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Carotid inflammation is unaltered by exercise in hypercholesterolemic swine

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Abstract

Introduction—Reduction of vascular inflammation might contribute to the beneficial effects of exercise. We hypothesized that 1) exercise would reduce carotid endothelial vascular cell adhesion molecule-1 (VCAM-1) and that 2) *in vivo* detection of carotid inflammation can be achieved in a large animal model using contrast-enhanced ultrasound (CEU) with VCAM-1 targeted microbubbles.

Methods—Familial Hypercholesterolemic (FH) swine were divided into sedentary (Sed) and exercise-trained (Ex) groups. Ex pigs underwent 16–20 wk of treadmill aerobic exercise. At the end of the study, *in vivo* CEU with VCAM-1 targeted microbubbles and assessment of endothelial-dependent dilation (EDD) were performed in carotid arteries. VCAM-1 mRNA and protein expression were compared to markers of atherosclerotic disease and health, and *in vitro* EDD was assessed in carotid arteries.

Results—Exercise training did not reduce inflammation nor did it improve EDD in carotid arteries of FH swine. Markers of atherosclerosis including VCAM-1 were prominent in the bifurcation compared to the proximal or distal common carotid artery (CCA) and inversely associated with phosphorylated- and total-eNOS. Signal intensity from VCAM-1-to-control microbubbles positively correlated with carotid VCAM-1 protein expression, validating our technique.

Conclusion—These results first demonstrate that aerobic exercise has no effect on carotid endothelial inflammatory markers and EDD in FH swine. Second, our findings indicate that CEU using VCAM-1 targeted microbubbles can detect inflammation *in vivo*, providing strong foundations for longitudinal studies examining the effect of therapeutic interventions on the inflammatory status of the endothelium.

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Keywords

VCAM-1; targeted microbubbles; exercise; Familial Hypercholesterolemia; carotid bifurcation

INTRODUCTION

During the last three decades, exercise has been increasingly used as a therapeutic intervention for patients with coronary artery disease (41), heart failure (3, 30) and type II diabetes (24), prescribed alone or in combination with medical therapy. Exercise is thought to be beneficial, in part, through direct effects on the vasculature (11), resulting in improvement of endothelial function in patients with risk factors for atherosclerosis or diagnosed coronary artery disease (12, 13, 46). Moreover, exercise has been shown to retard coronary plaque progression assessed by angiography (29). Improved endothelial function is the prevailing initiating mechanism invoked to explain the reduction in atherosclerosis with increased physical activity. Improved endothelial formation and release of nitric oxide, EDHF and reductions in vasoconstrictor prostanoids have been proposed to lead to reductions in adhesion molecule expression, e.g. VCAM-1, and subsequent lesion formation (25, 49). Indeed, inflammation is recognized as not only the major initiating factor of atherogenesis, but is also associated with plaques in all stages of disease and proposed to play a key role in acute coronary events (23, 32). Increased physical activity is associated with reductions in cerebrovascular events, i.e. stroke, as well as coronary and peripheral artery disease. However, while improved EDD with exercise is widely observed in peripheral and coronary arteries, the only reported effects of exercise on endothelial function in carotid arteries have been obtained in rabbits and are negative (7, 51, 52). Thus, evidence is lacking that exercise-induced reductions in cerebrovascular events can be linked to improved EDD and reduced adhesion molecule expression. Thus, we sought to determine whether chronic exercise training reduced carotid artery endothelial inflammation, i.e. VCAM-1 expression, and improved EDD using a swine model of atherosclerosis.

Assessment of endothelial function and inflammation *in vivo* could facilitate identification of active plaques at risk for rupture. Current methods of assessment of endothelial function *in vivo*, such as flow-mediated dilation and endothelial-dependent dilation (EDD), do not discriminate between the presence and absence of coronary artery disease (CAD) in dyslipidemic patients (36) and do not correlate with the extent of coronary artery stenosis, carotid intima-media thickness (IMT), or intima-media (I/M) ratio (35). In addition, atherosclerotic plaque can be found without impairment of EDD (45).

Non-invasive, direct assessment of endothelial inflammation could provide a tool to monitor the response to treatment, as the reduction of inflammation could signify stabilization of the atherosclerotic plaques (34). Since inflammation of the endothelium is characterized by early expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), several human studies have focused on linking exercise to levels of soluble adhesion molecules, with inconsistent results (15, 37). Therefore, we sought to examine the effects of exercise on inflammation using contrast-enhanced ultrasound (CEU) with VCAM-1 targeted microbubbles, a novel non-invasive approach to detect inflammation *in*

vivo. We chose to examine these effects on carotid arteries, a vascular bed commonly used in humans to predict the extent of both cerebrovascular and CAD. We hypothesized that (1) exercise would reduce VCAM-1 expression in carotid arteries and that (2) VCAM-1 targeted microbubbles can detect carotid inflammation *in vivo*. To increase the translational potential of the study, we tested these hypotheses in the Rapacz Familial Hypercholesterolemic swine (33), an animal model that both develops spontaneous atherosclerosis and is of sufficient size to allow direct application of CEU as in humans.

MATERIALS AND METHODS

Experimental animals

Fourteen male castrated Rapacz familial hypercholesterolemic swine (33) were purchased from the University of Wisconsin Swine Research and Teaching Center. Pigs were individually housed in rooms maintained at 20–23°C with a 12:12-h light-dark cycle and had *ad libitum* access to water. Pigs were fed the University of Wisconsin Gestation (UWG) diet consisting of a corn- and soybean-based cholesterol-free, 3% fat diet for the entire duration of the study. At 10–11 months of age, pigs were familiarized with the treadmill and then randomly assigned into exercise trained (Ex, n = 7) or cage confined/sedentary (Sed, n = 7) groups.

Approximately one week before the end of the 16- to 20-wk training program, pigs were sedated (telazol-xylazine) and underwent dual energy X-ray absorptiometry (DEXA) scanning (QDR 4500A, Hologic, Inc., Bedford, MA, USA) to assess body composition and to collect blood from the right external jugular vein. Animals were placed supine on the DEXA table in a fixed position and scanned once. Body composition (head, left and right thoracic and pelvic limbs and trunk) was determined by an experienced technician using computer software (QDR Software for Windows XP, version 12.4, Hologic, Inc., Bedford, MA). Percent body fat was expressed as a ratio of fat mass (g) divided by the total mass (g) $\times 100$.

At the end of the training program, swine were sedated with intramuscular telazol-xylazine, induced and maintained under general anesthesia with isoflurane inhalation (~1.5–2%). Approximately 20 mL of blood was collected from the right external jugular vein shortly after induction. An ear vein catheter was placed followed by the administration of an initial loading dose of heparin (300 U/kg IV) with maintenance doses of 100 U/kg IV each hour. Heart rate, saturation of peripheral oxygen, rectal temperature and electrocardiography were monitored during anesthesia. Contrast-enhanced ultrasound with microbubbles (targeted and non-targeted) was performed first followed by *in vivo* measurement of EDD using acetylcholine (ACH) infusion. At the end of the experiments, the animal was euthanized and the common carotid artery (CCA), including the proximal carotid bifurcation [referred to as the bifurcation later in the text] from the brachiocephalic trunk, were harvested and placed in cold physiologic saline solution. The left CCA and mid portion of the right CCA were cleaned of fat and connective tissue and divided into several segments for immunohistochemistry/histology, western blot, qRT-PCR, and *in vitro* endothelial function assessment with consistency between animals.

Lastly, forelimb skeletal muscles including the triceps brachii lateral head [TltH; both deep (D) and superficial (S) portions], triceps brachii accessory head (TAH), triceps brachii medial head (TMH), triceps brachii long head (TLH; both D and S portions), and deltoid, were harvested and frozen for measurement of citrate synthase activity. Experimental protocols were approved by the University of Missouri Animal Care and Use Committee and in accordance with the “Principles for the Utilization and Care of Vertebrate Animals used in testing, Research and Training.”

Training program

Experimental treatments lasted for 16–20 wk during which Sed pigs were restricted to their pens and Expigs underwent an exercise-training regime on treadmills as previously described (6). Briefly, pigs assigned to the Ex group completed a 16–20 wk endurance training program consisting of 5 exercise bouts/wk. At the start of the training program, each exercise bout consisted of a 5-min warm-up, followed by 15 min of run at 5 mph and a 20–30 min run at 3 mph. The intensity and duration of the exercise bouts were increased weekly to maximize the training stimulus so that by *week 10* of training, each exercise bout lasted 85 min and consisted of 5-min warm-up, a 15-min sprint run at 6–8 mph, a 60-min endurance run at 4–6 mph, and a 5-min cool down. Efficacy of the training program was determined from measurements of endurance time (from the treadmill exercise before and after test), forelimb skeletal muscle citrate synthase activity and pig heart weight-to-body weight ratios.

Contrast-enhanced ultrasound (CEU)

Microbubbles (MBs) targeted to VCAM-1 antibody (VCAM-1 MBs) were prepared by Targeson Inc. (San Diego, CA) using a purified VCAM-1 antibody produced by 6G10 hybridoma (ATCC, Manassas, VA, USA). Control microbubbles (control MBs) conjugated with rat immunoglobulin G antibody used to evaluate non-specific attachment of microbubbles were also obtained from Targeson Inc. Pigs (Ex, $n = 6$, Sed, $n = 6$) were placed in dorsal recumbency. Proximal and distal portions of the left CCA were localized and the skin marked to ensure consistent transducer placement. Both proximal and distal segments of the left CCA were first imaged using 2D brightness mode (HDI 5000, Philips, Andover, MA, USA), followed by capture of background images using contrast mode (mechanical index of 0.06). Next, a bolus of 5 units ($1.8\text{--}2.0 \times 10^9$ particles/mL) of VCAM-1 targeted or control microbubbles ($2\text{--}3 \mu\text{M}$ in size, Targeson, San Diego, CA) was randomly administered intravenously. Approximately 3–4 and 6–7 min following administration, postcontrast images of both portions of the left CCA were captured. At 9 min, the mechanical index was increased to 0.6 to obtain postdestruction images. A delay of 20 min between injections was provided to allow dissipation of the first type of microbubbles administered. Images were reviewed offline using Image J software (NIH, Bethesda, MD). Signal intensity from microbubbles present within the carotid arterial wall was measured using a region of interest (ROI) drawn around the near vessel wall including a minimal portion of the lumen adjacent to the wall. Post hoc review of the images demonstrated that the destruction process did not effectively destroy the microbubbles present in the field thus we normalized the signal intensity measured in each ROI to the signal intensity measured on background images prior to each subsequent injection.

***In vivo* measurement of endothelial-dependent relaxation (EDD)**

Following CEU, pigs underwent assessment of carotid endothelial function using the endothelial-agonist, acetylcholine (ACH). To achieve local concentration of ACH of 10^{-6} and 10^{-5} M in the left CCA, total blood flow was determined as described previously by Tsutsui *et al* (44) using the following equation: time-average velocity (cm/s) $\times \pi \times$ (vessel diameter (cm)/2)² $\times 60$. The vessel diameter was first measured in a longitudinal plane with ultrasound calipers (HDI 5000, Philips) at R wave using 2D brightness mode. With the transducer still in place, we switched to pulsed-wave Doppler ultrasonography and obtained the time-average velocity (TAM) based on three cardiac cycles. A JR SH guide catheter (Boston Scientific, Natick, MA) was introduced through a 7F sheath into the right femoral artery, and the distal tip of the guide catheter was positioned within the most proximal portion of the left CCA. The infusion protocol consisted of 2.5 min of saline, 5 min of ACH 10^{-6} M, 5 min of ACH 10^{-5} M and 7.5 min of saline at a constant infusion rate of 1 mL/min in twelve pigs (Ex, n = 6 and Sed, n = 6). In two pigs, one of each group, only the first concentration of ACH (10^{-6} M) was infused. Before starting the infusion, gain settings and depth using 2D-brightness mode were optimized for both carotid portions and kept constant during the entire length of infusion. Immediately following initiation of the infusion, two ultrasound images of the proximal and distal segments of the left CCA were captured at R wave (baseline). The infusion protocol lasted 20 min during which, two images per min of each carotid portion were captured, for a total of 84 images per subject. Systolic and diastolic blood pressure was also recorded at baseline and every minute of the infusion protocol. Off-line analyses of proximal and distal CCA diameters were performed using a custom-designed edge-detection and wall tracking software developed by M.J. Davis (Vision development module, Labview, National Instruments, Austin, TX). In brief, images were spatially calibrated using the original image file, and three regions of interest were placed along the longitudinal axis of the carotid. The internal edges of the artery were detected by thresholding and iterative regression procedures similar to a method previously described (9, 39). Location of the ROIs was maintained for all images of the same pig. ROIs from each image and time point were averaged and diameters were then normalized to mean arterial pressure and expressed as a percent change in carotid diameter from saline infusion. Carotid arterial diameters following infusion of saline and acetylcholine were normalized to mean arterial pressure to account for the systemic effect of acetylcholine resulting in 3–11% decrease in mean arterial pressure. Reduction in mean arterial pressure to up to 20% has been reported in rats using similar concentration of acetylcholine (10). Heart rate was only marginally affected by the acetylcholine infusion. To determine the maximal dilator response of the carotid artery, we attempted to infuse sodium nitroprusside, an endothelium-independent agonist, in the carotid artery. However, the dramatic decrease in blood pressure prevented us from repeating this protocol in other pigs.

***In vitro* assessment of EDD**

Isometric tension was determined on arterial segments 3 to 4.5 mm in length from the mid portion of the left and right CCA from five pigs in each group in a 20 mL bath filled with Krebs bicarbonate buffer maintained at 37°C with a gas mixture of 95% O₂ and 5% CO₂. Arterial segments were allowed 1 h to equilibrate to Krebs bicarbonate buffer. Before

concentration-response curves, carotid rings were stretched to optimal length (L_o) using 30 mM KCl as described previously (50). Carotid rings were precontracted with $\text{PGF}_{2\alpha}$ (30 μM) and endothelium-dependent relaxation was assessed using acetylcholine (ACH; 10^{-10} – 10^{-4} M) and bradykinin (BK; 10^{-11} – 10^{-6} M). Sodium nitroprusside (SNP; 10^{-10} – 10^{-4} M) was used to measure endothelium-independent relaxation. A total of five carotid rings from each animal were studied in parallel. In three rings (CTL), vasorelaxation to agonists only was measured by adding cumulatively increasing doses of the selected drug to the organ bath while measuring changes in force. Two rings received N^G -nitro-L-arginine methyl ester (L-NAME; 300 μM) to determine the role of nitric oxide (NO) in vasoactive responses by blocking NO synthase (NOS). Dose-response curves to ACH were studied first, followed by BK and SNP. At the end of each dose-response protocol, arterial rings were washed out several times by replacing bicarbonate buffer solution and arterial rings were allowed the necessary time to stabilize before initiation of the next protocol.

qRT-PCR

Carotid arterial segments were cut longitudinally. Trizol solution (TRI Reagent, Molecular Research Center, Inc.) was deposited on the luminal side of the opened arterial segment in enough amounts to cover the entire surface. After 30 seconds, endothelial cells were scraped with a sterile scalpel and collected in one ml of trizol solution. Endothelial cell scraps were quick frozen in liquid nitrogen and stored at -80°C until processing. Total RNA was isolated according to the manufacturer's published protocol. cDNA was transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems). A minus RT reaction was also performed to ensure no genomic DNA contamination. qRT-PCR was performed on a MyiQ Single-Color real time PCR detection system (Biorad) using reaction conditions optimized for each set of primers: VCAM-1 (sense, TCTGGAATTTACGTGTGCGAGGGA, antisense, AGCTGGAAAGCCATCACTAGAGCA), eNOS (sense, GCCTGAACAGCACAGGAGTT, antisense, GCTCTTCTAGCCGTGTGTCC), and β -actin (sense, GGACTTCGAGCAAGAGATGG, antisense, AGCACTGTGTTGTTGGCGTACAG) and gene expression was normalized to β -actin using the 2^{-C_t} method (22). Linearity and efficiency of each PCR condition were verified by creating a standard curve plotting the critical threshold vs. log of the dilution of cDNA.

Western Blot

Shortly after harvest, carotid arterial segments were opened with the luminal surface side-up, covered with approximately 80–100 μL of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% mercaptoethanol, 1 mM PMSF, 2 mM leupeptin, 1 mM pepstatin A, 10% vol/vol glycerol, 0.1% Triton-X-100) and scraped with the dull surface of an X-Acto blade. Equal protein amounts (10 μg) of sample were electrophoresed on 4–20% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane, blocked for 1 h and probed with anti-eNOS phosphorylated at serine residue 1177 (1:400, BD Transduction Labs #cat: 612392), anti-VCAM-1 antibody (1:100; from hybridoma 6G10), total eNOS (1:5000, BD Transduction Labs) and β -actin (1:2000; Sigma). Membranes were stripped between VCAM-1 and eNOS and between eNOS and β -actin using a Blot Restore Solution kit (Millipore). Goat-anti-mouse (1:1000; Santa-Cruz

Biotechnologies) and sheep anti-mouse (1:2500–10,000, GE Healthcare) were used as secondary antibodies and were incubated for 1 h at room temperature. Immunoreactive bands were detected by chemiluminescence (ECL, GE Healthcare) and quantified using NIH Image J software (Bethesda, MD).

Immunohistochemistry

Formalin-fixed tissue was embedded into paraffin and consecutive sections were prepared and stained for eNOS phosphorylated at serine residue 1177 (1:800, BD Transduction Labs #cat: 612392), total eNOS (1:800, BD Transduction Labs), VCAM-1 (no dilution, from hybridoma 6G10), SRA-E5 (1:100, TransGenic Inc), and nitrotyrosine (1:400, Chemicon) using a LSAB+ kit from Dako. Photomicrographs of each stained-sections from the bifurcation, as well as the proximal and distal CCA of all pigs were captured at 10x magnification using Olympus MicroSuite Biological Suite Software connected to an Olympus BX61 motorized system microscope (Leeds Precision Instruments, Minneapolis, MN).

Immunostaining quantification

VCAM-1, Scavenger-Receptor A (SRA), nitrotyrosine, phospho-eNOS and eNOS staining were analyzed using Image-Pro Plus Software (Version 6.2, Media Cybernetics, Inc., Bethesda, MD). Positive staining for each marker (except SRA) was expressed as the sum of positively stained endothelial areas divided by the luminal circumference of each arterial section. SRA staining was quantified by determining the percent of intimal area positively stained in each arterial section.

Histology

Paraffin sections from the bifurcation, proximal and distal CCA were stained for elastic fibers with Verhoeff-Van Gieson (VVG). Each arterial section was then captured on Olympus BX61 microscope as previously described for other markers. Maximal intima-media thickness (max IMT), intimal and medial areas were measured using Image-Pro Plus. IMT was defined as the distance between the external elastic lamina and the internal luminal border of the artery. Max IMT was measured three times at the widest part of the arterial section and averaged. Intimal area was defined as the area between the internal elastic lamina (IEL) and the luminal border of the artery while the area between the IEL and external-elastic lamina (EEL) was referred to as the medial area. Intima-media ratio (I/M) was calculated as the ratio of intimal- over medial-areas.

Biochemical analysis

Fresh whole blood was submitted to the Veterinary Medical Diagnostic Laboratory (VMDL) of the University of Missouri, the day of DEXA scanning for standard chemistry profiles including glucose. Frozen plasma collected the day of euthanasia was later submitted for quantification of triglycerides, total-, low- and high-density lipoprotein cholesterol blood content. Results from FH swine were compared to values measured in non-FH domestic swine. Citrate synthase activity was determined using a method described by Srere (40).

Statistical analysis

Unless otherwise stated, outcome variables were compared using 2-way ANOVA with repeated measures when appropriate, followed by post hoc testing for individual comparisons when justified. CEU signal intensity and animal characteristics were analyzed using two-tailed Student's *t* test or Mann-Whitney Rank Sum test where appropriate. Concentration-response curves for carotid arterial rings were analyzed with non-linear regression curves to determine the EC₅₀ and then, compared between groups using Student's *t* test. Maximal percent relaxation was used to compare the effect of L-NAME between groups using 2-way ANOVA on repeated measures. Statistical analyses were performed with Sigma Stat Version 3.5 for Windows (Dundas Software, Erkrath, Germany) except linear regression for which GraphPad Prism 5.0d for Mac OS X (GraphPad Software, San Diego, California) was used.

Results

Pig characteristics

Clinical characteristics of exercise-trained (Ex) and sedentary (Sed) Familial Hypercholesterolemic (FH) pigs are illustrated in Table 1. Efficacy of exercise training was demonstrated by a 33% increased endurance time between pre- and post-stress tests in Ex as well as 34% and 36% increased muscle citrate synthase activity in the deltoid and TAH muscles in the exercise-trained pigs, respectively. With exercise training, heart weight and heart weight-to-body weight ratio showed an increasing trend, which reached significance when heart weight were normalized to lean mass. Body weight and lean mass (total and individual anatomic regions) were not affected by training. Both Ex and Sed FH pigs were characterized by increased blood levels of total and LDL cholesterol and triglycerides, as shown previously (14); however, exercise training did not significantly alter overall or regional percent body fat, triglycerides, LDL-, HDL- or total-cholesterol.

Effect of training on *in vivo* VCAM-1 expression

Three minutes after injection of either type of microbubbles, the arterial wall was better visualized than on background images and microbubbles were no longer seen freely circulating within the arterial lumen (Fig. 1). Signal intensity derived from specific attachment of VCAM-1-targeted microbubbles was determined as the ratio of signal intensity from VCAM-1-to-control MBs. Since no difference was seen between signal intensity measured at 3 and 6 min, we combined data from both time points. Irrespective of group, mean VCAM-1-to-control signal intensity was greater than 1.0 indicating elevated VCAM expression in the FH carotids. However, exercise training did not result in a significant decrease in VCAM-1-to-control signal intensity in either proximal (Ex, 1.46 ± 0.55 ; Sed, 1.94 ± 0.30 ; $P = 0.24$) or distal (Ex, 1.55 ± 0.65 ; Sed, 1.85 ± 0.46 ; $P = 0.71$) CCA (Fig. 2A–B). We validated the assessment of carotid inflammation *in vivo* by examining the correlation between the signal intensity derived from VCAM-1-to-control microbubbles ratio and VCAM-1 protein expression in the proximal and distal CCA. A linear relationship was found between VCAM-1-to-control MBs signal intensity and VCAM-1 protein expression in the proximal ($r^2 = 0.50$; $p < 0.01$) and distal ($r^2 = 0.52$; $P < 0.01$) CCA (Fig. 2E–F).

Effect of training on VCAM-1 and indices of disease

A 2-fold increase in VCAM-1 mRNA was found in Ex versus Sed in both proximal ($P<0.05$; data not shown) and distal CCA ($P<0.05$; data not shown) although this increase was not reflected at the protein level (Fig. 2C–D). Exercise training had no significant effect on any of the parameters studied including endothelial VCAM-1 protein expression, intimal macrophage infiltration, endothelial oxidative stress, carotid maximal IMT and carotid intima-media ratio. Conversely, a main effect of location of carotid segment was found for all immunohistochemical markers of disease severity. Indeed, endothelial VCAM-1 expression and intimal macrophage accumulation were approximately 2 to 5-fold greater in the bifurcation compared to the proximal and distal segments of the CCA (main effect of segment: eVCAM-1, $P<0.05$; SRA, $P<0.001$ Fig. 3).

Effect of training on expression of markers of endothelial cell health

Expression of eNOS mRNA and protein was not affected by exercise training in the proximal and distal CCA, although a trend for an increase in eNOS protein expression was found in Ex in the proximal CCA (2.66 ± 0.85 vs. 1.0 ± 0.18 in Sed; $P = 0.20$; Fig. 4C). Similar findings were obtained for eNOS phosphorylation, an important regulator of eNOS activity (Ex, 2.23 ± 1.06 vs. Sed, 1.0 ± 0.27 ; $P = 0.62$; Fig. 4A). The complex anatomy of the bifurcation precluded obtaining protein, therefore we used immunohistochemistry to directly compare eNOS in the bifurcation, proximal and distal CCA. Interestingly, the proportion of phosphorylated- and total-eNOS staining throughout the vessel circumference was greater in the distal CCA (2–4 fold) compared to the bifurcation and proximal CCA, although the ratio of phosphorylated-to-total eNOS was similar throughout the carotid arteries (data not shown).

In vivo and *in vitro* assessment of endothelial-dependent vasoreactivity

Baseline carotid diameter, velocity and blood flow as well as mean arterial pressure were similar between groups (Table 1). Acetylcholine infusion resulted in a significant increase in diameter of the proximal and distal CCA, relative to saline, in both groups ($P<0.01$), however this response was not affected by exercise training (Fig. 5A, B). A trend for a greater increase in carotid diameter with the highest dose of ACH (10^{-5} M) was seen in both portions of the vessel, although it did not reach statistical significance. When studied *in vitro*, internal diameter, length, resting tension, percent stretch and PGF2- α were not different between Ex and Sed in this study (Table 1). ACH and BK elicited dose-dependent relaxation in Ex and Sed (Fig. 5C, E). A significant interaction was found between training and concentration dose of either ACH ($P<0.05$) or BK ($P<0.05$), however pairwise comparison procedures (Holm-Sidak method) failed to demonstrate a significant difference between Ex and Sed at any of the concentrations used. Moreover, the EC_{50} for neither ACH nor BK was affected by training. Maximal EDD was observed at mid-range concentrations of acetylcholine, similar to those used during local infusion. L-NAME blocked the majority of relaxation to both agonists suggesting that nitric oxide is the major contributor to EDD in carotid arteries of FH pigs, an effect that was not modified by training (Fig. 5D,F). Carotid rings from both groups exhibited a dose-dependent relaxation to sodium nitroprusside (SNP) that was not affected by training (data not shown).

Discussion

The main goals of this study were to investigate the effects of training on carotid inflammation and to evaluate the use of VCAM-1-targeted microbubbles for detection of inflammation *in vivo* in a swine model of atherosclerosis. The major findings of this study are: 1) contrary to our hypothesis, exercise training did not result in reduction of VCAM-1 protein expression along the endothelial surface of the carotid arteries; and 2) *in vivo* detection of inflammation using VCAM-1 targeted microbubbles correlated with the gold standard measure of *in vitro* VCAM-1 protein expression.

Unaltered carotid VCAM-1 expression with exercise training

To our knowledge, there are no human and very few animal studies (6, 8, 28) that have looked at the effect of regular exercise in patients with FH or animal models with FH. Exercise has been previously shown to exert effects that may vary depending on the intensity, type [aerobic vs. resistance training] and duration of the training program (16). In our institution, efficacy of the training program has been traditionally assessed by increased forelimb muscle citrate synthase activity, longer endurance time on the treadmill, increased heart weight and increased heart weight-to-body weight ratio and our results for these variables are in line with those reported in previous studies using this training protocol and performed in FH pigs of similar age and in miniature swine (6, 20). It is possible that 20 weeks of exercise training is insufficient to induce a measurable change in VCAM-1 expression. In humans, it has been suggested that exercise alters arterial wall thickness over time frame of several months and years (42). Longitudinal studies using non-invasive assessment of VCAM-1 expression over longer exercise training program may be necessary to detect measurable attenuation in pro-inflammatory pathways leading to changes in VCAM-1, such as maintenance of nitric oxide availability and repression of NF- κ B activation, an important transcription factor involved in VCAM-1 gene regulation (38)(48).

The lack of exercise effect on VCAM-1 expression in the carotid arteries may also be associated with the unaltered blood lipid profile in FH pigs. These results are in line with those obtained in diet-induced hypercholesterolemia in miniature swine submitted to a similar training protocol and, thus not unique to familial hypercholesterolemia (43). In a previous study by our group (unpublished data), carotid endothelial VCAM-1 was directly correlated with blood glucose and LDL cholesterol in sedentary FH pigs. Perhaps, the absence of impact of chronic exercise on the lipid profile or blood glucose may have contributed to similar levels of inflammation in this study.

Another factor that might have contributed to the lack of exercise effects on the expression of VCAM-1 is the early stage of atherosclerosis present in the carotid arteries in our subjects. Early in the development of atherosclerosis, the vessel wall may undergo remodeling changes such as outward remodeling to maintain normal shear rate and homeostatic functions of the endothelium. It is possible that in our pigs, activation of anti-inflammatory mechanisms may have still be sufficient to counteract the hypercholesterolemic state in both Ex and Sed, thus limiting a generalized switch towards an endothelial inflammatory phenotype and maintaining relatively low levels of VCAM-1

expression in the carotid arteries. In this early state of compensating mechanisms, exercise may not provide additional benefits.

Moreover, exercise has been previously shown to exert effects that may differ between various vascular beds (16). In humans, endurance training may result in improvement of IMT in peripheral active regions (e.g. femoral arteries) while carotid arteries are unaffected(42). We limited our investigation to the CCA to increase the translational potential of our findings since this vessel is already commonly used in humans for measurements of IMT. The superficial and easily accessible location of the human carotid artery makes this arterial bed ideal for obtainment of high-resolution images non-invasively using percutaneous ultrasound in humans. An additional advantage of carotid arteries over other peripheral arteries such as the brachial, is that the former can not only predict the extent of coronary artery disease but can assess local disease and therefore, contribute to the stratification of risk for ischemic stroke(2). In our model, the effect of exercise on VCAM-1 expression in vascular beds other than the carotid arteries still needs to be investigated. In the miniature swine model with early coronary artery disease, 20 weeks of exercise did not alter the expression of inflammatory markers including VCAM-1 in the coronary arteries whereas antioxidants capacity was improved in the coronary arterioles (1) supporting regional differences for at least the antioxidant pathways in the coronary vasculature. VCAM-1 expression in the carotid arteries was not investigated in that study. It is plausible that exercise has limited effects on pathways directly involved in regulation of inflammation but rather contributes to activation of compensatory mechanisms such as up-regulation of antioxidant pathways.

Consistent with our finding that VCAM-1 is not reduced with exercise; we found no training effect on protein expression of other markers of atherosclerotic disease such as intimal macrophage accumulation, oxidative stress, IMT, and intima-media ratio in carotid arteries. The only significant effect of exercise found in this study was an increase in VCAM-1 mRNA, which did not translate to the protein level. The cause of discrepancy between mRNA and protein expression of VCAM-1 in CCA is unknown, but could include transcriptional inhibition, post-transcriptional mechanisms, e.g. protein degradation, or inability to measure small differences in protein levels using Western blot or immunohistochemistry. Contrary to our expectations, KLF2, a transcription factor upregulated by laminar flow and involved in atheroprotective mechanisms (47), was decreased with exercise. KLF2 is an important regulator of VCAM-1 gene expression in endothelial cells, through regulation of NF- κ B nuclear accumulation (21), thus it is possible that the increase in VCAM-1 mRNA observed with exercise were mediated in part through KLF2.

Since controversy exists as to whether atherosclerosis plaque initiation has already begun prior to observation of altered EDD and since several studies have shown restoration of EDD with exercise, we concurrently measured EDD and VCAM-1 expression in carotid arteries. We found no evidence for impaired EDD in either group, despite the presence of early atherosclerosis, i.e. VCAM-1 expression and macrophage infiltration, providing evidence that endothelial inflammation precedes dysfunction. However, exercise training did

not increase *in vivo* EDD as evidenced by the finding that carotid diameter following ACH/saline infusion was similar between Ex and Sed.

The lack of training effect on *in vivo* EDD is in accordance with the absence of a significant training effect on markers of endothelial NO production (phosphorylated and total-eNOS) and on *in vitro* ED vasomotor function to ACH and BK performed on carotid artery rings. L-NAME almost abolished EDD in carotid arteries, indicating that in our study, relaxation is primarily dependent on nitric oxide release from a NOS dependent pathway, which is in agreement with a previous study (45).

Previously reported effects of exercise on endothelial function in carotid arteries are limited to rabbits and have failed to demonstrate an exercise response in either normal or hypercholesterolemic animals (7, 51, 52). We are unaware of other studies examining chronic exercise on carotid EDD in large mammals such as swine. The invasive nature of the procedure has limited evaluation of carotid endothelial function in humans. In our study, we did not evaluate EDD in other arterial beds, and thus are unaware if the lack of exercise effects in FH is unique to the carotid arteries or uniformly distributed throughout the arterial tree. In a previous study from our institution and performed on a different subset of FH pigs of similar age, exercise did not improve EDD in coronary arteries but reduced maximal contractile response to endothelin-1 (6). More studies would be needed to determine if exercise has beneficial effects on carotid arteries that were not assessed in this study.

Another factor to consider for the lack of exercise effects on carotid EDD is that carotid endothelial function may have been unimpaired in our FH pigs. In a diet-induced swine model of early vascular disease, hypercholesterolemia was shown to impair ED vasomotor function in carotid arteries (45). While we did not directly compare our FH swine to non-FH, the relative relaxation to ACH and BK obtained in our study was greater to that reported for non-FH hypercholesterolemic swine and comparable to that of pigs fed a chow diet (45), leading us to conclude that EDD was not impaired in carotid arteries in our FH swine. The impact of exercise training on vasomotor function of healthy individuals is equivocal (31). Indeed, in healthy exercise-trained pigs, 20 weeks of exercise training does not result in further enhancement of peripheral conduit arteries [brachial and femoral arteries] compared to their sedentary but otherwise healthy counterparts. Evidence indicates that EDD is transiently enhanced early after the onset of training in response to increased shear rate and then lost with continuation of the training and structural remodeling (16, 31). Since the goal of our study was not to directly assessed arterial remodeling over time, we did not measure carotid luminal diameter and wall thickness prior or during the course of training. However, exercise-trained animals did not reveal a greater carotid diameter nor decreased wall thickness compared to sedentary pigs at the end of the training program.

The relatively small number of animals per group and the variability of the response to either agonist limits the statistical power of these analyses, thus there should be caution in interpreting negative findings. While expensive, long-term studies with a larger number of animals in which EDD could be assessed over time would be needed to definitely conclude that exercise training has no effect on carotid arterial EDD or eNOS protein (19, 31).

Validation of *in vivo* detection of inflammation

Our data from CEU with VCAM-1-targeted microbubbles were positively correlated protein expression in both portions of the CCA, validating our method and minimizing the impact of the transducer location on the interpretation of the *in vivo* results. This is consistent with studies using rodent models of atherosclerosis in which preferential attachment of VCAM-1-targeted agents to regions of early plaque formation was demonstrated (17, 18, 27). Interestingly, despite the early stages of atherosclerosis in the CCA as indicated by less than 10% of the intima infiltrated by macrophages and intima/media ratios of only 1.5 to 3.5%, the greater signal intensity obtained with the VCAM-1 targeted microbubbles relative to the control microbubbles suggest that CEU is a very sensitive approach for detection of initiation of disease. If these results are proven to be similar in humans, this could dramatically impact our ability to detect early signs of carotid atherosclerosis in patients at risks, resulting in earlier and more adapted treatment and may ultimately help reduce the incidence of cerebrovascular events.

An important limitation of our study was the inability to distinguish microbubbles attached to the endothelium apart from those potentially adhered to endothelial cells of the vasa vasorum. In pigs, the depth of the carotid arteries requires increased penetration of the ultrasound beam at the expense of the axial resolution. In addition, the early stage of atherosclerosis in our pigs limited any substantial intimal thickening limiting our ability to easily identify the intima, media and adventitia individually. Since it was not possible to accurately isolate endothelial-specific signal, we measured the signal intensity in the carotid arterial wall including a minimal portion of the lumen. We therefore may have potentially included signal from VCAM-1-targeted microbubbles adhered to activated endothelial cells within vessels belonging to the most inner portion of the adventitia. We did not attempt to measure VCAM-1 staining in the adventitia, although we believe that adhered VCAM-1-targeted microbubbles in the adventitia would have contributed only a small portion of the signal detected if any since VCAM-1 staining in the adventitia of proximal and distal carotid arterial sections was either absent or minimal in all pigs except one with moderate adventitial staining. Nonetheless, none of pigs had more VCAM-1 staining in the adventitia compared to the endothelium.

In retrospect, it would have been interesting to obtain CEU data from the bifurcation of the carotid arteries since this region had the greatest expression of endothelial VCAM-1 and would have provided a wider range of VCAM-1 expression for our regression analysis. However, ultrasound imaging of the bifurcation requires removal of the first rib and therefore not suited for the development of a non-invasive approach designed to be used in longitudinal studies.

Non-uniformity of markers of atherosclerosis and endothelial cell health within the CCA

A consistent finding in our study is the increased levels of markers of atherosclerotic disease in the bifurcation compared to the proximal and distal segments of the CCA. Indeed, increased levels of carotid VCAM-1 and intimal infiltrated macrophages were associated with increased IMT, a commonly used index of disease severity previously shown to predict cardiovascular event (5). These findings are consistent with the preferential development of

atherosclerotic lesions at bifurcations and curvatures of the arterial tree, regions associated with low shear stress, and oscillatory or turbulent flow. Similarly, eNOS was significantly increased in the distal CCA compared to the bifurcation and proximal CCA, both in its phosphorylated and non-phosphorylated forms. On the other hand, oxidative stress was unexpectedly greater in the distal CCA, as shown by a 2-fold increase in nitrotyrosine staining compared to the proximal CCA. It is generally accepted that oxidative stress is associated with atherosclerosis and increased levels are usually found at regions of disturbed flow (4). Given the close proximity between the proximal CCA and the flow divider, we would predict more disturbed flow at the proximal than the distal CCA and consequently lower levels of oxidative stress in the distal versus proximal CCA. Although the reason for the discrepant finding is unknown, nitrotyrosine is a marker of peroxynitrite, which is formed by the reaction of superoxide with NO. Therefore, paradoxically, the greater nitrotyrosine may result from more abundant NO in the distal compared to the proximal CCA. Overall, these results support an inverse relationship between markers of disease and endothelial NO production and are consistent with the preferential development of atherosclerotic lesion at regions of disturbed flow such as bifurcations.

In conclusion, this study suggests that exercise training has no effect on endothelial inflammation or atherosclerosis and does not contribute to improve EDD in carotid arteries of FH swine. These conclusions cannot be extrapolated to the entire arterial tree as exercise effects are known to be complex, vascular bed specific and may involve broader endpoints than those we examined. Of importance, this study validated the use of contrast-enhanced ultrasound with VCAM-1-targeted microbubbles to detect carotid inflammation *in vivo* and provides the foundation for the use of VCAM-1-targeted microbubbles to non-invasively determine the inflammatory response to treatment over time.

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Figure 1. Representative contrast-enhanced ultrasound images of the left distal common carotid artery (CCA)

A, Longitudinal image of the distal CCA using 2D-brightness mode. The distal CCA was first located and depth optimized. The large white arrows delimit the external border of the artery. The lumen and near wall of the artery are indicated. B–C: Image of the same arterial segment before (B) and after injection of VCAM-1-targeted microbubbles (C), using ultrasound contrast mode. The presence of microbubbles adhered to the vessel wall resulted in increased signal intensity (small white arrows). Gain settings and depth were kept constant throughout the procedure. The scale located at the right of each sonographic image indicates centimeters of depth. MBs, microbubbles.

Figure 2. Effect of training on contrast-enhanced ultrasound signal in the proximal and distal common carotid arteries (CCA)

Signal intensity of VCAM-1 and control microbubbles measured at 3 and 6 min after injection were normalized to background and averaged. A–B, No difference in signal intensity was found between Ex and Sed in the proximal (A) or distal (B) CCA. The signal intensity of VCAM-1-to-control microbubbles was greater than 1.0 for Seds in the proximal CCA. Mean VCAM-1-to-control microbubbles signal intensity $\pm$ SE are represented. C–D, Endothelial VCAM-1 protein expression for Ex and Sed in the proximal and distal CCA. Mean VCAM-1 protein expression normalized to beta-actin in each group $\pm$ SE are represented. Similar levels of endothelial VCAM-1 protein were found in Ex and Sed. E–F, Relation between *in vivo* and *in vitro* detection of endothelial VCAM-1 protein expression. For each animal ($n = 14$), *in vivo* detection of VCAM-1-targeted microbubbles using contrast-enhanced ultrasound was compared to Western Blot measure of VCAM-1 protein expression. The signal intensity (SI) of VCAM-1-to-control microbubbles ratio was positively correlated with endothelial VCAM-1 protein expression in both proximal (E) and distal (F) CCA. * $P < 0.05$; CCA, common carotid arteries; Bckg, background; SI, signal intensity; MBs, microbubbles; EX, exercised-trained; SED: Sedentary animals.

Figure 3. Effect of training on endothelial VCAM-1, macrophage infiltration and oxidative stress staining in the bifurcation, proximal and distal common carotid arteries (CCA)

Training did not result in a significant difference in endothelial VCAM-1 (A), percent intimal macrophage infiltration (B) and oxidative stress (C) protein expression. A–B, A 3 to 5-fold increase in endothelial VCAM-1 expression and macrophage infiltration was seen in the bifurcation compared to the proximal and distal CCA. C, Oxidative stress as measured by nitrotyrosine staining was approximately 1.5-fold greater in the distal CCA compared to proximal CCA, whereas nitrotyrosine staining was similar in the bifurcation vs. proximal or distal CCA. Values are means $\pm$ SE. CCA, common carotid artery. * $P < 0.05$ vs. Bifurcation, † $P < 0.05$ vs. Proximal CCA.

Figure 4. Effect of training on protein levels of phosphorylated- and total-eNOS and phosphorylated-to-total eNOS ratio in proximal and distal common carotid arteries (CCA) Phosphorylated- (A, B), total-eNOS (C, D) and as phosphorylated-to-total eNOS ratio (E, F) protein levels assessed by immunoblot were not significantly different between Ex vs. Sed; however, a trend for increase in phosphorylated- and total-eNOS protein expression was seen in the proximal CCA. Values are means $\pm$ SE (n = 7 in each subgroup).

Figure 5. Effect of training on endothelial agonist-induced dilation in proximal and distal common carotid arteries (CCA)

A–B, Proximal and distal carotid luminal diameter was measured *in vivo* during 5 min of acetylcholine (10^{-6} and 10^{-5} M) infusion. Ex and Sed demonstrated similar acetylcholine induced dilation in proximal (A) and distal (B) CCA. For each group (Ex vs. Sed) and carotid segment (Proximal and Distal CCA), mean percent changes in diameter after each dose of acetylcholine compared to saline $\pm$ SE are represented ($n = 7$ in each group for ACH 10^{-6} M, and $n = 6$ in each group for ACH 10^{-5} M). C–F, Concentration-dependent acetylcholine (ACH) - and bradykinin (BK)-induced relaxation in mid CCA *in vitro*. Relaxation was expressed as percent reduction in force from PGF $_{2-\alpha}$ (30 μ M)-induced tension. Values are means $\pm$ SE ($n = 5$ in each subgroup). C, Relaxation to acetylcholine was similar between Ex and Sed. D, N^G-nitro-L-arginine methyl ester (L-NAME) significantly inhibited acetylcholine- (E) and BK-induced (F) relaxation to a similar extent in both groups.

Table 1

Clinical characteristics of Familial Hypercholesterolemic (FH) swine studied

Variable	Sed (7)	Ex (7)	Analysis
Heart weight (g)	215.7 ± 10.4	230.1 ± 11.6	ns
Body weight (kg)	58.7 ± 4.1	55.8 ± 2.6	ns
Lean mass (kg)	41.1 ± 2.6	38.3 ± 1.4	ns
Heart weight/body weight (g/kg)	3.7 ± 0.2	4.1 ± 0.1	ns
Heart weight/lean mass (g/kg)	5.3 ± 0.2	6.0 ± 0.2	†
Body fat (%)	26.2 ± 1.5	26.8 ± 1.9	ns
Glucose (mg/dL)	87.0 ± 6.7	88.1 ± 4.5	ns
Triglyceride (mg/dL)	28.6 ± 3.5	21.7 ± 1.8	ns
Cholesterol (mg/dL)	351.6 ± 39.5	315.7 ± 11.5	ns
LDL cholesterol (mg/dL)	280.1 ± 35.4	242.7 ± 10.4	ns
HDL cholesterol (mg/dL)	42.3 ± 2.1	40.4 ± 1.8	ns
Endurance time:			
Prestress test (min)	19.4 ± 0.7	20.9 ± 0.8	ns
Poststress test (min)	21.3 ± 0.9	27.7 ± 1.4	*†
Citrate synthase activity:			
TAH	14.9 ± 1.2	20.3 ± 1.6	†
TMH	10.9 ± 0.7	13.3 ± 0.9	ns
TLH	12.1 ± 0.7	13.4 ± 1.6	ns
TLtH	10.1 ± 1.2	12.3 ± 1.8	ns
Deltoid	11.4 ± 0.6	15.3 ± 1.0	†
Intima-media thickness (microns):			
Bifurcation	1787 ± 129.2	1619 ± 92.3	ns
Proximal CCA	914.2 ± 36.1	890.1 ± 52.0	ns
Distal CCA	870.2 ± 28.3	903.2 ± 72.4	ns
Baseline flow characteristics:			
Diameter (cm)	0.44 ± 0.02	0.46 ± 0.02	ns
Velocity (cm/sec)	22.2 ± 2.0	21.6 ± 2.2	ns
Blood flow (mL/min)	205.1 ± 24.7	212.7 ± 23.9	ns
Mean Arterial Pressure (mm Hg)	69.1 ± 8.0	57.9 ± 7.0	ns
Isometric tension characteristics:			
Internal diameter (mm)	1.5 ± 0.2	1.79 ± 0.1	ns
Length (mm)	3.9 ± 0.2	3.5 ± 0.3	ns
Resting tension (g)	21.1 ± 2.7	18.9 ± 2.3	ns
Wall Thickness (mm)	0.95 ± 0.04	0.94 ± 0.04	ns
Percent stretch	202.0 ± 9.3	190 ± 3.5	ns
PGF2α-induced tension (g)	59.3 ± 6.0	59.4 ± 3.0	ns

Data are means ± SE; Sed, sedentary familial hypercholesterolemic (FH) pigs; Ex, exercise-trained FH pigs; TLtH, triceps brachii lateral head; TAH, triceps brachii accessory head; TMH, triceps brachii medial head; TLH, triceps brachii long head.

† P < 0.05 for Sed vs. Ex;

* $P < 0.05$ for Ex prestress test vs. Ex poststress test comparison.

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