Exercise preserves endothelium-dependent relaxation in coronary arteries of hypercholesterolemic male pigs


Department of Biomedical Sciences and The Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211; and Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

Submitted 24 July 2003; accepted in final form 12 November 2003

HYPERCHOLESTEROLEMIA HAS been reported to cause decreased endothelium-dependent relaxation (EDR) of conduit arteries (endothelial dysfunction) in humans (2), monkeys (11, 17), and pigs (1, 3, 13, 30, 31, 40). Blunted EDR is present early in the progression of coronary artery disease, even before evidence of lesions (11, 17, 18, 20). There is a growing body of evidence that disruption of the nitric oxide (NO) synthase (NOS) pathway (6, 21, 24) and/or reduced availability of NO contribute to this dysfunction (35). Exercise training has been shown to improve endothelial function in normal coronary (22, 37) and peripheral arteries (4, 12, 19), at least, in part, due to increased release of NO by the endothelial NOS (eNOS) pathway (15, 16, 22, 38) and increased expression of superoxide dismutase (SOD) (28, 29). These opposing effects of hypercholesterolemia and exercise interact, as Hambrecht et al. (10) reported that exercise training increased endothelium-dependent dilation of conduit and resistance coronary arteries of patients with coronary artery disease (CAD) (10). Also, Woodman et al. reported that exercise training preserved EDR of brachial (40) and coronary arteries (39) of hypercholesterolemic female pigs early in the progression of artery disease (i.e., did not exhibit complex arterial lesions). The purpose of the present study was to determine whether exercise training preserves endothelial function in hypercholesterolemia similarly in coronary arteries of male pigs. Our hypothesis was that endurance exercise training would attenuate or reverse endothelial dysfunction produced by hypercholesterolemia in male porcine coronary arteries through increased NO-mediated EDR, resulting from increased eNOS expression and/or NO bioactivity. We examined the effects of hypercholesterolemia and exercise training on endothelial function by beginning exercise training 1 mo after the animals were placed on the high-fat diet. Thus our experimental design allowed examination of the effects of exercise training during the initial stages of the development of coronary disease, matching the design of Woodman et al. (39). Results confirm that high-fat male pigs were in the early stages of coronary artery disease as foam cells and limited fatty streaks were present, with little to no evidence of atherosclerosis.

METHODS

Experimental Animals

Ninety-one adult, male Yucatan miniature swine, 8–12 mo of age, 25–40 kg body wt (Charles River, Maine and Sinclair Research Farm, Columbia, MO), were used with protocols approved by the Animal Care and Use Committee at the University of Missouri. Pigs were housed in rooms maintained at 20–23°C with a 12:12-h light-dark cycle. One-half of the pigs were provided a normal-fat (NF) diet (Purina Lab Mini-pig Chow; 8% of daily caloric intake derived from fat), and one-half of the pigs were fed a high-fat (HF) diet (46% of their daily caloric intake from fat) consisting of pig chow supplemented with cholesterol (2.0%), coconut oil (17.1%), corn oil (2.3%), and sodium cholate (0.7%) (5, 32, 39). Pigs were exercise trained (Ex) or remained sedentary (Sed) for 16–20 wk, beginning 4 wk after the animals were placed on the high-fat diet. Thus our experimental design allowed examination of the effects of exercise training during the initial stages of the development of coronary disease, matching the design of Woodman et al. (39). Results confirm that high-fat male pigs were in the early stages of coronary artery disease as foam cells and limited fatty streaks were present, with little to no evidence of atherosclerosis.

Address for reprint requests and other correspondence: M. H. Laughlin, Dept. of Biomedical Sciences, E102 Vet. Med. Bldg., Univ. of Missouri, 1600 E. Rollins Rd., Columbia, MO 65211 (E-mail: laughlinm@mizzou.edu).
dietary intervention was initiated. Pigs continued to consume the HF or NF diet during the entire protocol. The experimental design used four groups of pigs: 1) NF-Sed, 2) NF-Ex, 3) HF-Sed, and 4) HF-Ex. Plasma lipid data from the pigs used in this study have been reported previously (32). The HF diet induced elevated plasma cholesterol (NF-Sed = 59 ± 4 mg/dl, HF-Sed = 404 ± 44 mg/dl), triglyceride (NF-Sed = 30 ± 6 mg/dl, HF-Sed = 40 ± 4 mg/dl), HDL-C (NF-Sed = 32 ± 1 mg/dl, HF-Sed = 96 ± 6 mg/dl), and LDL-C (NF-Sed = 24 ± 3 mg/dl, HF-Sed = 232 ± 23 mg/dl) in HF pigs. Importantly, Ex did not significantly alter the lipid profiles of these pigs (32).

Training Program

All pigs were familiarized with running on a motorized treadmill and randomly assigned to Ex or Sed groups for 16–20 wk. The Ex group completed the 16- to 20-wk endurance training program described previously (14, 22). Pigs assigned to the Sed group were restricted to their enclosures (2 × 4 m pens) and did not exercise.

Vascular Ring Preparation

At the end of the protocol, pigs were sedated with ketamine (30 mg/kg im) and anesthetized with pentobarbital sodium (35 mg/kg iv). Segments of the left anterior descending coronary artery (LAD) were removed by gentle rubbing of the luminal surface with Filar calibrated micrometer eye piece. Vasomotor reactivity was assessed by using ACh (10–6 to 10–4 M), and SNP was assessed by using 10–5 to 10–4 M). One subset of animals underwent measures of coronary structure and function in the cardiac catheterization laboratory before isolation of coronary arteries, as outlined above. There was no effect of these procedures on responses of isolated arteries, so the data are pooled.

Experimental Design

Direct and interactive effects of HF and Ex on contractile responses, endothelium-independent and endothelium-dependent responses (EDR), were examined. Because there are reports that HF diets produce impaired EDR in response to some, but not all, endothelium-dependent agonists (13, 30), whereas others report generalized endothelial dysfunction (6, 21, 24), we determined whether HF produced generalized or selective endothelial dysfunction in adult male pigs by examining responses to three different endothelium-dependent agonists [aggregating platelets, 5-HT, and bradykinin (BK)]. Three different protocols were used.

Vascular reactivity protocol 1. Protocol 1 examined relaxation responses produced by 5-HT, aggregating platelets, and sodium nitroprusside (SNP). One intact and one denuded LAD ring were treated with 30 μM PGF2α, to precontract the vascular rings for examination of relaxation responses. Rings were treated throughout the experiment with indomethacin (Indo; 5 μM) to block production of products of cyclooxygenase (COX) and ketanserin tartrate (1 × 10−6 M) to block smooth-muscle 5-HT2 receptors (13, 30). Concentration-response relationships for vasodilators were determined by cumulative addition of 5-HT (10−10 to 10−5 M) and SNP (10−10 to 10−4 M), in half-log increments to the bath (26). Concentration-response relationships for aggregating platelets were determined by cumulative addition of platelet-rich solution (25, 50, 75, and 100 × 106 platelets/μl) to the bath (13, 30, 31). Platelets were harvested from 300 ml of autologous blood collected before removal of the heart and prepared as described (13, 30, 31). For denuded LAD experiments, the endothelium was removed by gentle rubbing of the luminal surface with fine-tipped forceps and confirmed when the ring produced <5% relaxation in response to 10−6 M BK (26). Endothelium dependency of responses was evaluated by comparing responses of intact and denuded rings. SNP responses were examined after all other dose-response relationships were completed.

Vascular reactivity protocol 2. Protocol 2 was designed to examine contractile responses produced by KCl, 5-HT, and aggregating platelets (in the absence of Indo and ketanserin tartrate). This experiment was designed to determine the importance of smooth-muscle 5-HT2, serotonicergic receptor and second-messenger system, interactions between the effects of 5-HT on smooth muscle and endothelium in vascular dysfunction caused by HF, and the effects of Ex on these processes. Concentration-response relationships were determined by cumulative addition of KCl (20–100 M), 5-HT (10−10 to 10−5 M), and aggregating platelets (cumulative addition of platelet-rich solution: 25, 50, 75, and 100 × 107 platelets/μl) to the bath (13, 30, 31).

Vascular reactivity protocol 3. The third protocol examined the relative contributions of NO, prostacyclins (PGI2), and/or non-COX and non-NOS pathways by using procedures outlined in detail previously (39, 40). Four LAD rings were obtained from each pig: one untreated control, one treated with 0.3 mM G2-nitro-L-arginine methyl ester (1-NAME) to block NO release from NOS, one treated with 5 μM Indo to block COX, and one treated with both blockers (1-NAME + Indo) to evaluate the role of endothelium-derived hyperpolarizing factor (EDHF) and/or other endothelium-derived mediators. All four rings were precontracted with PGI2 (30 μM), and EDR was assessed by using ACh (10−10 to 10−4 M) and BK (10−11 to 10−6 M). One subset of animals underwent measures of coronary structure and function in the cardiac catheterization laboratory before isolation of coronary arteries, as outlined above. There was no effect of these procedures on responses of isolated arteries, so the data are pooled.

Solutions and Drugs

Kreb’s bicarbonate buffer solution contained (in mM) 131.5 NaCl, 5.0 KCl, 1.2 Na2HPO4, 1.2 MgCl2, 2.5 CaCl2, 11.2 glucose, 20.8 NaHCO3, 0.003 propranolol, and 0.025 EDTA. Solutions were aerated with 95% O2–5% CO2 (pH 7.4) and maintained at 37°C. All drugs and chemicals were purchased from Sigma Chemical.

Immunohistochemistry

Samples of LAD were dissected and immersed in 10% formalin for a minimum of 24 h, embedded routinely in paraffin, and sectioned serially at 5-μm thickness. Immunohistochemistry was performed, as reported previously (16, 29, 39), by using mouse monoclonal antibodies against scavenger receptor A (SRA-ES 1:100, Cosmo Bio), eNOS (1:800, BD Transduction Laboratories), rabbit polyclonal antibodies against caveolin-1 (Cav-1; 1:200, Santa Cruz Laboratories), SOD-1 (1:800, Stressgen Biotechnology), and SOD-2 (1:800, Stressgen Biotechnology). For negative controls, histological sections were prepared without primary antibodies. Sections were examined and photographed with an Olympus BX40 photomicroscope.

Immunoblot Analysis

LAD rings were homogenized in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 6 M urea, and 2% SDS by using a ground-glass homogenizer. After a 2-h incubation at 45°C and centrifugation (10,000 g, 1 min), protein concentration of the supernatants was determined by using the bicinchoninic acid assay (Pierce). Before electrophoresis, aliquots of these samples were supplemented with 150 mM dithiothreitol and boiled for 1 min. Gels were loaded with 30 μg of protein, electrophoresed, and electroblotted to polyvinylidene difluoride membranes. Membranes were analyzed for eNOS and Cav-1 (Transduction Laboratories, 1:3,500 and 1:2,000, respectively) and SOD-1 and SOD-2 (Stressgen, both 1:2,000). Secondary antibodies were conjugated with horseradish peroxidase. Antibody and blocking solutions contained 5% nonfat milk and 0.1% Tween in Tris-buffered saline. Chemiluminescent (Amersham) signals were captured and quantified by using the Syngene Chemi-Genius2 Bio-Imaging System and software (Syngene).
Statistical Analysis

All values are means ± SE. Concentration-response curves were evaluated by using mixed-factor, repeated-measures ANOVA. Data were analyzed with each animal counted as one observation for comparisons between groups with respect to each vasoactive agent or combination of agents. Data for the contractile responses were analyzed as developed tension (grams of tension) and as specific tension. Specific tension was calculated by dividing developed tension by the cross-sectional area of vessel wall (OD – ID × length). EC₅₀ was defined as the vasodilator concentration that produced 50% inhibition of a PGF₂α preconstriction. EC₅₀ was determined for each arterial ring with linear interpolation between the log concentrations that produced responses just below and above 50%. Significance of differences between mean values of citrate synthase activity, heart weight-to-body weight ratio, endurance times during treadmill performance test, protein content (immunoblot results), maximal effect, and EC₅₀ values were assessed with one-way ANOVA. P values of <0.05 were considered significant.

RESULTS

Average heart weight-to-body weight ratio was greater in Ex (NF-Ex = 5.6 ± 0.1 g/kg and HF-Ex = 5.6 ± 0.2 g/kg) than in Sed (NF-Sed = 4.7 ± 0.2 g/kg and HF-Sed = 4.5 ± 0.1 g/kg) pigs. Treadmill endurance times were significantly longer (25–30% longer times) in Ex pigs after training, and there were no between-group differences before training. Citrate synthase activity of the long and lateral heads of the triceps brachii muscle and the deltoid muscle of Ex pigs was 25–50% greater than Sed values, confirming the shift in skeletal muscle oxidative capacity that characterizes effective Ex (14–16, 23).

Vascular Ring Characteristics

LADs from HF animals had similar ODs, but smaller IDs, than LADs from NF pigs (Table 1). There were no statistically significant differences in LAD wall thickness among groups. Axial length, resting tension at Lmax, and percent stretch to Lmax were not significantly altered by diet, exercise, or the diet × exercise interaction.

Contractile Responses

KCl, ACh, and 5-HT. LADs from HF pigs developed significantly more force in response to increasing doses of KCl than NF LADs (Fig. 1A). Training did not alter KCl responses in LADs from pigs on either diet. As reported previously (25), ACh did not induce EDR, but caused increases in developed tension that were similar in LADs from all four groups (Fig. 1B). Treatment of the rings with l-NAME, Indo, and l-NAME + Indo did not significantly alter responses to ACh in any group (data not shown). 5-HT elicited a dose-dependent increase in developed tension in LADs from all groups of pigs

![Fig. 1. Effects of diet and exercise training on contractile responses of left anterior descending coronary arteries (LADs) in normal-fat (NF)-diet sedentary (Sed), NF-diet exercise-trained (Ex), high-fat (HF)-diet Sed, and HF-Ex pigs. Values are means ± SE for specific tension in g/mm² artery wall. Specific tension was calculated by dividing developed tension by the cross-sectional area of vessel wall (outside diameter – inside diameter × length). A: KCl-induced contraction of coronary arteries. Mixed-factor, repeated-measures ANOVA revealed that LADs from both HF groups exhibited greater contraction to KCl than did those from both NF diet groups (P < 0.001). B: ACh-induced contraction of coronary arteries. B, baseline tension (during PGF₂α-induced precontraction) before addition of ACh. Mixed-factor, repeated-measures ANOVA revealed that the contractile responses of the LADs to ACh were similar among groups. Ex did not induce significant changes in the ACh-induced contractions.]

Table 1. Characteristics of left anterior descending coronary arteries from normal-fat and high-fat-fed pigs

<table>
<thead>
<tr>
<th>Variable</th>
<th>NF-Sed</th>
<th>NF-Ex</th>
<th>HF-Sed</th>
<th>HF-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Outer diameter, mm</td>
<td>2.90±0.1</td>
<td>3.00±0.12</td>
<td>2.71±0.08</td>
<td>2.74±0.13</td>
</tr>
<tr>
<td>Inner diameter, mm</td>
<td>1.65±0.07</td>
<td>1.73±0.08</td>
<td>1.40±0.06†</td>
<td>1.39±0.09†</td>
</tr>
<tr>
<td>Wall Thickness, mm</td>
<td>0.63±0.03</td>
<td>0.64±0.03</td>
<td>0.66±0.03</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>Axial length, mm</td>
<td>3.16±0.13</td>
<td>2.96±0.07</td>
<td>3.05±0.08</td>
<td>2.91±0.13</td>
</tr>
<tr>
<td>Resting tension at Lmax, g</td>
<td>4.86±0.46</td>
<td>4.04±0.38</td>
<td>4.72±0.48</td>
<td>4.21±0.26</td>
</tr>
<tr>
<td>Percent stretch to Lmax, %</td>
<td>178±1.7</td>
<td>179±1.7</td>
<td>179±2.0</td>
<td>178±2.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of pigs. NF-Sed, normal-fat sedentary; NF-Ex, normal-fat exercise trained; HF-Sed, high-fat sedentary; HF-Ex, high-fat exercise trained; Lmax, optimal circumferential length. One-way ANOVA indicates that inner and outer diameters of NF left anterior descending coronary arteries are significantly greater than HF diameters. Significantly different from *NF-Sed and †NF-Ex, P < 0.05.
Contraction induced by high doses of 5-HT tended to be greater in LADs from HF pigs ($P = 0.06$). There was not a significant effect of Ex on contractile responses to 5-HT.

Aggregating platelets and PGF$_{2\alpha}$. Aggregating platelets elicited a dose-dependent increase in developed tension in intact LADs from all groups of pigs (Fig. 3A). Mixed-factor, repeated-measures ANOVA revealed a significant main effect of diet, indicating that contraction induced by aggregating platelets was greater in LADs from pigs fed the HF diet while Ex had no effect on responses to aggregating platelets. We also noted in results from protocol 3 that LADs from HF-Ex pigs often exhibited greater developed tension during preconstriction with 30 µM PGF$_{2\alpha}$ than those from NF groups [PGF$_{2\alpha}$ developed tension (in g): NF-Sed = 6.5 ± 1.1, NF-Ex = 6.7 ± 0.8, HF-Sed = 5.9 ± 1.2, HF-Ex = 9.3 ± 0.8]. ANOVA revealed a significant diet dose interaction, indicating that 5-HT-induced contraction was greater in LADs from pigs fed the HF diet ($P = 0.05$). There was not a significant effect of Ex on contractile responses to 5-HT.

**Fig. 2.** Effects of diet and Ex on dose-dependent responses of LADs to cumulative addition of 5-HT. Results are presented for LADs isolated from NF-Sed ($n = 10$), NF-Ex ($n = 9$), HF-Sed ($n = 10$), and HF-Ex ($n = 12$) pigs. Values are means ± SE. A: developed tension in grams. Mixed-factor, repeated-measures ANOVA revealed a significant main effect of diet, indicating that 5-HT-induced contraction was greater in LADs from pigs fed the HF diet ($P < 0.05$). There was not a significant effect of Ex on contractile responses to 5-HT. B: %relaxation of PGF$_{2\alpha}$-induced precontraction after treatment with indomethacin (Indo) and ketanserin, as described in the text. Mixed-factor, repeated-measures ANOVA revealed a significant main effect of diet ($P = 0.03$), indicating that 5-HT-induced relaxation was impaired in LAD from HF pigs. In addition, statistical analysis revealed a significant diet × Ex interaction ($P = 0.05$), indicating that 5-HT-induced relaxation was impaired more in LADs from HF-Ex than in LADs from HF-Sed pigs. Indeed, responses to 5-HT in LADs from NF-Sed and NF-Ex were changed from contraction to relaxation by blocking cyclooxygenase with Indo and smooth-muscle 5-HT$_2$ receptors with ketanserin tartrate (10$^{-6}$ M). This was in contrast, in the presence of Indo and ketanserin tartrate, 5-HT did not significantly affect tension in LADs from HF-Ex pigs, and 5-HT only produced relaxation at high doses in HF-Sed.

**Fig. 3.** Dose-dependent responses of LADs to cumulative addition of aggregating platelets (no. of aggregating platelets/µl) to the bath in NF-Sed ($n = 9$), NF-Ex ($n = 6$), HF-Sed ($n = 7$), and HF-Ex ($n = 10$) pigs. Values are means ± SE. A: developed tension in grams. Mixed-factor, repeated-measures ANOVA revealed a significant main effect of diet, indicating that aggregating platelet-induced contraction was greater in LADs from pigs fed the HF diet. There was not a significant effect of Ex on contractile responses to aggregating platelets. B: %relaxation of PGF$_{2\alpha}$-induced precontraction after treatment with Indo and ketanserin, as described in the text. Responses to aggregating platelets in LADs from all groups were changed from contraction to relaxation by blocking cyclooxygenase with Indo and smooth-muscle 5-HT$_2$ receptors with ketanserin tartrate (10$^{-6}$ M). Mixed-factor, repeated-measures ANOVA revealed that LADs from both HF groups exhibited blunted relaxation responses to aggregating platelets compared with responses from NF-Sed and NF-Ex. C: relaxation response induced by aggregating platelets observed during treatment with Indo and ketanserin is indeed endothelium dependent, because nearly all relaxation as well as group differences in responses of LADs to aggregating platelets were abolished by denudation.
revealed that this difference was not statistically significant ($P = 0.09$).

**Relaxation Responses**

5-HT. In the presence of Indo and ketanserin, 5-HT elicted a concentration-dependent relaxation of LADs (Fig. 2B). Mixed-factor, repeated-measures ANOVA revealed a significant main effect of diet ($P = 0.03$), indicating that 5-HT-induced relaxation was impaired in LAD from pigs fed the HF diet. Furthermore, statistical analysis revealed a significant diet × Ex interaction ($P = 0.05$), indicating that 5-HT-induced relaxation was impaired more in HF-Ex than in HF-Sed.

Aggregating platelets. In all groups, responses of the LADs to aggregating platelets were changed from contraction (Fig. 3A) to relaxation by treatment with Indo and ketanserin tartrate (Fig. 3B), with the relaxation in the NF LADs being greater than relaxation observed in the HF LADs. Relaxation to aggregating platelets was not altered by Ex. There was no longer a difference between relaxation to aggregating platelets in NF and HF groups after denudation (Fig. 3C).

BK. BK elicted a dose-dependent relaxation of LAD rings from all groups of pigs (Fig. 4). Mixed-factor, repeated-measures ANOVA did not reveal a significant main effect of diet on BK-induced relaxation. However, there was a significant diet × exercise × dose interaction ($P = 0.04$), indicating that relaxation produced by intermediate and high doses of BK was less in LADs from HF-Sed pigs. BK-induced relaxation was improved in HF-Ex LADs such that relaxation was not significantly different from that in NF-Sed or NF-Ex LADs.

The effects of L-NAME treatment on BK-induced relaxation are summarized in Fig. 5. Mixed-factor ANOVA revealed a significant main effect of L-NAME (Fig. 5), indicating that NOS inhibition decreased BK-induced relaxation. ANOVA did not reveal a significant L-NAME × diet interaction, indicating that L-NAME treatment had similar effects on BK-induced relaxation in NF and HF arteries (Fig. 5, A and B). Also, statistical analysis did not reveal a significant L-NAME × Ex interaction, indicating that L-NAME treatment had similar effects in LADs from Sed and Ex pigs (Fig. 5, C and D). The percent BK-induced relaxation inhibited by L-NAME treatment was 36% in HF-Ex and 17% in HF-Sed (Table 2), suggesting...
that Ex increased the importance of NOS-generated NO in HF LADs.

Treatment with Indo did not significantly alter responses to BK in any group (Table 2). L-NAME + Indo inhibited BK-induced relaxation in NF-Sed, NF-Ex, and HF-Ex LADs such that relaxation was no longer greater than that in HF-Sed arteries (Fig. 6A). Exercise did not alter the effects of double blockade on BK-induced relaxation in NF pigs (Fig. 6B), and L-NAME + Indo had similar effects on LADs from HF-Ex and

<table>
<thead>
<tr>
<th>Variable</th>
<th>NF-Sed</th>
<th>NF-Ex</th>
<th>HF-Sed</th>
<th>HF-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, untreated</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>ED50 (log M)</td>
<td>8.56 ± 0.13</td>
<td>8.76 ± 0.14</td>
<td>8.59 ± 0.35</td>
<td>8.80 ± 0.11</td>
</tr>
<tr>
<td>L-NAME</td>
<td>33 ± 10</td>
<td>33 ± 9†</td>
<td>17 ± 5</td>
<td>39 ± 9†</td>
</tr>
<tr>
<td>ED50 (log M)</td>
<td>8.39 ± 0.08</td>
<td>8.70 ± 0.25</td>
<td>8.38 ± 0.36</td>
<td>8.50 ± 0.14</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>61 ± 8</td>
<td>66 ± 8 ‡</td>
<td>42 ± 10*</td>
<td>81 ± 6‡</td>
</tr>
<tr>
<td>ED50 (log M)</td>
<td>8.69 ± 0.11</td>
<td>8.99 ± 0.13</td>
<td>8.58 ± 0.16</td>
<td>8.89 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of pigs. Percent relaxation was calculated as percent reduction in PGF2α-induced contractile tension (30 μM). L-NAME, Nω-nitro-L-arginine methyl ester; SNP, sodium-nitroprusside. Significantly different from *all other groups, †the untreated control value of this group, ‡the L-NAME value of this group, and §the indomethacin value of this group: P < 0.05.

Table 2. Maximal bradykinin-induced relaxations and doses of bradykinin that produced ED50: effects of inhibitors

SNP Responses

LADs from all four groups exhibited a dose-dependent relaxation in response to SNP. Neither HF nor Ex altered LAD responses to SNP (Table 2).

Immunoblot Analysis

Immunoblot analyses of LAD ring homogenates revealed that eNOS protein content was similar in all groups (Fig. 8A). ANOVA revealed a main effect of diet on Cav-1 protein content, indicating that HF diet produced an increase in Cav-1 content (Fig. 8B). SOD-1 protein content was lower in NF-Ex and HF-Sed than in NF-Sed (Fig. 8C). SOD-2 protein content was lower in NF-Ex and HF-Sed than in HF-Ex (Fig. 8D). Ex
Results of this study indicate that the endothelial dysfunction produced by this HF diet as well as the effects of Ex in preserving endothelial function in male coronary arteries are generally similar to those previously described in female coronary arteries (39). However, endothelial function appears to be more severely impaired in male coronary arteries, and there are some differences in adaptive mechanisms activated by HF and Ex in male coronary arteries. The primary findings of this study are as follows. 1) LADs from HF pigs exhibited greater contractile tension in response to stimulation with KCl, aggregating platelets, and 5-HT. 2) Ex did not significantly alter contractile responses of HF LADs. 3) Blockade of the smooth-muscle 5-HT2 receptors revealed blunted EDR stimulated by 5-HT and/or aggregating platelets in LADs from HF pigs. 4) BK-induced relaxation was impaired in LADs from HF-Sed pigs. 5) The decreased BK-induced relaxation in HF LADs is the result of decreased release and/or activity of NO combined with release of an Indo-sensitive vasoconstrictor. 6) In LADs from HF-Ex pigs, BK-induced relaxation was similar to responses of NF LADs. 7) The training-induced increase in BK-induced relaxation in HF-Ex LADs appears to be the result of an increased contribution of NOS and decreased production of an Indo-sensitive vasoconstrictor. 8) The increased contribution of NOS to BK-induced relaxation in HF-Ex LADs is not the result of increased expression of eNOS protein, but was associated with increases in SOD-1 and SOD-2 content of LADs. Collectively, these data indicate that EDR is blunted in HF by impaired NO release and/or activity and increased release of an Indo-sensitive vasoconstrictor. Ex attenuated the effects of HF on EDR in coronary arteries by reversing or preventing these effects.

Influence of HF Diet on EDR

Role of NO release and NO bioactivity. HF produced endothelial dysfunction in the LADs of these male, adult pigs (Figs. 3 and 4). Our results provide insight into the potential role of the following mechanisms for decreased EDR (8): decreased eNOS expression and protein content, decreased NOS activity, decreased NO bioavailability, decreased production of PGI2, decreased production of EDHF or other endothelium-derived relaxing factors, and increased production of constrictor substances.

The percent EDR inhibited by l-NAME treatment was less in the HF-Sed LADs (17%) than in LADs from NF-Sed and NF-Ex pigs (27 and 34%) (Table 2), indicating that the contribution of NO release to EDR was decreased by HF, consistent with our hypothesis. However, HF did not decrease eNOS protein expression, as we had proposed. Immunoblot results indicate similar eNOS protein content in LADs from all groups (Fig. 8). Interestingly, this HF diet increased eNOS protein content of LADs from HF-Sed female pigs (39). Fault eNOS-positive staining of macrophage foam cells was revealed by immunohistochemical analysis of LADs from both HF-Sed and HF-Ex male pigs (Fig. 9), similar to eNOS staining in foam cells of LADs from female pigs on this HF diet (39). This suggests the possibility that endothelial cell eNOS content may have been decreased by HF, even though total eNOS content of the arteries was not decreased because of eNOS in other cells.

**DISCUSSION**

There was positive immunoreactivity for eNOS in the endothelium of all groups. Importantly, faint positive staining also was present in macrophage foam cells in HF-Sed and HF-Ex pigs (Fig. 9). Positive immunoreactivity for Cav-1, SOD-1, and SOD-2 was present in the endothelium and smooth muscle in all groups of pigs (Figs. 10–12). Staining for SOD-2 was punctate and consistent with staining of mitochondria (Fig. 12). Foam cells in LADs from HF-Ex stained well for SOD-1, whereas those in LADs from HF-Sed did not (Fig. 11). Foam cells were identified in the intima of LADs from both HF groups by staining for SRA (Fig. 13). There was no positive SRA macrophage staining in LADs from NF groups (Fig. 13). In the absence of primary antibody against eNOS, SOD-1, SOD-2, Cav-1, or SRA, no immunoreactivity was detected (data not shown).

**Immunohistochemistry**

appeared to increase SOD-1 and SOD-2 protein content in LADs of HF animals, because SOD-1 and SOD-2 content in HF-Ex LADs was not different from that in NF-Sed.

**Fig. 7. Effects of treatment with l-NAME and/or Indo on dose-dependent relaxation of LADs after cumulative addition of BK. Values are means ± SE. A: effects of l-NAME + Indo (Both) treatment on relaxation of LADs from HF-Sed and HF-Ex pigs. Mixed-factor ANOVA revealed an interaction between l-NAME + Indo and exercise (i.e., treatment with both produced a greater decrease in BK-induced relaxation in HF-Ex than in HF-Sed). B: results demonstrate that Indo treatment alone did not have a significant effect on BK-induced relaxation in LADs from either HF-Sed or HF-Ex pigs.**
Fig. 8. Immunoblot analyses of LAD rings. Endothelial nitric oxide synthase (eNOS; A), caveolin-1 (Cav-1; B), and superoxide dismutase (SOD)-1 (C) and SOD-2 (D) are illustrated. Top: representative immunoblot illustrates 3 different samples (animals) from each indicated treatment group. Bottom: summary data from \( n = 9–15 \) animals/group, with each analyzed with duplicate immunoblots. Values are means \( \pm \) SE, normalized to set the mean NF-Sed group results to unity for each protein analyzed. A: there were no significant differences in eNOS content of LADs from the 4 groups of pigs. B: ANOVA revealed a significant main effect of diet on Cav-1 protein content of LADs, but no main effect of exercise. C: *NF-Ex and HF-Sed SOD-1 protein contents are significantly less than NF-Sed SOD-1 content. HF-Ex content is not different from that of NF-Sed. D: #NF-Ex and HF-Sed SOD-2 protein contents are significantly less than HF-Ex SOD-2 content. HF-Ex content is not different from that of NF-Sed.

Fig. 9. Representative panels of immunohistochemistry performed for eNOS in the LAD branch of the coronary artery. Note staining of endothelium in all sections and faint staining of foam cells in the sections from HF-Sed and HF-Ex (arrows). Bars = 100 \( \mu \)m.
We did not anticipate that other vascular cells would express eNOS protein in HF LADs.  
We examined Cav-1 expression in our arteries to evaluate the possibility that NO-mediated EDR of HF LADs was impaired due to increased expression of Cav-1 (8, 9) as hypercholesterolemia increases Cav-1 expression in cultured endothelial cells (7). Consistent with this hypothesis, our results reveal a main effect of diet, indicating that Cav-1 protein content was increased by HF (Fig. 8). However, immunohistochemical results suggest that both endothelial and smooth-muscle cells of HF LADs have more Cav-1 than do those of low-fat-diet pigs (Fig. 10). Therefore, whereas our results are consistent with the notion that EDR is decreased in HF due to decreased activity of eNOS, resulting from increased Cav-1 and eNOS-Cav-1 binding (7–9), it will be necessary to quantify Cav-1 content specifically in the endothelial cells to be sure. Of
interest, this HF diet did not produce increased LAD Cav-1 content in female pigs (39).

Decreased maximal BK-induced relaxation without significant changes in sensitivity in HF-Sed LADs (Table 2) is consistent with a decrease in NO bioavailability resulting from increased reactive oxygen species (ROS). SOD-1 content was less in HF-Sed LADs than in NF-Sed LADs (Fig. 8). Decreased SOD-1 content in HF-Sed LADs may be the result of low expression of SOD-1 in foam cells in HF-LADs (Fig. 11). The observation of SRA-positive macrophage foam cells in HF-LADs in addition to endothelial and smooth-muscle cells is another important difference between HF LADs and NF LADs (Fig. 13). Macrophage foam cells from atherosclerotic rabbit aorta have been shown to produce superoxide, NO, and hydrogen peroxide (27). Thus immunoblot and immunohistochemical results are consistent with a contribution of increased ROS

---

Fig. 12. Representative panels of immunohistochemistry for SOD-2 in the LAD branch of the coronary artery. Note punctuate staining in endothelium and smooth-muscle cells in all sections and foam cells in HF-Sed and HF-Ex insets (arrows). Bars = 100 μm.

Fig. 13. Representative panels of immunohistochemistry for scavenger receptor A in the LAD branch of the coronary artery. Note the lack of staining in NF-Sed and NF-Ex and positive staining of foam cells in HF-Sed and HF-Ex. Bars = 100 μm.
in HF LADs, causing reduced NO-mediated EDR (34). These results are also different from those reported in female pigs on this diet (39). Female pig LADs exhibited no change in SOD-1 among groups but did exhibit increased SOD-2 in response to HF.

In summary, our results indicate that NOS-mediated, BK-induced relaxation is decreased in LADs of male pigs on this HF diet. The immunoblot and immunohistochemical results are consistent with the notion that NO-mediated relaxation is decreased due to the actions of ROS and suggest that not all of the eNOS protein in the HF arteries is located in endothelial cells. We conclude that decreased NO-mediated relaxation contributes to blunted EDR in LADs from adult male HF-Sed pigs.

**Role of PG_{I2} and other non-NOS mechanisms.** Indo treatment increased BK-induced relaxation only in HF-Sed LADs, suggesting the presence of an Indo-sensitive vasoconstrictor. Furthermore, t-NAME alone and t-NAME + Indo had similar inhibitory effects on EDR in arteries from NF-Sed, NF-Ex, and HF-Ex pigs. In contrast, BK-induced relaxation was not inhibited by treatment with t-NAME + Indo in HF-Sed LADs (Fig. 6D). These results indicate an Indo-sensitive vasoconstrictor substance present in HF-Sed LADs.

Finally, because responses to BK were similar in all groups during treatment with t-NAME + Indo (Fig. 6A), it does not appear that BK-induced relaxation in HF-Sed LADs was reduced because of decreased release of EDHF or other non-NOS, non-COX vasodilator mechanisms. Therefore, we conclude that blunted EDR observed in LADs of HF-Sed male pigs is the result of two mechanisms: 1) production of an Indo-sensitive vasoconstrictor substance; and 2) decreased NO-mediated relaxation resulting from decreased SOD-1 expression and increased Cav-1 content.

**Influence of Exercise Training on EDR**

**Role of NO release and NO bioactivity.** Ex improved BK-induced relaxation of LADs from HF-Ex (Fig. 4), but not EDR stimulated by aggregating platelets or 5-HT. The relatively small magnitude of EDR responses to aggregating platelets and 5-HT, compared with EDR produced by BK, may explain the lack of effect of Ex on these responses.

Because t-NAME treatment caused 36% inhibition of BK-induced EDR in HF-Ex LADs (Table 2) vs. only 17% in HF-Sed LADs (Table 2), results indicate that Ex in HF pigs increased the importance of NO, generated by NOS to EDR. Although there was not a significant interaction between t-NAME treatment and exercise (P = 0.1) (Fig. 5D), t-NAME did decrease maximal BK-induced relaxation in HF-Ex LADs, but not HF-Sed LADs (Table 2), also suggesting an increased contribution of NOS to EDR after Ex. This exercise-induced increased NO-mediated relaxation of NO was not the result of increased eNOS protein content or decreased Cav-1 protein content of HF-Ex LADs (Fig. 8B). However, SOD-1 and SOD-2 content were greater in HF-Ex LADs than in HF-SEDs, indicating that improved NO-mediated relaxation in LADs from HF-Ex pigs is, in part, the result of improved NO bioavailability (Fig. 8, C and D).

The increased SOD-1 content in HF-Ex vs. HF-Sed LADs (Fig. 8C) is consistent with previous observations that Ex increased SOD-1 expression in coronary arterioles (28) and in aortic endothelial cells of female Yucatan pigs trained with this same training program (29). It is interesting that Ex decreased SOD-1 content in LAD of male pigs on the NF diet (Fig. 8C), but increased SOD-1 content in HF-Ex female pigs, relative to NF-SEDs (39).

**Role of PG_{I2} and other non-NOS mechanisms.** The protective effect of exercise on EDR in HF LADs does not appear to be the result of enhanced release of PG_{I2} because Indo did not significantly alter BK-induced relaxation in HF-Ex or HF-Ex LADs (Fig. 7). The Indo-sensitive constrictor substance in HF-Sed LADs, discussed above, was not evident in the LADs of HF-Ex pigs. Ex decreased or prevented production of this vasoconstrictor substance. Consistent with this interpretation, statistical analysis revealed an interaction between double blockade and exercise, indicating that t-NAME + Indo produces a greater decrease in BK-induced relaxation in HF-Ex than in HF-Sed (Fig. 7). During treatment with t-NAME + Indo, BK-induced EDR was similar in all groups (Fig. 7A), indicating that NOS- and COX-independent vasodilator mechanisms (EDHF) have similar effects in LADs from all groups of pigs. The observation that EDHF-mediated EDR was not altered by HF or Ex is similar to results from female pigs (39).

Because HF LADs tended to develop greater tension in response to several agonists, it is important to evaluate the potential role of greater tension on EDR in these arteries. PG_{F2\alpha} induced similar amounts of tension in HF-Sed and NF LADs. Therefore, blunted EDR in HF-Sed LADs is not likely, due to greater constriction of HF-Sed arteries. Similarly, the fact that HF-Ex exhibited ~20% greater PG_{F2\alpha}-induced force (P = 0.09) argues against the possibility that HF-Ex LADs exhibited greater EDR in response to BK because of less preconstriction. Furthermore, the facts that EDR to aggregating platelets was similar in HF-Ex and HF-Sed LADs and that there were no differences among the BK-induced relaxations during double blockade or among the SNP responses of these arteries also argue that differences observed in BK-induced responses of these LADs are predominantly the result of differences in the endothelium.

**Gender Perspectives**

These results indicate that this HF diet has similar effects on EDR of coronary arteries of male and female pigs (39), except that LADs from HF-Sed males appeared to be more impaired than those from female pigs. Consistent with this sex difference, the immunohistochemistry results of this study also suggest that foam cells are more apparent in male LADs than in female pigs on the same diet (39). Thus the severity of endothelial dysfunction and perhaps the degree of vascular disease produced by this HF diet are influenced by sex, but the primary effects and mechanisms responsible for said effects appear similar between males and females. Thus, in both sexes, blunting of BK-induced EDR in coronary arteries was the result of a combination of decreased release and activity of NO and increased production of an Indo-sensitive constrictor. Also, the contributions of non-NOS and non-COX systems do not appear to be altered by HF or Ex in either sex of pig (39). Finally, Ex attenuated these deleterious effects of HF on endothelial function in male LADs by increasing NO-mediated EDR and by reducing the Indo-sensitive constrictor substance in a manner similar to exercise effects observed in female pigs on the same HF diet (39). These effects of exercise in HF males...
were not mediated by changes in eNOS protein content of the LADs. Interestingly, Ex and HF appeared to have different effects on Cav-1, SOD-1, and SOD-2 contents of male LADs (Fig. 8) than in female LADs (39). Thus there are some differences in adaptive mechanisms between male and female pigs exposed to HF and Ex, even though the net effect of these different adaptations produces similar effects on endothelial function.

Conclusions

Results of this study indicate that EDR was impaired by HF in coronary arteries of adult male pigs. The detrimental effects of HF were characterized by blunted EDR responses to BK, 5-HT, and aggregating platelets. BK-induced relaxation appeared to be blunted in HF-Sed LADs due to the presence of an Indo-sensitive vasoconstrictor substance and decreased NO-mediated relaxation. The decreased NO-mediated relaxation appears to be the result of decreased SOD content and increased Cav-1 content of the HF-Sed LADs. Results indicate that endurance Ex prevented or reversed the deleterious effects of hypercholesterolemia on BK-induced EDR. The Ex-induced preservation and/or improvement of endothelial function in HF-Ex arteries is partially the result of enhanced relaxation by NO (perhaps due to increased SOD content) and removal of an Indo-sensitive vasoconstrictor. Training did not appear to alter the role of non-NOS, non-COX factors such as EDHF.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of Pam Thorne, Denise Holiman, Jennifer Casati, and Tammy Strawn.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-52490 and HL-36088 (to M. H. Laughlin), National Institute on the role of non-NOS, non-COX factors such as EDHF.

REFERENCES


