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Adaptations of the endothelin system after exercise training in a porcine model of ischemic heart disease

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Abstract

Objective—Test the hypothesis that exercise training would increase endothelin-mediated vasoconstriction in collateral-dependent arteries via enhanced contribution of ET_A.

Methods—An ameroid constrictor was surgically placed around the proximal LCX artery to induce gradual occlusion in Yucatan miniature swine. Eight-weeks postoperatively, pigs were randomized into sedentary or exercise-training (treadmill; 5 days/wk; 14 wks) groups. Subsequently, arteries (~150 μm diameter) were isolated from collateral-dependent and nonoccluded myocardial regions and studied.

Results—Following exercise training, ET-1-mediated contraction was significantly enhanced in collateral-dependent arteries. Exercise training induced a disproportionate increase in the ET_A contribution to the ET-1 contractile response in collateral-dependent arteries, with negligible contributions by ET_B. In collateral-dependent arteries of sedentary pigs, inhibition of ET_A or ET_B did not significantly alter ET-1 contractile responses in collateral-dependent arteries, suggesting compensation by the functionally active receptor. These adaptations occurred without significant changes in ET_A, ET_B, or ECE mRNA levels but with significant exercise training-induced elevations in endothelin levels in both nonoccluded and collateral-dependent myocardial regions

Conclusions—Taken together, these data reveal differential adaptive responses in collateral-dependent arteries based upon physical activity level. ET_A and ET_B appear to compensate for one another to maintain contraction in sedentary pigs, whereas exercise-training favors enhanced contribution of ET_A.

Keywords

coronary artery disease; near-resistance arteries; coronary artery occlusion; stenosis

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Disclosures

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Introduction

ET-1 is cleaved from its precursor, big endothelin, by ECE in vascular endothelial cells and binds to ET_A and ET_B to elicit its vasoactive effects. Functionally, ET-1 binds with its receptors in vascular smooth muscle and induces vasoconstriction. ET_A is found mainly on vascular smooth muscle and is predominantly responsible for ET-1-mediated constriction in pig coronary arteries (2, 38). ET_B is found not only in vascular smooth muscle, where it induces ET-1-mediated constriction but also on the endothelium where they contribute to vasodilation by promoting endothelial release of nitric oxide and prostacyclin (7, 26, 49), as well as clearance of ET-1 via internalization (3). Several studies have shown increased ET-1 levels in the presence of cardiovascular disease states (14, 27, 30) as well as alterations in expression and function of endothelin receptor subtypes (9, 20, 30, 54). Others have reported that exercise training can induce a reduction in plasma ET-1 levels in patients with vascular disease (4, 34, 41). Interestingly, Duncker, Merkus, and colleagues have demonstrated that both systemic and coronary ET-1-induced vasoconstriction in pigs is mediated primarily through ET_A and that this response is significantly blunted during acute exercise, particularly in the coronary circulation (5, 36, 38, 39). These findings suggest that the vasoconstrictor effect of ET-1 wanes during acute exercise. However, the adaptations of an extended exercise-training regimen in progressive coronary occlusion to the endothelin system have received limited evaluation.

Given that the number one recommendation for patients with ischemic coronary disease is therapeutic lifestyle changes that include an exercise-training regimen, we sought to determine adaptations in the endothelin system of the coronary vasculature in response to a 14-week exercise-training regimen in a swine model of chronic coronary occlusion. Our laboratory has recently reported exercise training-enhanced ET-1 vasoconstriction in collateral-dependent small coronary arteries mediated by increased Ca²⁺ sensitization and up-regulation of the PKC signaling pathway (46). To further explore potential underlying mechanisms, we tested the hypothesis that an enhanced role for ET_A would contribute to increased endothelin-mediated vasoconstriction in collateral-dependent arteries after a persistent exercise-training regimen.

Materials and Methods

Experimental animals and surgical instrumentation

All animal protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee and conformed to the NIH Guide for Care and Use of Laboratory Animals (NIH publication 85-23, revised 2010). As illustrated in Figure 1, the experimental protocol was initiated when adult female Yucatan miniature swine (6–7 mo of age) were surgically instrumented with an ameroid constrictor around the proximal LCX artery as described previously (21, 22, 24, 25, 46). Animals were pre-anesthetized with glycopyrrolate (0.004 mg·kg⁻¹, i.m.), midazolam (0.5 mg·kg⁻¹, i.m.) and ketamine (20 mg·kg⁻¹, i.m.). Anesthesia was induced with 3% isoflurane. Animals were intubated and anesthesia was maintained with 2–3% isoflurane and supplemental O₂ during aseptic surgery. During surgery, animals received the following drugs as necessary: pancuronium (0.1 mg·kg⁻¹; neuromuscular blocker) or vecuronium bromide (0.1 mg·kg⁻¹; neuromuscular

blocker) and lidocaine ($1 \text{ mg}\cdot\text{kg}^{-1}$, i.v.; anti-arrhythmic). Immediately following surgery, pigs received ketofen ($3.0 \text{ mg}\cdot\text{kg}^{-1}$, i.v.; NSAID). Prior to surgery and during surgical recovery, animals received either buprenorphine hydrochloride ($0.1 \text{ mg}\cdot\text{kg}^{-1}$, i.v.; analgesic) or butorphanol tartrate ($0.5 \text{ mg}\cdot\text{kg}^{-1}$, i.v.; analgesic) every 3–6 hr, as needed for pain relief. Antibiotics (ceftiofur $4 \text{ mg}\cdot\text{kg}^{-1}$, i.m.) were administered 24 hours before surgery, immediately prior to surgery and for two days following surgery. As indicated in Figure 1, previous studies have demonstrated that gradual narrowing to complete occlusion of the left circumflex coronary artery in this model is achieved generally at about three weeks after surgical placement of the ameroid ring (47). Previous reports have also revealed that collateral development in this model levels off by 7–8 weeks postoperatively (47, 57). For this reason, we allow the animals eight weeks of surgical recovery prior to initiation of the sedentary or exercise training regimen (Figure 1). This recovery period allows us to distinguish collateral development as a result of occlusion alone from that of exercise training in the presence of occlusion.

Sedentary and exercise protocols

Following surgical recovery, pigs were randomly assigned to either a sedentary or exercise-training group. Exercise-trained ($n=30$) pigs underwent a progressive treadmill program (5 days/wk for 14 wks) as described previously (11, 46). Speed and duration of the exercise training sessions were progressively increased so that during the last week 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. Sedentary ($n=31$) pigs were confined to their pens. Effectiveness of the exercise-training program was determined by comparing heart-to-body weight ratio and skeletal muscle citrate synthase activity as previously described (17, 25).

Preparation of coronary arteries

Following completion of the 14-wk exercise-training protocol or sedentary confinement, pigs were anesthetized using xylazine ($2.25 \text{ mg}\cdot\text{kg}^{-1}$, i.m.), ketamine ($35 \text{ mg}\cdot\text{kg}^{-1}$, i.m.) and pentothal sodium ($30 \text{ mg}\cdot\text{kg}^{-1}$, i.v.), followed by administration of heparin ($1000 \text{ U}\cdot\text{kg}^{-1}$, i.v.). Pigs were intubated and ventilated with room air and a left lateral thoracotomy was performed in the fourth intercostal space. Hearts were removed, placed in Krebs bicarbonate buffer ($0\text{--}4^\circ\text{C}$) and weighed. Visual inspection of the ameroid occluder during dissection of the LCX artery indicated 100% occlusion in all pigs used for this study. Size-matched arteries ($\sim 150 \mu\text{m}$ luminal diameter) were isolated from myocardial regions distal to both the collateral-dependent LCX (distal to occlusion) and the nonoccluded LAD arteries as previously described (21, 46). Isolated arteries were used for either same day microvascular function studies or flash frozen with liquid nitrogen and stored at -80°C until processed for mRNA or protein quantification.

Microvascular function studies

Arterial rings were studied using specialized isometric microvessel myographs (Danish Myograph Technology) as previously described (21, 46). Briefly, following establishment of

optimal vessel length and equilibration, arterial rings were pretreated with endothelin receptor subtype antagonists for 15 min. Subsequently, an ET-1 concentration-response curve was performed by cumulative additions of ET-1 to the tissue bath to achieve the desired concentrations. Control ET-1 curves were also generated in the absence of endothelin receptor antagonists. The contractile response was measured at steady state after addition of each concentration of ET-1. Developed tension (T) was calculated as the millinewton (mN) of force generated (F) per axial vessel length (g; in mm), where $T = F/2g$ (40). The selective inhibitors, BQ123 (ET_A inhibitor; 1 μ M), BQ788 (ET_B inhibitor; 1 μ M) or both, were used to assess the contribution of these receptors to the ET-1-mediated tension development. To avoid residual effects of the pharmacologic agents used in these studies only one curve was completed in each arterial segment.

Endogenous endothelin tissue measurements

Myocardial tissue (~200 mg) from collateral-dependent and nonoccluded regions was collected from both sedentary and exercise treatment groups, flash frozen, and stored at -80°C until processed as described previously (14) with modifications. Samples were homogenized in 10X volumes of 0.1 M acetic acid, sonicated, and boiled. The homogenates were centrifuged at 12 g for 10 min at 4°C . Supernatant was analyzed for endothelin content through a commercially available immunometric assay (Endothelin EIA kit; Cayman Chemical) as per manufacturer's instructions.

Real-time PCR quantification of ET_A, ET_B, and ECE mRNA

Total RNA was extracted from small coronary arteries (~150 μ m luminal diameter; ~5–10 mm total length) isolated from nonoccluded and collateral-dependent myocardial regions of sedentary and exercise-trained groups using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total isolated RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad), in a final volume of 20 μ L. Transcription reactions without reverse transcriptase were performed to verify that DNA contamination was below the level of detection. Real-time quantitative PCR was performed using CFX96 Touch Thermal Cycler (Bio-Rad). Primers for swine ET_A, ET_B, and ECE were selected from a previous report (14). Quantitative real-time RT-PCR was performed using iQ SYBR Green Supermix (5 μ L; Bio-Rad), sense and anti-sense primers (5 pmol each), water (2 μ L), cDNA (2 μ L) in 10 μ L of total reaction volume. The real-time PCR protocol that was employed included initial denaturation for 5 min at 95°C , 40 cycles of 95°C for 10 s and 60°C for 20 s, followed by a melting step with a slow heating from 55 to 95°C with a rate of 0.05°C/s . All samples per investigated gene were detected in one run to eliminate inter-assay variance and each sample was amplified in triplicate for each gene. Real-time efficiency for each primer set was acquired by amplification of a standardized cDNA dilution series. The specificity of the amplified PCR products was verified by analysis of the melting curve, which is sequence-specific and by agarose gel electrophoresis to confirm detection of a single product. GAPDH was used to normalize the amount of mRNA. The relative amount of the target genes isolated from each myocardial region was calculated using the $2^{-\text{CT}}$ method and expressed relative to the amount from the nonoccluded myocardial region of sedentary pigs.

Immunoblots

Additional coronary arteries (~150 μ m; 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. Arteries were homogenized in 40 μ l of 2X lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 3 mM EGTA, 0.1% Triton-X100, 1% protease and 1% phosphatase inhibitor cocktails by freeze-thaw cycles and vortexed ~6–8 times. Protein concentration was determined by BCA protein assay kit (Thermo Scientific Pierce). Arterial lysate (10 μ g of total protein) was subjected to 10 % SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed overnight with primary antibody. Primary antibody dilutions were as follows: ET_A (1:250), ET_B (1:200), and GAPDH (1:5,000) at 4°C overnight. After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated species-specific anti-IgG (1:50,000–1:100,000 depending on primary antibody) for two hours at 25°C. Peroxidase activity was detected using SuperSignal West Dura Substrate. Scanning densitometry was used to quantify signal density from luminograms. Normalization for potential loading differences was accomplished using the ratio of densitometry signals for the receptor protein to GAPDH.

Solutions and drugs

Krebs bicarbonate buffer 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. All drugs were obtained from Sigma Chemical unless otherwise noted. Endothelin-1 was purchased from Bachem. BQ123 was purchased from EMD Millipore and BQ788 was purchased from American Peptide Company. Primary antibodies directed against the following proteins were utilized for these studies: ET_A (sc-33536) and ET_B (sc-33537; sc-21199; sc-21196) receptors from Santa Cruz Biotechnology, ET_B (ab50658) from Abcam, and GAPDH (RGM2-200) from Advanced Immunochemical.

Data analysis

Endothelin levels in myocardial samples and real time PCR and immunoblot data were analyzed using two-way ANOVA. Concentration-response relationships were analyzed using repeated-measures two-way ANOVA. When a significant main effect was identified by ANOVA, mean differences were ascertained using Bonferroni correction for multiple comparisons. Apparent ET-1 EC₅₀ was calculated based on previously reported mathematical calculations (1). 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 analyses were conducted. Body weight, heart-to-body weight ratio, citrate synthase values, dimensional characteristics of coronary arterial rings, and EC₅₀ values were analyzed using Student's *t*-test. For all analyses, a *P* value of 0.05 was considered significant. Data are presented as means $\pm$ SEM, and *n* values reflect the number of pigs studied.

Results

Efficacy of the exercise-training program

The effectiveness of the 14-wk exercise-training program was demonstrated by a significant increase in the heart-to-body weight ratio and increased skeletal muscle oxidative enzyme activity in exercise-trained compared with sedentary animals (Table 1). Although body weight did not differ between sedentary and exercise-trained animals at the time of death, the heart-to-body weight ratio was significantly greater in exercise-trained compared with sedentary pigs. Citrate synthase activity increased significantly in the deltoid muscle and the medial and long heads of the triceps brachii muscle in exercise-trained compared with sedentary pigs.

Coronary artery dimensions and characteristics

No significant differences in dimensional characteristics were observed between arterial rings from the nonoccluded or collateral-dependent regions of either sedentary or exercise-trained animals (Table 2).

ET-1 concentration-response curves

We examined concentration-dependent contractile responses to ET-1 in small coronary arterial rings isolated from the nonoccluded and collateral-dependent regions of sedentary and exercise-trained pigs. In sedentary animals, contractile responses to ET-1 were not different in arteries isolated from the collateral-dependent compared with the nonoccluded region (Fig 2A). In contrast, exercise training induced significantly enhanced ET-1-mediated contractile response in collateral-dependent compared to nonoccluded arteries (Fig 2B). Similarly, EC₅₀ data revealed that exercise training increased the sensitivity to ET-1 in collateral-dependent compared with nonoccluded arteries (Table 3; no inhibitor column).

ET-1 concentration-response curves in the presence of endothelin receptor inhibition

The effect of endothelin receptor antagonism on ET-1-mediated tension development was compared in arteries isolated from the nonoccluded and collateral-dependent myocardial regions of sedentary and exercise-trained pigs. Arteries were pretreated with antagonists for ET_A (BQ123; 1 μ M) or ET_B (BQ788; 1 μ M) or combined receptor antagonism. As illustrated in Fig 3A, inhibition of either ET_A or ET_B significantly attenuated the ET-1 contractile response in arteries from the nonoccluded region of sedentary pigs. These findings indicate that the ET-1 contractile response is mediated through both ET_A and ET_B. Inhibiting both receptors simultaneously decreased the ET-1 response to a significantly greater degree compared with single receptor antagonism. In collateral-dependent arteries of sedentary pigs (Fig 3B), single blockade of either ET_A or ET_B had a negligible effect on the contractile response to ET-1; however, dual blockade significantly decreased ET-1 contractile responses compared with no inhibitors or with single receptor blockade.

We also completed receptor inhibition studies in nonoccluded and collateral-dependent arteries of exercise-trained pigs. As revealed in Fig 3C, inhibition of either ET_A or ET_B did not significantly reduce the contractile response to ET-1 in arteries from the nonoccluded myocardial region; however, dual blockade significantly decreased this response when

compared with no inhibitors. In collateral-dependent arteries of exercise-trained pigs (Fig 3D), blockade of ET_A significantly attenuated the ET-1 response. Conversely, blockade of ET_B did not alter the ET-1 contractile response. Simultaneous inhibition of ET_A and ET_B decreased the ET-1 response to a degree not statistically different from that of single blockade of ET_A and to a significantly greater extent compared to blockade of ET_B alone.

ET_A, ET_B, and ECE mRNA expression

To determine the effects of occlusion and exercise training on the mRNA expression of the endothelin receptor subtypes and ECE, we performed quantitative RT-PCR experiments on small coronary arteries isolated from the nonoccluded and collateral-dependent myocardial regions from both sedentary and exercise-trained pigs. These experiments demonstrated that the mRNA expression levels of ET_A (Fig 4A), ET_B (Fig 4B), and ECE (Fig 4C) were not significantly altered by chronic coronary artery occlusion or exercise training.

ET_A and ET_B protein content

We examined the protein levels of the ET_A receptor subtype in nonoccluded and collateral-dependent arteries from sedentary and exercise-trained pigs as shown by the immunoblot image of ET_A with GAPDH as loading control (Fig 5A). These experiments revealed that ET_A protein levels were not altered significantly by occlusion or exercise training (Fig 5A). We also initiated studies to examine protein levels of the ET_B receptor subtype. In preliminary studies, we discovered that while we were able to detect ET_B receptor protein in pig lung, the protein levels of ET_B receptor in pig arteries were of very low abundance and not detectable via immunoblot, even when the total protein load for the coronary arteries was increased to 40 µg (Fig 5B). Although four ET_BR antibodies were screened as indicated in the methods section, results of one representative trial (Santa Cruz no. sc-33537) are shown in Figure 5B.

Regional tissue endothelin levels

Endothelin levels were measured in tissue sections from both nonoccluded and collateral-dependent myocardial regions. These experiments revealed that exercise training significantly elevated regional endothelin levels when compared to sedentary pigs, independent of chronic coronary occlusion (Fig 6).

Discussion

Alterations to the endothelin system in cardiovascular disease states have been examined previously and have formed the basis for the use of endothelin receptor antagonists clinically. However, clinical outcomes with the use of such pharmacological agents have resulted in variable degrees of success depending on the cardiovascular disease targeted (reviewed in (29, 31, 45)). Few studies have evaluated the combined effects of chronic coronary occlusion and the implementation of a progressive exercise-training regimen on this system (46). Results of the current study reveal several key novel findings that highlight unique vascular adaptations pertaining to the endothelin system in small coronary arteries of an ischemic heart disease animal model. We demonstrate that in nonoccluded arteries of sedentary pigs, single endothelin receptor inhibition of either receptor subtype significantly

reduces the ET-1 contractile response to comparable levels suggesting that in these vessels both receptors contribute similarly to ET-1 vasoconstriction. We also show that although the ET-1 responses in collateral-dependent arteries are similar to their nonoccluded counterparts under control conditions, individual endothelin receptor antagonism has little effect on the ET-1 contractile responses. These data suggest an occlusion-mediated increase in compensation by the functionally active receptor after inhibition of either ET_A or ET_B. In addition, we demonstrate that in collateral-dependent arteries, exercise training produces a disproportionate increase in the ET_A contribution to the ET-1 contractile response with negligible contribution by ET_B. Finally, these functional adaptations occurred without significant changes in endothelin receptor mRNA expression and ET_A protein content but with significant increases in endothelin levels of both nonoccluded and collateral-dependent myocardial regions in exercise-trained pigs.

Our findings in the different arterial treatment groups evaluated in this porcine model indicate that there may be a significant degree of “cross-talk” or compensation by the endothelin receptor subtypes. This concept of compensation has been previously described as the limited ability of a blocked endothelin receptor subtype to decrease an endothelin-mediated response due to compensation by the other functionally active receptor (8, 43). In sedentary pigs, it is clear that single antagonism of either ET_A or ET_B results in a significant decrease in the ET-1 contractile response in nonoccluded (Fig 3A) but not collateral-dependent (Fig 3B) arteries. Furthermore, combined inhibition of both receptor subtypes nearly abolished contraction in both nonoccluded and collateral-dependent arteries. Taken together, these data suggest that the receptor subtypes in collateral-dependent arteries reveal a unique adaptation in that the functionally active receptor appears to effectively compensate upon single receptor antagonism of ET_A or ET_B. Similar compensatory interactions by the endothelin receptor subtypes have been previously reported in small pulmonary resistance arteries isolated from a rat model of myocardial infarction (50, 51). It is possible that this synergistic inhibition with dual receptor antagonism is due to a functional heterodimerization between ET_A and ET_B as previously suggested in other disease states, such as pulmonary arterial hypertension (12). Indeed, endothelin receptors have been shown to form heterodimers (10, 13, 16) and it has been suggested that the pharmacology of these receptors may differ from that of monomers (55), potentially requiring both selective ET_A and ET_B receptor antagonists to inhibit the function of the heterodimeric receptor (35). However, a role for heterodimers in the coronary vasculature of our model is speculative as the basic understanding of endothelin receptor functional heterodimerization is a relatively novel concept and thus limited (55). Nevertheless, our finding in collateral-dependent arteries provides support for consideration of the use of dual endothelin receptor antagonism clinically rather than selective inhibition in patients with ischemic coronary disease in an attempt to improve blood flow to compromised myocardium. Indeed, various studies have reported the benefits of the use of dual endothelin receptor antagonism in coronary artery disease patients (32, 56).

Evaluation of the ET-1 contractile response in arteries from the nonoccluded regions in exercise-trained pigs (Fig 3C) revealed that single antagonism of either ET_A or ET_B did not significantly reduce the ET-1 contractile response, while dual antagonism significantly attenuated ET-1-mediated contraction. These findings are statistically similar to that

observed in the collateral-dependent arteries of sedentary pigs, although the impact of dual receptor antagonism did not appear as extensive in the nonoccluded arteries of exercise-trained pigs (Fig 3B). The basis for the slightly more persistent contraction in the presence of dual receptor antagonism is unknown. The ET-1 contractile response of arteries from the collateral-dependent region in exercise-trained pigs (Fig 3D) was unique in that inhibition of ET_A resulted in a significant decrease in the ET-1 contractile response whereas inhibition of ET_B did not alter ET-1 contraction. While it appears that simultaneous ET_A and ET_B blockade inhibited the ET-1 response even further than ET_A blockade alone, this comparison did not achieve statistical significance. Despite the lack of statistical difference, the greater effect of dual receptor antagonism than ET_A blockade alone suggests that ET_B may play a role during ET_A antagonism. Notably, these adaptations were exclusive to these arteries, indicating that the combination of occlusion and exercise training stimulated novel changes that were not observed with occlusion or exercise training alone.

A multitude of studies have highlighted increases in coronary blood flow when an exercise-training regimen is implemented in patients (18, 19, 52, 58) and animal models with ischemic coronary disease (6, 48). Bloor and colleagues measured regional myocardial blood flow distribution in a similar porcine model of chronic coronary artery occlusion and exercise training (48). In these studies, blood flow into the collateral-dependent region during exercise was significantly increased in the subendocardium, midmyocardium, and subepicardium after the exercise training regimen compared with pre-training levels (48). Accordingly, the exercise-trained animals also displayed enhanced regional left ventricular wall thickening during exercise relative to pre-training levels. Pigs that remained sedentary did not demonstrate significant changes in blood flow or myocardial contractility in the collateral-dependent region over the same time period (48). Enhanced blood flow into the collateral-dependent region would be mediated through growth of the collateral circulation and/or structural/functional adaptations in the microcirculation of the collateral-dependent region. Thus, it is difficult to reconcile the enhanced ET-1/ET_A contractile response in collateral-dependent arteries of exercise-trained pigs with the observation of increased blood flow into that region of this porcine model. In a previous report, use of an ET_A receptor blocker revealed that endogenous endothelin contributed to coronary vascular tone under resting conditions in a porcine model (38). Interestingly, during acute exercise, the vasoconstrictor influence of endogenous endothelin on the coronary vasculature decreased (38). These investigators proposed that ET_A receptor sensitivity to endothelin may have decreased during exercise through an increase in nitric oxide production, which can directly modulate the binding of endothelin to the ET_A receptor (38). Indeed, we have previously reported that NOS inhibition results in a significant increase in sensitivity (EC₅₀) to ET-1 in collateral-dependent but not nonoccluded arteries of exercise-trained pigs (46). These data are consistent with our more recent findings that eNOS protein is significantly increased in collateral-dependent compared with nonoccluded arteries of exercise-trained pigs (23). We have also reported enhanced bradykinin-mediated relaxation in collateral-dependent arteries of exercise-trained pigs that is reversed by NOS inhibition (23). Furthermore, others have reported that nitric oxide synthase inhibition decreases blood flow into the collateral-dependent region more so during exercise than at rest (53), providing evidence that the contribution of nitric oxide increases as coronary metabolic demand rises in the

compromised myocardial region. Thus, we propose that the adaptive response of enhanced ET-1/ET_A contraction in collateral-dependent arteries of exercise-trained pigs may serve to oppose increased nitric oxide signaling and thereby, maintain coronary vascular resistance under resting conditions. Accordingly, as metabolic demand increases with exercise, enhanced nitric oxide production may function to overcome ET-1/ET_A contraction and thereby, promote perfusion into the compromised myocardial region.

Despite functional adaptations in the endothelin receptors, our data indicate that there were no significant changes to endothelin receptor or ECE mRNA expression (Fig 4) or ET_A protein levels (Fig 5A) between treatment groups. These data suggest that adaptations in the contribution of endothelin receptors to ET-1 contractile responses with occlusion and exercise training may result from adaptations in receptor interaction with the downstream signaling mechanisms rather than changes in receptor density. Indeed, our previous study documented an increased contribution of the PKC signaling pathway to ET-1-mediated contraction as well as enhanced Ca²⁺ sensitization in collateral-dependent arteries from exercise-trained pigs (46). Taken together, our previous and current findings suggest that exercise training increases ET-1-mediated Ca²⁺ sensitization in collateral-dependent small coronary arteries likely by way of an enhanced ET_A/PKC signaling pathway that leads to augmented contractile responses. In addition, the functional vascular adaptations observed in exercise-trained pigs occurred concurrently with increased regional levels of endothelin. Therefore, collateral-dependent small coronary arteries of exercise-trained pigs, in addition to the disproportionately enhanced contribution of ET_A to the ET-1-mediated contractile response, are also exposed to higher endothelin levels than regionally matched vessels from the sedentary control group. Such conditions have been shown to induce endothelin receptor desensitization (15), although this phenomenon was not tested in the current study. We did not see an elevation in endothelin levels in collateral-dependent myocardial regions of sedentary pigs, although others have reported increased plasma endothelin levels in sedentary porcine models of coronary thrombosis (54) and acute myocardial infarction (37). However, the rapid induction of these previously studied disease states may differentially alter endothelin levels compared to that observed with the progressive nature of the coronary occlusion used for the current study (59). Additionally, it is possible that these acute coronary injury models produce adaptations that would potentially change over the time course associated with our chronic disease model.

Conclusions

Our studies reveal that the enhanced ET-1-induced contractile response in collateral-dependent arteries after exercise training appears to be mediated by an enhanced contribution subtype. These adaptations of the ET_A receptor subtype with little involvement from the ET_B protein occurred independently of changes in ET_A and ET_B receptor mRNA expression or ET_A levels. Taken together with our previous data (46), these findings suggest that upregulation of a receptor, such as that mediated by PKC, may be the signaling pathway initiated at the ET_A primary mechanism underlying exercise training-enhanced ET-1 contractile responses in collateral-dependent arteries. Previous data from our laboratory also demonstrate enhanced vasodilatory mechanisms in these small collateral-dependent arteries of exercise-trained pigs (23, 46), suggesting adaptations in both vasoconstrictor and

vasodilator pathways that may interact to optimize control of coronary blood flow into the compromised myocardium.

Perspective

After ET-1 was discovered and characterized as a potent vasoconstrictor, its role in various cardiovascular diseases was evident, eventually leading to the development of endothelin receptor antagonists. Unfortunately, these agents have not had the degree of clinical success that was initially expected in diseases like heart failure (28, 33, 42). However, there have been encouraging findings in patients with atherosclerotic coronary disease (44). One important clinical implication that we can derive from the current study is that by understanding adaptations in endothelin signaling mechanisms in response to chronic occlusion and exercise training, we can tailor anti-endothelin medications in order to achieve a more desirable outcome for the patient. For example, endothelin receptor antagonists could be used acutely to prevent endothelin-mediated vasomotor reactivity. As the patient implements an exercise-training regimen, these could be faded out in order to allow for exercise-mediated adaptations in the compromised myocardium that may be hindered in the presence of endothelin receptor antagonists. Further studies are needed to validate this proposed concept.

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Abbreviations

EC₅₀	agonist concentration producing 50% of the maximal contractile response
ECE	endothelin converting enzyme
ET_A	endothelin receptor subtype A
ET_B	endothelin receptor subtype B
ET-1	endothelin-1
LAD	left anterior descending coronary artery
LCX	left circumflex coronary artery
NIH	National Institutes of Health
NSAID	nonsteroidal anti-inflammatory drug
PVDF	polyvinylidene fluoride

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Figure 1. Time line of experimental protocol

Miniature swine were instrumented with ameroid constrictors around the proximal LCx artery to initiate the experimental protocol. Gradual narrowing of the LCx typically develops into complete occlusion by around three weeks postoperatively. Animals were allowed eight weeks of surgical recovery before being randomly assigned to the exercise training or sedentary regimens. Following 14 weeks of exercise training or being confined to pens, coronary arteries and myocardium were harvested for functional assessment and additional assays.

Figure 2. Endothelin-1 concentration-response relationships

ET-1 concentration-response curves between nonoccluded and collateral-dependent arteries in sedentary (A) and exercise-trained (B) pigs. Concentration-response curves for ET-1 were truncated at the concentration (3 nM) because arteries generally displayed reduced ET-1-mediated contraction at subsequent concentrations. A subset of these data has been published previously (46). * $P < 0.05$; n in parentheses equals number of animals.

Figure 3. Effect of endothelin receptor subtype inhibition on endothelin-1-mediated constriction
 ET-1 concentration-response curves were examined in the absence of any inhibitors or in the presence of BQ123 (1 μ M), BQ788 (1 μ M), or combined antagonism in nonoccluded (nonocc) and collateral-dependent (coll-dep) arteries from sedentary and exercise-trained pigs. * $P < 0.05$ compared with no inhibitor. † $P < 0.05$ compared with BQ788. ‡ $P < 0.05$ compared with BQ123. n in parentheses equals number of animals.

Figure 4. Effects of chronic occlusion and exercise training on mRNA expression of endothelin receptor subtypes A and B and endothelin converting enzyme

Expression of ET_A , ET_B , and ECE mRNA was not altered significantly by chronic occlusion or exercise training. Values are means $\pm$ S.E.M. of the number of animals indicated in parentheses and reported relative to expression level in nonoccluded arteries of sedentary pigs (SED nonoccl).

Figure 5. Effect of chronic occlusion and exercise training on protein levels of endothelin receptor subtypes

Evaluation across artery treatment groups revealed that neither occlusion nor exercise training produced significant alterations in ET_AR protein levels. Assessment of ET_BR protein revealed that ET_BR content in coronary arteries was below the level detectable by immunoblot. Protein was quantified by densitometry analysis, normalized to GAPDH, and expressed relative to the density of nonoccluded arteries of sedentary pigs. SED nonocc, lanes 1, 3, 9, 15, 19, 21; SED coll-dep, lanes 2, 4, 10, 16, 20, 22; EX nonocc, lanes 5, 7, 11, 13, 17, 23; EX coll-dep, lanes 6, 8, 12, 14, 18, 24. PL, pig lung; PA, pig artery. Values are means ± S.E.M. of the number of animals indicated.

Figure 6. Effects of chronic occlusion and exercise training on regional myocardial endothelin levels

Myocardial endothelin levels were increased after exercise training in both nonoccluded (nonocc) and collateral-dependent (coll-dep) myocardial regions. * $P < 0.05$ compared with nonocc myocardial regions of SED pigs. † $P < 0.05$ compared with coll-dep myocardial regions of SED pigs. n in parentheses equals number of animals.

Table 1

Efficacy of the exercise-training program

		CS Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)			
	BW (kg)	HW/BW (g/kg)	DM	TMH	TLH
Sedentary (n = 31)	35.1 $\pm$ 0.9	4.43 $\pm$ 0.09	37.2 $\pm$ 1.3	36.0 $\pm$ 1.5	31.3 $\pm$ 1.4
Exercise (n = 30)	33.6 $\pm$ 0.7	5.31 $\pm$ 0.12 *	45.7 $\pm$ 1.6 *	44.4 $\pm$ 2.0 *	36.8 $\pm$ 1.6 *

Values are mean $\pm$ S.E.M. Numbers in parentheses represent number of animals; CS, citrate synthase; BW, body weight; HW/BW, heart-to-body weight ratio; DM, deltoid muscle; TMH, triceps medial head; TLH, triceps long head

* P<0.05 vs. sedentary.

Table 2

Coronary artery dimensions and characteristics

	ED (μm)	ID (μm)	WT (μm)	AL (mm)	RT L95 (mN/mm)
Sedentary (n = 31)					
nonoccluded	240 ± 7	151 ± 7	44 ± 1	1.52 ± 0.02	0.50 ± 0.04
collateral-dependent	246 ± 7	135 ± 9	45 ± 1	1.52 ± 0.02	0.62 ± 0.07
Exercise (n = 30)					
nonoccluded	240 ± 8	158 ± 6	47 ± 1	1.51 ± 0.02	0.45 ± 0.02
collateral-dependent	262 ± 9	179 ± 9	45 ± 1	1.51 ± 0.02	0.58 ± 0.07

Values are mean ± S.E.M. Numbers in parentheses represent number of animals; ED, external diameter; ID, internal diameter; WT, wall thickness; AL, axial length; RT L95, arterial resting tension (RT) where L95=0.95*L100 and L100 is the internal circumference that the artery would have under a transmural pressure of 100 mmHg, as previously (23). No significant differences exist.

Table 3

Apparent EC₅₀ values for ET-1 -mediated constriction in the absence and presence of inhibition of select endothelin receptor subtypes

Group	No inhibitors	BQ123	BQ788	BQ123 + BQ788
Sedentary	(n=14–16)	(n=9)	(n=7)	(n=6)
nonoccluded	−8.65 ± 0.02	−8.67 ± 0.02	−8.52 ± 0.02 [*]	−8.43 ± 0.05 [*]
collateral-dependent	−8.61 ± 0.03	−8.68 ± 0.02 [*]	−8.63 ± 0.03 [†]	−8.45 ± 0.02 [*]
Exercise-Trained	(n=13–14)	(n=10)	(n=7)	(n=6)
nonoccluded	−8.61 ± 0.03	−8.67 ± 0.02	−8.63 ± 0.05	−8.52 ± 0.06
collateral-dependent	−8.76 ± 0.06 [†]	−8.72 ± 0.04	−8.69 ± 0.04	−8.53 ± 0.03 [*]

Values (log M) are means ± SEM. n values represent number of animals studied and are indicated in parentheses under column headers.

^{*} P<0.05 vs. corresponding EC₅₀ from control (no inhibitors).

[†] P<0.05 vs. corresponding EC₅₀ from nonoccluded artery.