Endothelial function in coronary arterioles from pigs with early-stage coronary disease induced by high-fat, high-cholesterol diet: effect of exercise

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Departments of 1Biomedical Sciences, 2Veterinary Pathobiology, 3Medical Pharmacology and Physiology and 4Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211; and 5Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

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Henderson, Kyle K., James R. Turk, James W. E. Rush, and M. Harold Laughlin. Endothelial function in coronary arterioles from pigs with early-stage coronary disease induced by high-fat, high-cholesterol diet: effect of exercise. J Appl Physiol 97: 1159–1168, 2004. First published June 18, 2004; 10.1152/japplphysiol.00261.2004.—Because hypercholesterolemia can attenuate endothelial function and exercise training can augment endothelial function, we hypothesized that exercise training would improve endothelial function of coronary arterioles from pigs in the early stages of cardiovascular disease induced by a high-fat, high-cholesterol (HF) diet. Yucatan miniature swine were fed a normal-fat (NF) diet or HF diet (2% cholesterol) for 20 wk in which 8 and 46% of their calories were derived from fat, respectively. Both groups were subdivided into sedentary (Sed) or exercise-trained (Ex) groups. This resulted in four experimental groups: NFSed, NFEx, HFsed, and HFEx. Endothelial function was assessed in coronary arterioles 75–100 μm in diameter dissected from the left ventricular apex. Responses to endothelial-dependent dilation induced by bradykinin (BK), ADP, and flow were similar in all four groups, whereas dilation to aggregating platelets was attenuated in Ex groups (P < 0.05) and increased in Ex groups (P < 0.05). Interestingly, the relative contribution of nitric oxide to BK-induced dilation, as assessed with nitro-L-arginine methyl ester, was similar in arterioles in the NF, HF, Sed, and Ex groups. These results suggest that, in the early stages of cardiovascular disease, a high-fat, high-cholesterol diet has modest effects on endothelial-dependent dilation in coronary arterioles; nonetheless, these effects are prevented or reversed with exercise training.

porcine; nitric oxide; prostanoid; platelets

SEVERAL STUDIES SUGGEST HYPERCHOLESTEROLEMIA attenuates endothelial function by altering the nitric oxide synthase (NOS) pathway and/or reducing the bioavailability of nitric oxide (1, 16, 25, 28, 30). Furthermore, there is evidence that endothelial dysfunction occurs in humans before atheroma formation (12, 13). Together, these data suggest that a reduction in nitric oxide release by the endothelium may set the stage for cardiovascular disease.

In the coronary circulation, where oxygen extraction is near maximal, changes in endothelial-dependent dilation can have significant consequences if other mediators fail to compensate. Indeed, the primary mediator of flow-induced dilation in the coronary microcirculation shifts from nitric oxide in healthy patients to an endothelial-dependent hyperpolarizing factor in patients with coronary artery disease (14). It is possible that the shift in mediator balance to endothelial-dependent hyperpolarizing factor is a compensation for the loss of nitric oxide. As nitric oxide inhibits platelet aggregation and thrombus formation, responses to aggregating platelets may also change with coronary artery disease. Indeed, endothelial-dependent dilation to aggregating platelets is attenuated in iliac arteries of swine with advanced vascular disease produced by feeding a high-fat, high-cholesterol diet (6). Furthermore, in the presence of advanced stages of coronary artery disease, sensitivity to ADP and serotonin, two factors released by aggregating platelets, is attenuated in coronary arterioles of swine (7).

Evidence shows that exercise has beneficial effects in the prevention and treatment of coronary artery disease through maintenance or restoration of endothelial function by enhancing nitric oxide production (9, 11, 15, 29) and extending nitric oxide bioavailability by the increased expression of superoxide dismutase (18, 19). In human coronary artery disease patients with coronary endothelial dysfunction, exercise training improves mean peak flow velocity and the coronary blood flow reserve responses to acetylcholine (5). This study suggests that endothelial function in coronary conduit and resistance arteries improves with exercise training. From these data, we hypothesized that exercise training would allow a more normal endothelial function in coronary arterioles of pigs consuming a high-fat, high-cholesterol diet. Importantly, we used a model of early coronary disease induced by feeding pigs a diet with high fat (17% coconut oil by weight) and high cholesterol (2% cholesterol by weight) (HF diet). Previous results have indicated that these pigs are in Stary stage I–III in the progression of vascular disease (23). In addition, results from our laboratory have previously shown that exercise training preserves endothelial function in conduit coronary arteries (25, 30) and in brachial arteries (31) of pigs with this model of early vascular disease.

A program of exercise training (16 wk) was initiated 4 wk after pigs started the HF diet. This allowed us to examine the effects of exercise training on coronary arterioles in this model...
of early-stage cardiovascular disease, in which Stary type I–III accumulations of foam cells and fatty streaks are found in coronary arteries (26). This stage of disease generally occurs at the first to second decade of life in humans and would present no clinical symptoms (22, 23).

METHODS

Experimental Animals and Diet

One hundred and seventeen adult male Yucatan miniature swine (7 mo) were obtained from the breeder (Charles River) and maintained in accordance with the standards set forth by the American Association for Laboratory Animal Care and the University of Missouri Institutional Animal Care and Use Committee. At death, pigs weighed 42.6 ± 0.6 kg and were ~1 yr of age. Normal-fat (NF)-fed pigs received Purina Lab mini-pig breeder chow; for NF pigs, 8% of their daily caloric intake was derived from fat. Pigs on the HF diet received the same pig chow supplemented with (by weight) 2% cholesterol, 17.1% coconut oil, 2.3% corn oil, and 0.7% sodium cholate. This diet derives 46% of the daily caloric intake from fat. All pigs were fed once per day and were maintained on their respective diets for 20 wk until death. This HF diet has been shown to produce a similar amount of hypercholesterolemia in sedentary and exercise-trained pigs (24).

Training Program

All pigs were familiarized with running on a motorized treadmill, and performance tests were conducted to establish baseline exercise tolerance. Pigs were then randomly divided into sedentary (Sed) or exercise-trained (Ex) groups, resulting in four experimental groups: NF-Sed, NF-Ex, HF-Sed, and HF-Ex. Sed pigs were confined to their pens (2 × 4 m) for 20 wk. Ex pigs were subjected to a progressive treadmill training protocol, training 5 days/wk for 16 wk. By the 12th wk, training intensity reached a plateau and consisted of the following: 5-min warm-up at 2.5 mph, 15-min sprint at 5–8 mph, 60-min endurance run at 4–5 mph, and a 5-min cool down at 2.5 mph. Exercise tolerance tests were administered to all pigs at the end of the study to verify the efficacy of exercise training by measuring exercise duration and heart rate (17). Additionally, at death, heart weight-to-body weight ratios and skeletal muscle oxidative capacity were measured by citrate synthase activity (15). This training program leads to adaptations classically associated with the exercise-trained state in mammals.

Isolation of Coronary Arterioles

Pigs were sedated with ketamine (Fort Dodge; 35 mg/kg im) and xylazine (Bayer; 2.25 mg/kg im) and anesthetized with thiopental sodium (Abbott Laboratories; 10 mg/kg im). A tracheotomy was performed, and the animal was placed on mechanical ventilation. The right jugular vein was catheterized for blood sampling. Heparin (1,000 IU/kg) was administered intravenously, a left thoracotomy was performed, and the heart was rapidly removed and placed in ice-cold physiological saline solution (PSS). A portion of the left ventricular apex was removed and placed into a dissection chamber filled with PSS maintained at 4°C. With the aid of a dissecting microscope, epicardial coronary arterioles (75–100 μm in intraluminal diameter) were isolated from the surrounding tissue at a depth not exceeding 3.0 mm. Arterioles were transferred to a Plexiglas chamber containing PSS + albumin, equilibrated with room air at ambient temperature. Glass micropipettes, pulled to ~50 μm in outer diameter, were filled with filtered PSS + albumin and used to cannulate each vessel. Vessels were secured with 11-0 ophthalmic suture. The glass micropipettes were connected to independent reservoirs that had been raised 60 cm above the vessel chamber. This pressurized the vessel to 44 mmHg. This pressure was selected because in vivo studies have suggested that intravascular pressures in coronary arterioles of this size are approximately this pressure (4). Arterioles were allowed to equilibrate for 1 h during which the temperature of the chamber was raised and maintained at 37°C with a circulating water bath. During the 1-h equilibration period, the PSS + albumin solution was replaced two to four times, and 80 mM KCl was used to stimulate and verify functional vessels. Arterioles were required to develop at least 20% spontaneous tone. The cannulated vessel was viewed with an inverted microscope and displayed on a television monitor equipped with video micrometers to measure intraluminal diameter.

The PSS used in these experiments consisted of (in mM) 145 NaCl, 4.7 KCl, 2.0 CaCl2, 1.17 MgSO4, 1.2 Na2HPO4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS buffer. PSS pH was adjusted to 7.4 and filtered through 0.22-μm microfilters (Fisher Scientific). All drugs were obtained from Sigma Chemical (St. Louis, MO), unless otherwise specified. PSS + albumin contained 10 mg/ml BSA (US Biochem Bovine; fraction V; 98–99% albumin).

Fifteen animals underwent catheter laboratory procedures under isoflurane anesthesia to assess in vivo coronary arterial responses to bradykinin (BK) and adenosine. Four HF-Sed pigs received additional HF feed to determine whether caloric matching to HF-Ex pigs would augment atheroma formation. Arterioles from these pigs were compared with earlier studies, and no statistical effects of these interventions were detected.

Protocol 1: Receptor- and Nonreceptor-Mediated Dilation

To assess the effect of the HF diet and exercise training on endothelial-dependent dilation, BK (3 × 10^-14 to 1 × 10^-8 M) was added to the bath in half-log doses. To assess nonreceptor-mediated endothelial-dependent dilation, flow-induced dilation was used. For flow experiments, glass micropipettes were matched for electrical resistance, and flow was generated by raising and lowering the micropipette reservoirs equally (8). This technique generates flow while maintaining constant intraluminal pressure. When reservoirs were vertically spaced apart at 2, 4, 6, and 10 cm, flows of ~1.4, 3.2, 5.1, and 8.9 μl/min were generated, respectively.

Protocol 2: Assessing Endothelial Responses to Aggregating Platelets

Because a reduction in nitric oxide, secondary to the HF diet, could alter responses to aggregating platelets, a second series of experiments was conducted. The response to aggregating platelets provides a global view of how the endothelium interacts with several endogenous factors released by platelets. For these studies, arterioles were incubated with indomethacin (Indo; 5 μM) and ketanserin (1 μM) for 20 min. Indo inhibits the cyclooxygenase (COX) pathway, preventing the confounding influence of prostaglandin synthases and dilators, whereas ketanserin blocks serotonin receptors on vascular smooth muscle. These experiments allowed us to examine the role of nitric oxide and the effect of the HF diet and exercise training on aggregating platelet-induced endothelial-dependent dilation. Platelets were collected from 300 ml of autologous blood and prepared as previously described (6, 20, 21). Concentration-response curves were obtained by adding cumulative doses of aggregating platelets (25, 50, 75, 100, 150, 200, and 250 × 10^7 platelets/μl). Arterioles were then evaluated with ADP (1 × 10^-5 to 1 × 10^-4 M) and then sodium nitroprusside (SNP; 1 × 10^-6 to 1 × 10^-4 M) in the presence of Indo and ketanserin. ADP is a mediator released by aggregating platelets, and SNP assesses endothelial-independent dilation.

Protocol 3: Assessing NOS, COX, and non-NOS/non-COX Mediators

A third series of experiments was conducted to further examine the effects of the HF diet and exercise training on endothelial-dependent mediators. In these experiments, BK-induced dilation was further examined in the presence of nitro-L-arginine methyl ester (l-NAME;
300 μM), a nonspecific inhibitor of NOS and/or Indo (5 μM). Arterioles were incubated with inhibitors for 20 min before the BK dose-response studies were started. Some arterioles were exposed to different sequences of inhibitors with l-NAME or Indo preceding the combined inhibition of l-NAME + Indo. l-NAME + Indo responses were similar regardless of prior exposure to l-NAME or Indo. At the end of each experiment, arterioles were exposed to 100 μM SNP to determine maximal diameter.

**Immunohistochemistry**

Surrounding tissue and arterioles were assessed for the presence of eNOS with immunohistochemistry (IHC). This analysis was performed to determine whether immunoblot measures for eNOS reflected endothelial cell content or was influenced by other cells, such as macrophage foam cells, which may contain eNOS protein (25). A cubic centimeter of myocardium from the left ventricular apex was obtained for IHC analysis. Tissue was fixed by immersion in aqueous-buffered formalin (Anatech) for >6 h and processed through standard paraffin embedding. To confirm the localization of eNOS to the endothelium, sections from four hearts were floated onto positively charged slides (Fisher, St. Louis, MO). Sections were deparaffinized and steamed in citrate buffer at pH 6.0 (target retrieval solution S1699; Dako, Carpintera, CA) for 20 min to achieve antigen retrieval and then cooled for 20 min. An autostainer (Dako DC34000) was used for immunohistochemical staining. Sequential Tris buffer and water wash steps were performed after each step in the automated staining protocol. Sections were incubated with avidin–biotin two-step blocking solution (Dako X590) to inhibit background staining and in 3% hydrogen peroxide to inhibit endogenous peroxidase. Nonserum protein–binding (Dako S1699) was applied to inhibit nonspecific protein binding, and slides were incubated in primary mouse monoclonal IgG anti-eNOS antibody (Transduction Laboratories N30020) at 1:600 dilution for 60 min. After the appropriate washing steps were completed, slides were incubated with biotinylated anti-mouse link secondary antibody in PBS containing 15 mM sodium azide and peroxidase-labeled streptavidin (Dako LSAB+ kit, peroxidase, K0690). Diaminobenzidin (Dako) applied for 5 min allowed visualization of eNOS antibody staining. Slides were counterstained with Mayer's hematoxylin stain for 1 min, dehydrated, and coverslipped as described previously (10). Sections were photographed with an Olympus BX40 photomicroscope and Spot Insight color camera (Diagnostic Instruments).

**Immunoblots**

Protein content of eNOS was measured in isolated arterioles. Segments of coronary arterioles were carefully dissected from the left ventricular apex, and surrounding connective tissue and fat were removed. Dissection was made in PSS without albumin. Diameters and lengths were measured, and samples were placed into microcentrifuge tubes and frozen at −80°C. Diameters were calculated to correct for differences in maximal diameter possible. One-way ANOVA on relative diameters of spontaneously constricted arterioles revealed that HF arterioles had greater tone (NFsed vs. HFSed, P < 0.001; Table 1). Relative diameters were calculated to correct for differences in maximal diameter. One-way ANOVA on relative diameters of spontaneously constricted arterioles revealed that HF arterioles had greater tone (NFsed vs. HFSed, P = 0.0071). Furthermore, intraluminal diameters of arterioles with spontaneous tone were significantly different, with HF arterioles having greater tone (NFsed vs. HFSed and NFEx vs. HFX, P < 0.001; Table 1). Relative diameters were calculated to correct for differences in maximal diameter. One-way ANOVA on relative diameters of spontaneously constricted arterioles revealed that HF arterioles had greater tone (NFsed vs. HFSed, P = 0.006; NFEx vs. HFX, P = 0.017). There was no effect of exercise on relative spontaneous tone (NFsed vs. NFEx, P = 0.992; HFSed vs. HFX, P = 0.993; Table 1).

Interestingly, inhibition of NOS and/or COX with l-NAME and/or Indo, respectively, did not alter the amount of tone in HFX arterioles (P = 0.659). In NFsed, NFEx, and HFSed animals, l-NAME significantly reduced baseline relative arteriole diameter (P < 0.008), whereas Indo did not alter the amount of tone developed. Also of interest, the reduction in

**Asymmetrical Dimethyl Arginine Assay**

Plasma samples from male swine were shipped to Medical Science Labs (Palo Alto, CA) for an ELISA for asymmetrical dimethyl arginine (ADMA) assay.

**Gender**

Forty-six female pigs were subdivided into NFsed, NFEx, HFSed, and HFX groups. In these studies, female arterioles were preconstricted with endothelin if they did not develop >20% spontaneous tone. Responses to BK and SNP were assessed. Data from these studies are presented at the end of the RESULTS.

**Data Analysis**

Data for concentration-response relationships are presented as changes in relative diameter and percent possible dilation. Briefly, relative diameter is the ratio of the arteriole diameter relative to the maximal diameter possible (Dd/Dmax × 100). This measurement corrects for differences in maximal diameters. Percent possible dilation is the percent change in diameter relative to the maximal change in diameter possible [(Dd − Db)/(Dmax − Db) × 100]. This measurement corrects for differences in maximal and beginning diameters. Here, Db represents diameter at baseline with spontaneous tone, Dd represents diameter after a drug intervention, and Dmax represents maximal possible diameter.

All values are means ± SE, with statistical significance at P = 0.05. One-way ANOVA was used to identify between-group differences in beginning and ending arteriole diameters and EC50 values.

One-way ANOVA with repeated measures was used to determine differences in enzyme inhibitors for BK-induced dilation within each group. Two-way ANOVA with repeated measures on two variables, diet and exercise, was used to determine differences in concentration-response curves, followed by least square mean analysis to identify differences associated with diet or exercise.

**RESULTS**

**Arterioles**

Epicardial coronary arterioles, 75–100 μm in intraluminal diameter, were selected from the left ventricular apex. To be included in this study, male arterioles were required to develop ≥20% spontaneous tone. One-way ANOVA revealed significant variation in intraluminal diameters of maximally dilated arterioles (P = 0.007) (Table 1). Furthermore, intraluminal diameters of arterioles with spontaneous tone were significantly different, with HF arterioles having greater tone (NFsed vs. HFSed and NFEx vs. HFX, P < 0.001; Table 1). Relative diameters were calculated to correct for differences in maximal diameter. One-way ANOVA on relative diameters of spontaneously constricted arterioles revealed that HF arterioles had greater tone (NFsed vs. HFSed, P = 0.006; NFEx vs. HFX, P = 0.017). There was no effect of exercise on relative spontaneous tone (NFsed vs. NFEx, P = 0.992; HFSed vs. HFX, P = 0.993; Table 1).

Interestingly, inhibition of NOS and/or COX with l-NAME and/or Indo, respectively, did not alter the amount of tone in HFX arterioles (P = 0.659). In NFsed, NFEx, and HFSed animals, l-NAME significantly reduced baseline relative arteriole diameter (P < 0.008), whereas Indo did not alter the amount of tone developed. Also of interest, the reduction in
basal diameter with l-NAME + Indo was less than l-NAME alone, with basal diameter being significantly reduced with l-NAME + Indo in NFEx (P = 0.03) but not in NF Sed (P = 0.09) or HFSed (P = 0.20) arterioles (Table 1).

Diet

Plasma lipid data have been reported previously (24). Briefly, the following were significantly increased in HF pigs when NF Sed vs. HFSed groups were compared: 58.8 ± 4.0 vs. 403.5 ± 44.1 mg/dl total cholesterol, 29.8 ± 6.3 vs. 39.9 ± 4.3 mg/dl triglycerides, 31.6 ± 1.4 vs. 96.1 ± 6.3 mg/dl HDL-C, and 24.4 ± 3.1 vs. 231.6 ± 22.5 mg/dl LDL-C. Exercise training did not significantly influence plasma lipid profiles in these animals.

Exercise Training

The efficacy of the exercise protocol leading to the trained state in pigs has been reported previously (9, 15, 18, 29). Pigs used in the present study demonstrated increased exercise tolerance, which was measured as run time to exhaustion (in min): 23.9 ± 0.7 (NF Sed) vs. 33.5 ± 1.0 (NF Ex) and 24.0 ± 0.8 (HFSed) vs. 31.9 ± 0.8 (HFE x). Heart weight-to-body weight ratios (in g/kg) were increased with exercise 4.7 ± 0.1 (NF Sed) vs. 5.5 ± 0.1 (NF Ex) and 4.5 ± 0.1 (HFSed) vs. 5.6 ± 0.1 (HFE x). Additionally, citrate synthase activity in the deltoid muscle (in μmol·min⁻¹·g wet wt⁻¹) was increased with exercise: 15.0 ± 1.1 (NF Sed) vs. 21.0 ± 0.9 (NF Ex) and 15.8 ± 0.8 (HFSed) vs. 22.5 ± 0.9 (HFE x).

Protocol 1: Receptor- and Nonreceptor-Mediated Dilation

BK and flow-induced dilation were used to assess the effects of the HF diet and exercise training on endothelial-dependent dilation. To correct for differences in maximal diameters and basal tone, dose-response data are presented as percent possible dilation. BK elicited a significant dose-dependent dilation in myocardial arterioles; however, mixed-factor repeated-measures ANOVA revealed no significant effect of diet (P = 0.526) or exercise (P = 0.512) and no diet-by-exercise interaction (P = 0.655) (Fig. 1). Sensitivity to BK was not altered by diet or by exercise (P = 0.413). EC₅₀ values (in 10⁻¹⁰ M) were as follows: −12.36 ± 0.27 (NF Sed), −12.68 ± 0.26 (NF Ex), −12.21 ± 0.40 (HFSed), and −12.95 ± 0.35 (HFE x).

Increases in luminal flow elicited a significant dose-dependent dilation of myocardial arterioles in all groups of animals.

Mixed-factor repeated-measures ANOVA revealed a significant effect of diet (P = 0.015); pigs fed the HF diet have augmented flow-induced dilation in coronary arterioles. However, when the data were expressed as relative diameters, responses to flow-induced dilation were similar except for the degree of spontaneous tone in HF arterioles (Fig. 2). Thus, when data are expressed as percent possible dilation, flow-induced dilation appears to be increased in HF arterioles because of their greater spontaneous tone. Thus mixed-factor repeated-measures ANOVA of relative diameters was used; there were no significant effects of diet (P = 0.522) or of exercise (P = 0.823) and no interaction between diet and exercise (P = 0.439) on flow-induced dilation.

Protocol 2: Assessing Endothelial Responses to Aggregating Platelets

Aggregating platelets elicited a significant dose-dependent dilation of myocardial arterioles incubated with Indo and ketanserin. There were no significant effects of diet (P = 0.126). Additionally, citrate synthase activity in myocardial arterioles was increased with exercise: 15.0 ± 1.1 (NF Sed) vs. 21.0 ± 0.9 (NF Ex) and 15.8 ± 0.8 (HFSed) vs. 22.5 ± 0.9 (HFE x).
activity to aggregating platelets (HFsed vs. HFEx, \( P \) = 0.522) or Ex \( (P = 0.823) \) and no HF-by-Ex interaction \( (P = 0.439) \).

0.138) or of exercise \( (P = 0.275) \). However, there was a diet by exercise interaction \( (P = 0.026) \). Further examination of the data with a least square mean analysis revealed that platelet-induced dilation was attenuated in HFsed animals (NFsed vs. HFsed, \( P = 0.013 \)) and that this response was prevented or reversed in HFEx swine (HFEx vs. HFsed, \( P = 0.029 \)) (Fig. 3). Sensitivity to aggregating platelets was not changed among the four groups \( (P = 0.207) \). EC\( _{50} \) values (in \( 10^{-10} \) M) were \(-4.01 \pm 0.15 \) in NFsed, \(-4.62 \pm 0.49 \) in NFEx, \(-5.32 \pm 0.56 \) in HFsed, and \(-4.21 \pm 0.39 \) in HFEx animals. Results indicate that dilator sensitivity to aggregating platelets was reduced in sedentary arterioles from HF animals (NFsed vs. HFsed, \( P = 0.054 \)). Exercise training did not increase sensitivity to aggregating platelets (HFEx vs. HFsed, \( P = 0.119 \)).

There was a significant dose-dependent dilation to ADP in the presence of Indo and ketanserin; however, there was no effect of diet \( (P = 0.426) \) or exercise \( (P = 0.464) \) and no diet-by-exercise interaction \( (P = 0.855) \). Sensitivity to ADP was not altered by the HF diet or the exercise training protocol \( (P = 0.925) \). EC\( _{50} \) values (in \( 10^{-10} \) M) were \(-7.63 \pm 0.29 \) in NFsed, \(-7.50 \pm 0.14 \) NFEx, \(-7.53 \pm 0.36 \) in HFsed, and \(-7.38 \pm 0.16 \) in HFEx animals. Data are not shown.

SNP, a nitric oxide donor, elicited a dose-dependent dilation in the presence of Indo and ketanserin (Fig. 4). Mixed-factor repeated-measures ANOVA revealed a significant effect of diet \( (P = 0.027) \), no effect of exercise \( (P = 0.218) \), but a significant diet-by-exercise interaction \( (P = 0.041) \). Least square mean analysis revealed that exercise training in HF swine attenuates SNP-induced dilation (HFsed vs. HFEx, \( P = 0.019 \)) and that the HF diet attenuates SNP-induced dilation in Ex swine (NFEx vs. HFEx, \( P = 0.004 \)). Sensitivity to SNP was significantly different between groups \( (P = 0.036) \) and NFEx vs. HFEx \( (P = 0.051) \) but was not different between HFsed vs. HFEx \( (P = 0.102) \). EC\( _{50} \) values (in \( 10^{-10} \) M) were \(-7.05 \pm 0.12 \) NFsed, \(-7.13 \pm 0.18 \) NFEx, \(-7.02 \pm 0.17 \) HFsed, \(-6.44 \pm 0.22 \) HFEx animals.

**Protocol 3: Assessing Endothelial-Dependent Mediators**

**Effect of inhibitors in each group.** One-way ANOVA with repeated measures was employed to determine the effect of enzyme inhibition on BK-induced dilation in each group. In the presence of L-NAME, dilation to BK was significantly attenuated in all groups: NFsed \( (P = 0.007) \), NFEx \( (P = 0.023) \), HFsed \( (P = 0.008) \), and HFEx \( (P = 0.008) \) (Fig. 5). Indo did not alter BK-induced responses: NFsed \( (P = 0.999) \), NFEx \( (P = 0.999) \), HFsed \( (P = 0.748) \), and HFEx \( (P = 0.466) \) (Fig. 5). L-NAME + Indo significantly attenuated BK-induced dilation in all groups: NFsed \( (P = 0.013) \), NFEx \( (P = 0.039) \), HFsed \( (P = 0.003) \), and HFEx \( (P = 0.046) \) (Fig. 5).

![Fig. 2. Flow-induced dilations in male coronary arterioles represented by relative diameter. Mixed-factor repeated-measures ANOVA revealed no significant effects of HF \( (P = 0.522) \) or Ex \( (P = 0.823) \) and no HF-by-Ex interaction \( (P = 0.439) \).](https://www.jap.org/)

![Fig. 3. Platelet-induced dilations in male coronary arterioles in the presence of indomethacin (Indo) and ketanserin. Mixed-factor repeated-measures ANOVA revealed no significant effects of HF \( (P = 0.138) \) or Ex \( (P = 0.275) \) but a significant HF-by-ex interaction \( (P = 0.026) \). Least square mean analysis demonstrated a significant effect of HF in Sed arterioles (NFsed vs. HFsed, \( P = 0.013) \) and Ex in HF arterioles (HFEx vs. HFsed, \( P = 0.029) \). Sensitivity to platelets, as measured by EC\( _{50} \) values, was not changed \( (P = 0.207) \). However, sensitivity was attenuated by HF, but not significantly, in Sed arterioles (NFsed vs. HFsed, \( P = 0.054) \). Ex did not alter platelet sensitivity in HF arterioles \( (P = 0.119) \). Statistically different from NFsed and HFsed, respectively.](https://www.jap.org/)

![Fig. 4. Sodium nitroprusside (SNP)-induced dilations in male coronary arterioles in the presence of indomethacin (Indo) and ketanserin. Mixed-factor repeated-measures ANOVA revealed a significant effect of HF \( (P = 0.027) \) and no effect of Ex \( (P = 0.218) \) but a significant HF-by-Ex interaction \( (P = 0.041) \). Least square mean analysis demonstrated a significant effect of Ex in HF swine (HFEx vs. HFsed, \( P = 0.019) \) and a significant effect of HF in Ex swine (NFEx vs. HFEx, \( P = 0.004) \). Sensitivity to SNP, as measured by EC\( _{50} \) values, was statistically different between groups \( (P = 0.036) \) and NFEx vs. HFEx \( (P = 0.051) \) but not between HFsed vs. HFEx \( (P = 0.102) \). Statistically different from NFEx and HFsed, respectively.](https://www.jap.org/)
Effect of inhibitors between groups. Two-way ANOVA with repeated measures was used to determine whether there were differences between groups and their responses to enzyme inhibition. Responses to L-NAME were similar among groups and were not influenced by diet (P = 0.986) or exercise (P = 0.613) (data not shown). BK sensitivity in the presence of L-NAME was not altered by the HF diet or exercise training (P = 0.201). EC_{50} values (in 10^{-10} M) for BK dose responses in the presence of L-NAME were \(-11.29 \pm 0.47\) in NFSed, \(-10.24 \pm 0.25\) in NFEx, \(-10.15 \pm 0.33\) in HFSed, and \(-10.51 \pm 0.52\) in HFEx animals. Interestingly, although Indo responses were not influenced by diet (P = 0.980) or by exercise (P = 0.132), least square mean analysis revealed a significant difference between HFSed and HFEx animals (P = 0.039) (Fig. 6). In the presence of Indo, HFSed responses to BK were significantly greater than those of HFEx. However, sensitivity to BK in the presence of Indo was not different (P = 0.364); EC_{50} values (in 10^{-10} M) for BK dose responses in the presence of Indo were \(-12.04 \pm 0.40\) in NFSed, \(-12.26 \pm 0.40\) in NFEx, \(-13.13 \pm 0.51\) in HFSed, and \(-12.14 \pm 0.52\) in HFEx animals. *Statistically different from control.

Fig. 5. A: NFSed. B: NFEx. C: HFSed. D: HFEx. BK-induced dilations in the presence or absence of nitro-L-arginine methyl ester (L-NAME) and/or Indo in male coronary arterioles. One-way ANOVA with repeated measures was used to determine the effect of enzyme inhibition on BK-induced dilation in each group. L-NAME significantly attenuated BK-induced dilation in all groups: NFSed (P = 0.007), NFEx (P = 0.023), HFSed (P = 0.008), and HFEx (P = 0.008). Indo led to similar BK-induced dilation: NFSed (P = 0.999), NFEx (P = 0.999), HFSed (P = 0.748), and HFEx (P = 0.466). L-NAME + Indo (L-I) significantly attenuated BK-induced dilation in all groups: NFSed (P = 0.013), NFEx (P = 0.039), HFSed (P = 0.003), and HFEx (P = 0.046). *Statistically different from control.

Fig. 6. BK-induced dilations in the presence of Indo in male coronary arterioles (HFSed vs. HFEx). Least square mean analysis revealed a significant difference between HFSed and HFEx (P = 0.039). Sensitivity to BK in the presence of Indo, as measured by EC_{50} values, demonstrated no statistical effect of exercise in HF arterioles (HFSed vs. HFEx, P = 0.196). *Statistically different from HFEx.
in HFEx animals. Further sensitivity to BK dose responses in the presence of Indo was not different between HFSed vs. HFEx \((P = 0.196)\). BK-induced dilations in the presence of \(\text{L-NAME + Indo}\) were similar among groups and were not influenced by diet \((P = 0.935)\) or by exercise training \((P = 0.190)\) (data not shown). Sensitivity to BK dose responses in the presence of \(\text{L-NAME + Indo}\) was not different \((P = 0.354)\). EC\(_{50}\) values (in log M) for BK dose responses in the presence of \(\text{L-NAME + Indo}\) were \(11.28 \pm 0.65\) in NFSed, \(-11.07 \pm 0.53\) in NFEx, \(-10.04 \pm 0.34\) in HFSed, and \(-10.78 \pm 0.49\) in HFEx animals.

**IHC and Immunoblot Analysis**

IHC revealed staining for eNOS, confined to the endothelium, in arterioles from all groups of animals (Fig. 7). In the absence of primary antibody against eNOS, no immunoreactivity was detected (data not shown). The effects of the HF diet and exercise training on eNOS protein levels in coronary arteriole homogenates are shown in Fig. 8. ANOVA demonstrated that eNOS protein content was significantly attenuated by HF \((P = 0.0007)\) and significantly increased by exercise training \((P = 0.0001)\).

**ADMA Assay**

Values for ADMA, an endogenous inhibitor of eNOS, were low compared with values in humans with pathophysiological conditions \((3–15 \text{ M})\) (2, 3) and healthy/normal human values, which range from 0.5 to 1.2 \(\text{ M}\) (27). There was no effect of the HF diet \((P = 0.30)\) or exercise training \((P = 0.94)\) on ADMA values. Mean values (in \(\text{ M}\)) were 0.048 \(\pm\) 0.005 for NFSed, 0.036 \(\pm\) 0.020 for NFEx, 0.021 \(\pm\) 0.013 for HFSed, and 0.036 \(\pm\) 0.007 for HFEx animals.

**Gender Effects**

We conducted a second series of experiments using 46 female Yucatan miniature swine to examine the influence of gender on responses to the HF diet and exercise training. In these experiments, endothelin was used to generate tone if arterioles did not develop >20% spontaneous tone. Furthermore, SNP-induced dilation was assessed in the absence of Indo and ketanserin. Similar to the male responses, BK elicited a significant dose-dependent dilation in myocardial arterioles, and mixed-factor repeated-measures ANOVA revealed no significant effect of diet \((P = 0.835)\) or exercise \((P = 0.928)\) and...
no diet-by-exercise interaction ($P = 0.563$) (Fig. 9). Responses to SNP were not influenced by the HF diet or by the exercise training (data not shown).

**DISCUSSION**

Our hypothesis was that 20 wk of a high-fat, high-cholesterol diet would lead to endothelial dysfunction in coronary arterioles of adult male Yucatan miniature swine and that exercise training would prevent or attenuate the impairment of endothelial function by augmenting the relative contribution of nitric oxide. In this model of early cardiovascular disease, we found limited endothelial dysfunction and modest changes in the relative importance of different endothelial mediators of BK-induced dilation. Exercise training prevented or reversed the modest effects of the HF diet on endothelial-dependent dilation. The primary findings of this study are as follows: 1) coronary arterioles from HF pigs had greater spontaneous tone than arterioles from NF pigs; 2) neither the HF diet nor the exercise training appeared to alter endothelium-dependent dilation responses to BK, flow, or ADP; 3) endothelial-dependent dilation to aggregating platelets was attenuated in HFSed arterioles but not in HFSed arterioles; 4) sensitivity to SNP was attenuated in male HFSed arterioles; 5) the relative contribution of nitric oxide and non-NOS/non-COX mediators to BK-induced dilation was not altered by the HF diet or by exercise training; 6) HFSed arterioles, with BK stimulation, released an Indo-sensitive prostanoid constrictor; 7) in HFSed arterioles, BK stimulation led to the release of an Indo-sensitive prostanoïd dilator; 8) eNOS protein content was reduced in HFSed and increased in NFEx and HFSed arterioles; and 9) dilation responses of arterioles from female pigs to BK and SNP were similar among all four groups.

**Spontaneous Tone**

Arterioles isolated from HF swine exhibited greater spontaneous tone. NOS inhibition produced similar constriction in NFEx, NFEx, and HFSed arterioles, indicating similar and significant basal release of nitric oxide. These data suggest that changes in the basal release of nitric oxide are not causing the HFSed arterioles to have greater spontaneous tone. NOS inhibition did not alter tone in HFSed arterioles, but sensitivity to SNP was attenuated. These data suggest that basal release of nitric oxide could be reduced and/or that sensitivity to nitric oxide is attenuated, which might have contributed to the greater spontaneous tone in these arterioles.

**BK, Flow, ADP, and Platelet-Induced Dilation**

Kuo et al. (7) demonstrated attenuated BK-, flow-, serotonin-, histamine-, and ADP-induced dilations in coronary arterioles from pigs with more advanced coronary disease than the pigs used in this study. Blunted endothelial function in the pigs of Kuo et al. was reversed with l-arginine supplementation, suggesting substrate limitation and/or that competitive inhibition of eNOS caused blunt endothelial function in their model. The animals used in Kuo’s study had atherosclerotic lesions occluding 10–50% of the coronary artery lumen and had foam cells and calcium deposits in some arteries. There was even evidence of pathology in the arterioles, as regions of the internal elastic lamina appeared thickened and damaged, with the endothelium frequently containing lipid droplets and/or large vacuoles (7). The Stary scale (I–VII) for classifying atherosclerotic lesions indicated that these animals had advanced disease, stage V–VI (23). Importantly, pathology present in our animal model included foam cells, modest intima-medial thickening, and, rarely, non-symptom-producing fatty streaks in the conduit arteries, indicating that the disease was in the early stages of development (Stary stage I–III). There was no sign of pathology in the coronary arterioles of pigs used in the present study. In the arterioles of our HF pigs, endothelial function as measured by BK, flow, or ADP-induced dilation was similar to that of NF pigs. These observations suggest that the most reasonable explanation for different results between the present study and that of Kuo et al. is that different stages of atherosclerotic disease were studied. Other possible explanations include differences in breed (domestic vs. Yucatan) and/or diet (3% vs. 2% cholesterol and 24% lard vs. 17% coconut oil).

It is important to emphasize that the left anterior descending coronary arteries of the same pigs that supplied the arterioles used in this study exhibited blunted endothelial function in HFSed pigs and that exercise training reversed these effects, with HFSed left anterior descending coronary arteries exhibiting normal BK-induced relaxation (23). These results suggest that the HF diet produces endothelial dysfunction in conduit coronary arteries at this early stage of disease, whereas endothelial function seems to be preserved in coronary arterioles. Perhaps of equal importance, endothelial function in the coronary circulation of these animals, from conduit to resistance arteries, parallels the spatial pattern of pathology.

It is also of interest that platelet-induced dilation was attenuated in arterioles from HFSed pigs. Aggregating platelets release many factors, including but not limited to serotonin, ADP, platelet-activating factor, and thromboxane A$_2$ (6, 20, 21). Endothelial-dependent dilation was examined, without the confounding influence of prostanoïd constrictors and dilators, by adding Indo to the bath to block the COX pathway. Furthermore, we used ketanserin to block smooth muscle serotonin receptors to prevent platelet-induced constriction. Because ADP responses were not altered by the HF diet or by...
exercise training and because platelet-activating factor and thromboxane A$_2$ work primarily through the COX pathway, these results suggest endothelial sensitivity to serotonin may be altered with the HF diet.

**NOS, COX, and Non-NOS/Non-COX Mediators**

The results of the pharmacology experiments revealed the following. 1) Neither the HF diet nor the exercise-training treatment appeared to alter the contribution of NOS or non-NOS/non-COX pathways to BK-induced dilation because l-NAME and l-NAME + Indo attenuated BK-induced dilations to a similar extent in all groups of animals. 2) Indo treatment produced similar dilations in NFSed and NFEx arterioles. However, Indo treatment revealed that HFSed arterioles stimulated with BK released a prostanoid constrictor, whereas HFEx arterioles released a prostanoid dilator. These results suggest that the HF diet caused increased production of a prostanoid constrictor during BK treatment. Furthermore, this modest shift in the COX pathway activated by BK was altered by exercise training so that the COX pathway appeared to produce a prostanoid dilator in HFEx arterioles.

**IHC and Immunoblot**

IHC was used to assess eNOS localization within the artery wall. In these arterioles, staining for eNOS protein was limited to the endothelial cells. eNOS protein, as measured with immunoblot, was decreased in HF arterioles and increased in arterioles taken from Ex pigs. These data, by themselves, suggest that the HF diet may attenuate the relative contribution of nitric oxide to endothelium-dependent dilation. Also, these results suggest that exercise training would augment the relative contribution of nitric oxide to endothelial-dependent dilation. Interestingly, only responses to aggregating platelets were attenuated in HFEx arterioles. Indeed, BK-, flow-, and ADP-induced dilations were similar in NF and HF arterioles, and no differences were observed with exercise training. Furthermore, NOS inhibition with l-NAME significantly attenuated BK-induced dilation to a similar extent in all experimental groups. Thus changes in eNOS protein altered neither the endothelial-dependent dilation nor the relative contribution of nitric oxide to BK-induced dilation. This finding that statistically significant changes in eNOS protein did not translate into changes in nitric oxide-mediated dilation in response to BK was unexpected. These data suggest that the changes in eNOS protein content were not great enough to exert physiological effects and/or that other mechanisms associated with nitric oxide production-bioavailability were altered.

**Conclusions**

In this early model of coronary disease, we observed limited endothelial dysfunction and modest changes in the relative contribution of endothelial-derived mediators induced by the HF diet and by exercise training. The HF diet 1) resulted in increased spontaneous tone; 2) caused no changes in responses to BK-, flow-, or ADP-induced dilations; 3) attenuated endothelial-dependent dilation to aggregating platelets; 4) produced the release of Indo-sensitive prostanoid constrictor; and 5) reduced eNOS protein levels in coronary arterioles. Exercise training 1) did not alter BK-, flow-, or ADP-induced dilations; 2) restored dilator responses to aggregating platelets in HF arterioles; 3) abolished release of Indo-sensitive prostanoid constrictors and produced release of an Indo-sensitive prostanoid dilator in HFEx arterioles; and 4) increased eNOS protein levels. Overall, endothelial-dependent dilation was maintained in coronary arterioles at this early stage in the pathogenesis of atherosclerotic vascular disease.

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