameroid occluded animals, may be attributed to enhanced nitric oxide bioavailability rather than increased responsiveness of the vascular smooth muscle of these arterioles to nitric oxide.

Limitations

Our examination of the role of prostanoids was limited to experiments of sustained bradykinin-mediated relaxation in arteries of control (nonoccluded) pigs (Figure 2). In these studies, pretreatment with indomethacin did not significantly alter the time-dependent relaxation response to a single bradykinin concentration in sedentary or exercise-trained animals. Furthermore, our previous studies in coronary arteries from chronically occluded

pigs have revealed that treatment with the prostanoid inhibitor, indomethacin, did not significantly alter bradykinin-mediated relaxation responses compared with control (no inhibitor) conditions in any of the artery treatment groups (i.e., nonoccluded or collateral-dependent of sedentary or exercise-trained pigs) (5). We also reported recently that prostanoid inhibition had no effect on bradykinin-mediated relaxation until combined with nitric oxide synthase inhibition (5). These data suggested compensation for nitric oxide inhibition by prostanoids and thus, we included indomethacin in all experiments to control for this compensatory mechanism in arterial relaxation. Based on these findings and limitations in the number of chronically occluded pigs, we opted to forego further experiments using indomethacin alone.

CONCLUSIONS

Our results indicate that chronic exercise training preferentially produces prolonged endothelium-dependent relaxation in small coronary arteries from control animals, as well as collateral-dependent arteries from ameroid-occluded animals. Nitric oxide plays a major role in the exercise-enhanced sustained bradykinin-mediated relaxation, whereas a nitric oxide- and prostanoid-independent mediator also appears to contribute to both the peak response to bradykinin in all arteries studied and to a persistent sustained component in the arteries from exercise trained control pigs and the collateral-dependent arteries of exercise trained ameroid occluded pigs. Furthermore, sustained responses in bradykinin-stimulated endothelial Ca^{2+} levels and Ca^{2+} -dependent activation of eNOS may provide the underlying mechanism by which exercise training-enhanced, nitric oxide-dependent bradykinin-mediated relaxation persists over time. Our combined findings implicate the possibility that effects of training-enhanced nitric oxide production during both basal and agonist-stimulated conditions *in vivo* may contribute to sustained endothelium-dependent vasodilation and optimization of blood flow to the myocardium-at-risk in the diseased heart.

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Abbreviations

Akt	protein kinase B
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
EX	exercise-trained
Hsp90	heat shock protein of 90 kDa
INDO	indomethacin

LAD	left anterior descending coronary artery, nonoccluded
LCX	left circumflex coronary artery, collateral-dependent
L-NAME	N ^ω -nitro- <i>L</i> -arginine methyl ester
NOS	nitric oxide synthase
PSS	physiological saline solution
p-eNOS	phosphorylated eNOS
SED	sedentary

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PERSPECTIVES

Our data reveal that collateral-dependent arteries of exercise trained pigs demonstrate persistent bradykinin-mediated relaxation, whereas arteries from the nonoccluded region of exercise trained animals do not display increased relaxation compared with arteries from sedentary pigs. The reactivity of small coronary arteries likely assumes an enhanced role in the regulation of blood flow into the compromised region of an occluded/stenosed heart, where small arterioles have undergone ischemia-induced vasodilation (6). Increased agonist-mediated relaxation in the collateral-dependent region that is absent in the nonoccluded region after exercise training would increase blood flow into the compromised myocardium without the diversion of blood into the nonoccluded region. These diverse regional responses would potentially reduce the debilitating effects of coronary steal responses downstream to coronary occlusion/stenosis in human patients.

Figure 1. Relaxation responses of small coronary arteries from control pigs to increasing concentrations bradykinin (10⁻¹⁰ to 10⁻⁷ M)

A. Relaxation to bradykinin in the presence of endothelin-1 (ET-1) preconstriction plus indomethacin (5 μ M) pretreatment was significantly enhanced in arteries of exercise trained (EX) compared with sedentary (SED) animals. The concentration of bradykinin required to yield 50% maximum relaxation (IC₅₀; *inset*) was significantly lower in arteries from EX compared with SED pigs. B. In the presence of ET-1 preconstriction plus indomethacin and L-NAME (100 μ M) pretreatment, relaxation to bradykinin was not significantly different between arteries from SED and EX animals. IC₅₀ values (*inset*) also were not different in the presence of indomethacin plus L-NAME. C. In the presence of KCl (35 mM) preconstriction plus indomethacin and L-NAME pretreatment, relaxation to bradykinin was not significantly different between arteries from SED and EX pigs. IC₅₀ values (*inset*) also were not different under these conditions. Values are means $\pm$ S.E.M. of the number of animals in parentheses; *P < 0.05 EX vs. SED.

Figure 2. Time-dependent relaxation responses of small coronary arteries from control pigs in response to a single concentration of bradykinin (30 nM)

A. In the absence of inhibitors (no pretreatment), the sustained relaxation response to bradykinin was enhanced in exercise trained (EX) compared with sedentary (SED) animals. B. In the presence of indomethacin (5 μ M), the enhanced peak and sustained relaxation responses to bradykinin persisted in arteries from EX compared with SED pigs. C. In the presence of indomethacin plus L-NAME (100 μ M), relaxation responses to bradykinin were not significantly different between arteries from SED and EX animals. All arteries were precontracted with ET-1. *P = 0.05 EX vs. SED.

Figure 3. Time-dependent relaxation responses of small coronary arteries from pigs with ameroid-occluded hearts in response to a single concentration of bradykinin (30 nM)

A. In arteries isolated from the nonoccluded myocardial region of occluded hearts, the bradykinin-mediated relaxation responses are not different between sedentary (SED) and exercise trained (EX) animals in the presence of indomethacin (5 μ M). In the presence of indomethacin plus L-NAME (100 μ M), both the peak and sustained relaxation responses to bradykinin were markedly reduced in arteries from both SED and EX pigs compared with indomethacin alone. Relaxation in the presence of indomethacin and L-NAME was not significantly different between arteries from SED and EX animals. B. In arteries isolated from the collateral-dependent region of occluded hearts, the sustained portion of bradykinin-mediated relaxation was significantly increased in arteries from EX compared with SED pigs in the presence of indomethacin. This difference was corrected in the presence of indomethacin plus L-NAME (100 μ M). * $P < 0.05$ indo vs. indo + L-NAME for both SED and EX. † $P < 0.05$ EX indo vs. SED indo.

Figure 4. Relaxation responses of small coronary arteries to increasing concentrations of nitroprusside (10^{-10} to 10^{-4} mol/L)

A. In arteries from control pigs, nitroprusside-mediated relaxation was not different between sedentary (SED) and exercise trained (EX) pigs. B. Nitroprusside-induced relaxation was not different between arteries isolated from the nonoccluded and collateral-dependent myocardial regions of SED and EX ameroid-occluded pigs. N values in parentheses represent the number of animals studied.

Figure 5. Intracellular Ca^{2+} response to bradykinin stimulation in endothelial cells isolated from small coronary arteries

A. Representative trace showing intracellular free Ca^{2+} (fura-2) response during prolonged exposure to bradykinin. Endothelial cells were superfused with PSS for 3 min, followed by 20-min exposure to bradykinin (30 nM). B. Bradykinin-stimulated peak endothelial Ca^{2+} response was not altered by occlusion or exercise training. C. The plateau Ca^{2+} response (average of last 30 sec of 20-min bradykinin exposure) was significantly increased in endothelial cells from collateral-dependent arteries of exercise trained compared with sedentary pigs. Numbers in parentheses are animals, cells studied. Values are means $\pm$ S.E.M. of the number of animals in parentheses. *P < 0.05 vs. SED nonoccluded and SED collateral-dependent.

Figure 6. Effect of chronic occlusion and exercise training on eNOS protein levels and associated regulatory proteins in small coronary arteries

Protein levels for eNOS (A), caveolin-1 (B), Hsp90 (C), and Akt (D) were quantified by densitometry analysis, normalized to β -actin, and expressed relative to density of control arteries of sedentary pigs. SED, sedentary; EX, exercise trained. Membrane protein bands presented are: SED nonoccluded (lanes 1, 5, 9, 13), SED collateral-dependent (lanes 2, 6, 10, 14), EX nonoccluded (lanes 3, 7, 11, 15), and EX collateral-dependent (lanes 4, 8, 12, 16) for all panels. Values are means $\pm$ S.E.M. Number of animals studied is provided in parentheses. * $P < 0.05$ vs. SED nonoccluded.

Table 1

Dimensional characteristics of coronary arterial rings

	Outer Diameter (μm)	Inner Diameter (μm)	Wall Thickness (μm)	Axial Length (mm)	RT L ₉₅ (mN/mm)
Control pigs					
Sedentary (n=38)	279 ± 12	185 ± 10	44 ± 2	1.54 ± 0.02	1.13 ± 0.08
Exercise (n=34)	288 ± 15	187 ± 11	48 ± 2	1.57 ± 0.02	1.12 ± 0.10
Ameroid-occluded pigs					
Sedentary (n=22)					
Control	255 ± 11	159 ± 8	46 ± 2	1.56 ± 0.01	1.00 ± 0.07
Collateral-dependent	265 ± 15	171 ± 12	47 ± 2	1.56 ± 0.01	1.12 ± 0.10
Exercise (n=23)					
Control	236 ± 11	139 ± 9	48 ± 1	1.56 ± 0.01	0.95 ± 0.08
Collateral-dependent	251 ± 10	158 ± 9	46 ± 1	1.57 ± 0.01	1.09 ± 0.12

Values are mean ± SE. RT L₉₅, arterial resting tension (RT) at L₉₅, as described in text.
Numbers in parentheses indicate number of arteries studied. No significant differences exist.