Exercise training blunts microvascular rarefaction in the metabolic syndrome

Jefferson C. Frisbee, Julie Balch Samora, Jonathan Peterson, and Randall Bryner

1Center for Interdisciplinary Research in Cardiovascular Sciences, 2Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown, West Virginia

Submitted 1 June 2006; accepted in final form 21 June 2006

Frisbee, Jefferson C., Julie Balch Samora, Jonathan Peterson, and Randall Bryner. Exercise training blunts microvascular rarefaction in the metabolic syndrome. Am J Physiol Heart Circ Physiol 291: H2483–H2492, 2006. First published June 23, 2006; doi:10.1152/ajpheart.00566.2006.—Reduced skeletal muscle microvessel density (MVD) in the obese Zucker rat (OZR) model of the metabolic syndrome is a function of a chronic reduction in vascular nitric oxide (NO) bioavailability. Previous studies suggest that exercise can improve NO bioavailability and reduce chronic inflammation and that low vascular NO bioavailability may be associated with impaired angiogenic responses via increased matrix metalloproteinase (MMP)-2 and MMP-9 activity. As such, we hypothesized that chronic exercise (EX) would increase NO bioavailability in OZR and blunt microvascular rarefaction through reduced MMP activity, and potentially via altered plasma cytokine levels. Ten weeks of treadmill exercise (1 h/day, 5 days/wk, 22 m/min) reduced body mass and fasting insulin and triglyceride levels in EX-OZR vs. sedentary (SED) OZR. In EX-OZR, gastrocnemius muscle MVD was improved by 19 ± 4%, whereas skeletal muscle arteriolar dilation and conduit arterial methacholine-induced NO release were increased. In EX-OZR, functional hyperemia was improved vs. SED-OZR, and minimum vascular resistance within perfused gastrocnemius muscle was reduced, although no change in arteriolar stiffness was identified. Western blotting and gelatin zymography demonstrated that neither expression nor activity of MMP-2 or MMP-9 was altered in skeletal muscle of EX vs. SED animals. Plasma markers of inflammation associated with angiogenesis, monocyte chemoattractant protein-1 and IL-1β, were increased in SED-OZR and were reduced with training, whereas IL-13 was reduced in SED-OZR and increased with exercise. These data suggest that exercise-induced improvements in skeletal muscle MVD in OZR are associated with increased NO bioavailability and may stem from altered inflammatory profiles rather than MMP function.

Skeletal muscle microcirculation; regulation of skeletal muscle perfusion; microvessel density; vascular remodeling; vascular resistance

The obese Zucker rat (OZR) is a model of the metabolic syndrome based on chronic hyperphagia (5, 21). As such, this animal model rapidly becomes obese and develops profound insulin resistance and hypertriglyceridemia, with a clinically relevant hypertension and the genesis of a prothrombotic and proinflammatory state (2, 17, 24, 51). We have previously demonstrated that, with evolution of the metabolic syndrome in the OZR, a progressive reduction in skeletal muscle microvessel density develops that can result in 25% reduction in the total number of microvessels within this tissue compared with levels determined in control animals (14). This rarefaction of the vascular network contributes to an elevation in peripheral vascular resistance in OZR (12, 14, 15) and appears to be largely independent of any developing increase in mean arterial pressure (13). In contrast, ongoing studies in our laboratory have provided compelling evidence that this reduction in microvessel density may be most accurately predicted by the chronic reduction in nitric oxide (NO) bioavailability that accompanies the developing insulin-resistant state in OZR (12, 13). However, determining mechanisms through which this low NO bioavailability-induced reduction in skeletal muscle microvessel density evolves has not been elucidated.

Studies from Matsunaga et al. (35, 36) have suggested that chronic inhibition of nitric oxide synthase (NOS) can prevent development of coronary collateral microvessels in response to chronic intermittent ischemia. Furthermore, these authors have concluded that this abrogation of ischemic-collateralization under conditions of low NO bioavailability may be because of an increased activity of matrix metalloproteinases (MMP)-2 and -9 and the generation of angiotatin (6, 35, 36). However, our recent study suggests that this pathway does not contribute to the reduction in microvessel density in skeletal muscle of normal rats receiving chronic NOS inhibition, since patterns of MMP expression and activity were unaltered between normal and Nω-nitro-L-arginine methyl ester (L-NAME)-treated rats and plasma and skeletal muscle homogenates did not exhibit alterations in angiotatin production (J. C. Frisbee, J. Balch Samora, and D. P. Basile, unpublished observation). However, these results do not address a role for MMP-2 and MMP-9 in mediating microvascular rarefaction in skeletal muscle of OZR, where a myriad of other compromised conditions exist beyond NOS inhibition.

An additional area of intense investigation with regard to vascular dysfunction and the metabolic syndrome is that of the development of a chronic inflammatory state, which appears to evolve in parallel with reductions in vascular NO bioavailability (8, 18, 30). Furthermore, interventions that can reduce the severity of the inflammatory state in the metabolic syndrome are associated with improvements in vascular reactivity and improvements in NO bioavailability (2, 8, 30). As such, one of the mechanisms through which chronic reductions in NO bioavailability may be associated with microvessel rarefaction in OZR could be via alterations in the profile of inflammation inherent in the development metabolic syndrome. However, to date, this has not been investigated.

A growing number of previous studies have indicated that the imposition of a chronic exercise regimen will increase endothelial NOS (eNOS) expression and activity, with the cumulative result of increasing NO bioavailability under con- The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpheart.org 0363-6135/06 $8.00 Copyright © 2006 the American Physiological Society

H2483
control conditions (4, 19, 29, 52) and in animal models of type I diabetes mellitus (37), heart failure (55), aging (44, 49), and the metabolic syndrome (1). Taken together with our previous work and that from Matsunaga et al. (35, 36), the present study was designed to test the hypothesis that imposition of a chronic exercise regimen would increase vascular NO bioavailability in OZR and blunt peripheral microvascular rarefaction. Furthermore, it was also hypothesized that an attenuated reduction of skeletal muscle microvessel density would be manifested through a reduced expression and/or activity of MMP-2 and MMP-9 within skeletal muscle of exercise-trained OZR because of an increased inhibition of these enzymes from the elevated vascular NO bioavailability.

MATERIALS AND METHODS

Animals

Male lean Zucker rats (LZR) and OZR (Harlan) fed standard chow and drinking water ad libitum were used for all experiments. Rats were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal care facility at the West Virginia University Health Sciences Center and all protocols received prior Institutional Animal Care and Use Committee approval. At 6–7 wk of age, rats were divided into the following two groups: 1) sedentary, where animals were cage restricted for the subsequent 10-wk period and 2) exercised trained, where animals were subjected to treadmill running for 1 h/day, 6 days/wk at 22 m/min for the subsequent 10 wk.

At 16–17 wk of age, after an overnight fast, rats were anesthetized with injections of pentobarbital sodium (50 mg/kg ip), and received tracheal intubation to facilitate maintenance of a patent airway. In all rats, a carotid artery and an external jugular vein were cannulated for determination of arterial pressure and for intravenous infusion of supplemental anesthetic, as necessary. Blood samples were drawn from the jugular vein cannula for determination of glucose, insulin, and inflammatory cytokine profiles (Linco) as well as cholesterol and triglyceride levels (Stanbio).

Preparation of Isolated Skeletal Muscle Resistance Arterioles

In anesthetized rats, the intramuscular continuation of the right gracilis artery was identified, and the vessel was surgically removed and isolated as described previously (13, 15). Subsequent to the initial equilibration period, the reactivity of isolated arterioles was assessed after challenge with ACh (10⁻¹⁰ to 10⁻⁴ M) and sodium nitroprusside (10⁻¹⁰ to 10⁻⁴ M) to assess reactivity to NO from both endothelium-dependent (ACh) and -independent (sodium nitroprusside) agonists. After completion of the above procedures, the perfusate and superfusate were replaced with Ca²⁺-free physiological saline solution (PSS) and vessels were treated with 10⁻⁸ M phenylephrine to deplete the vessel of intracellular calcium stores until all reactivity and tone were abolished. At this time, intraluminal pressure within the isolated vessel was altered, in 20-mmHg increments, between 0 and 160 mmHg, and the inner and outer diameter of arterioles was determined at each pressure, as described previously (14). These data were used to calculate arteriolar wall mechanics, indicators of structural alterations to individual microvessels.

Preparation of In Situ Blood-perfused Skeletal Muscle

In all rats, the left gastrocnemius muscle was isolated in situ, as described previously (15), and the distal stump of the sectioned sciatic nerve was inserted in a stimulating electrode and tied in place. Finally, a microcirculation flow probe (0.5 or 0.7 pS; Transonic) was placed around the femoral artery, immediately distal to its origin from the iliac artery, to measure gastrocnemius muscle perfusion. The entire preparation was covered in PSS-soaked gauze and plastic film to minimize evaporative water loss and was placed under a lamp to maintain temperature at ~37°C. At this time, heparin (1,500 IU/kg) was infused via the jugular vein to prevent blood coagulation.

Measurement of Vascular NO Bioavailability

From each animal, the abdominal aorta was removed, and vascular NO production was assessed using commercially available amperometric sensors (World Precision Instruments). Briefly, aortas were isolated, sectioned longitudinally, pinned in a Silastic-coated dish, and superfused with warmed (37°C) PSS equilibrated with 95% O₂ and 5% CO₂. An NO sensor (ISO-NOPF 100) held in a micromanipulator was placed in close apposition to the endothelial surface of the aorta, and a baseline level of current was obtained. Subsequently, methacholine was added to the vessel bath to a final concentration of 10⁻⁶ M, and the changes in current were determined. To verify that the recorded data represented endothelium-dependent NO release, the responses were reevaluated after acute treatment of the aortas with 10⁻⁴ M L-NAME.

In addition, the ascending and thoracic aorta, along with the noncannulated carotid and femoral arteries, were removed and snap-frozen in liquid N₂. These vessels were then used for the determination of NOS activity using a commercially available kit (Cayman Chemical).

Determination of Minimum Vascular Resistance

After the completion of all procedures involving the blood perfused gastrocnemius muscle in the left leg, the femoral artery was cannulated immediately distal to its origin from the iliac, and the gastrocnemius muscle was perfused with a Ca²⁺-free PSS containing 10⁻⁵ M papaverine and sodium nitroprusside for 5 min, with the jugular vein cannula open to maximally dilate and flush the microvascular networks within the muscle. Subsequently, the gastrocnemius muscle was perfused at increasing flow rates for 3 min each (0.5–3.0 ml/min), and perfusion pressure was monitored continuously. These data were used to calculate the minimum vascular resistance across the gastrocnemius muscle. Gastrocnemius mass did not differ significantly across the different groups, averaging 2.3 ± 0.2 g.

Histological Determination of Microvessel Density

At the conclusion of the muscle contraction protocols, the gastrocnemius muscle was removed, rinsed in PSS, and fixed in 0.25% formalin. Muscles were embedded in paraffin and cut into 5-μm cross sections. Sections were incubated with Grifóonia simplicifolia I lectin (Sigma), as described previously (14, 20). From each gastrocnemius muscle, six individual cross sections were used for analysis, with six randomly selected regions within an individual cross section chosen for study. Each region of study had an area of ~1.47 × 10⁵ μm². All acquired images from individual sections were analyzed for microvessel number using Metamorph imaging software (Universal Imaging, Downingtown, PA).

MMP Expression

After tissue homogenization, total protein was quantified using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL), and 50 μg total protein were loaded in each lane. The resolving gel used for these procedures contained (for 2 gels) 5 ml buffer, 4 ml of 40% acrylamide (29:1), 10.92 ml dH₂O, 80 μl of 20% ammonium persulfate (APS), and 16 μl of tetramethylethylenediamine (TEMED). After being poured, the gel was overlaid with 0.1% SDS. The stacking gel used for these procedures contained (for 2 gels) 3 ml of buffer, 1.36 ml 40% acrylamide (29:1), 7.64 ml dH₂O, 60 μl of 20% APS, and 10 μl of TEMED. All wells were loaded with samples, with one well containing the protein ladder (no. 161–0324; Bio-Rad). After transfer at 50 volts for 8 h, the membrane was placed in Superblock T20 (no. 37516; H2484 EXERCISE, NITRIC OXIDE, AND MICROVESSEL RAREFACTION • VOL 291 • NOVEMBER 2006 • www.ajpheart.org

Downloaded from http://ajpheart.physiology.org/ by 10.220.33.3 on November 10, 2017
Pierce) at room temperature for 1 h with gentle rocking. After 1 h, the blocking buffer solution was replaced with new buffer containing the primary antibody (MMP-2, 1:5,000; MMP-9 1:1,000; Santa Cruz Biotechnology) and was incubated at room temperature for 1 h with gentle shaking when the antibody solution was replaced with PBS containing 0.0005% Tween 20 (PBS-T). After three PBS-T washes, the membrane was treated with blocking buffer containing the secondary antibody (1:100,000 for both MMP-2 and MMP-9) for 1 h at room temperature. The membranes were placed on a transparent sheet of plastic and covered with 1 ml of enhanced chemiluminescence reagent mixture (no. RPN-2106; Amersham Biosciences). The membranes were then exposed to X-ray film (Biomax Light Film catalog no. 178–8207; Kodak). Finally, membranes were then washed three times in PBS-T as before and exposed to the actin antibody (Santa Cruz Biotechnology).

Gelatin Zymography

The resolving gel (10% acrylamide) used for these studies contained 2.5 ml of 40% acrylamide (29:1), 1.875 ml of 2 M Tris (pH 8.8), 4.6 ml of dH2O, 100 µl of 10% SDS, 50 µl of 10% APS, 10 µl of TEMED, and 1 ml of gelatin (10 mg/ml, made immediately before pouring the gel). Upon completion, the resolving gel was overlaid with 0.1% SDS. The stacking gel (4% acrylamide) used contained 530 µl of 40% acrylamide (29:1), 700 µl of 1 M Tris (pH 6.8), 3.78 ml of dH2O, 50 µl of 10% SDS, 25 µl of 10% APS, and 5 µl of TEMED.

Samples were diluted in 2× sample buffer [1.25 ml of 1 M Tris (pH 6.8), 2 ml glycerol, 4 ml of 10% SDS, 0.5 ml of 0.1% bromphenol blue, and 2.25 ml dH2O] in a 1:1 ratio. After the gel was loaded, samples were run at 100 volts until dye front has just barely run off the bottom of the gel (~3.5–4 h). The separating gel was then incubated in 2.5% Triton X-100 (diluted with dH2O) for 30 min at room temperature with gentle rocking. The Triton solution was then replaced with 100 ml of 1× developing buffer (1.2 g Tris base, 6.3 g Tris-HCl, 11.7 g NaCl, 0.74 g CaCl2 and dH2O to 100 ml, with 200 µl Brij 35) and equilibrated for an additional 30 min at room temperature, after which the developing buffer was replaced with a fresh 100 ml and the gels were incubated at 37°C overnight. The next morning, the developing buffer was removed, and the gel was stained with 0.5% Coomassie blue R-250 for 30 min at room temperature, after which the gel was treated with a destaining solution (250 ml methanol-50 ml acetic acid-200 ml dH2O). Areas of protease activity appear as clear bands on a blue background.

Citrate Synthase Activity Determination

Portions of the contralateral gastrocnemius muscle from each rat (~20 mg) were homogenized on ice in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Citrate synthase activity was determined spectrophotometrically, as described previously (47). Briefly, homogenates were frozen under liquid N2 and thawed four times to disrupt the mitochondria and to expose the citrate synthase. The assay system contained in a total volume of 200 µl: 100 mM Tris buffer (pH 8.35) 5 mM 5,5-dithiobis(2-nitrobenzoate) (DTNB), 22.5 mM acetyl-CoA, 25 mM oxaloacetate (OAA), and 4 µM of homogenate of muscle. The principle of the assay was to initiate the reaction of acetyl-CoA with OAA and link the release of free CoA reductase (CoA-SH) to a colorimetric reagent, DTNB (acetyl-CoA + OAA + H2O → citrate + CoA-SH, then CoA-SH + DTNB → mercaptide ion). The rate change in color was monitored at a wavelength of 405 nm at 15-s intervals for a period of 3 min by using a Dynex MRX plate reader controlled through personal computer software (Revelation; Dynatech Laboratories). The solubilized protein extracts of the homogenates were quantified in duplicate by using BCA reagents (Pierce) and BSA standards. The enzymatic activity was normalized to total protein content and was reported in units of activity per milligram protein per minute.

Experimental Protocols

In all protocols, before preparation of the left gastrocnemius muscle, the right gracilis muscle resistance arteriole was surgically removed and isolated for the evaluation of vascular reactivity and passive mechanical characteristics of the vessel wall, as described above. Upon completion of the full surgical preparation, the gastrocnemius muscle was allowed 30 min of self-perfused rest and was then stimulated (via the sciatic nerve) to perform a bout of isometric twitch contractions (5 Hz, 0.4-ms duration, 5 volts) lasting for 3 min, with arterial pressure and femoral artery blood flow continuously monitored. After completion of the muscle stimulation protocols, the gastrocnemius muscle was perfused with Ca2+-free PSS as described above for the determination of minimum vascular resistance. At the conclusion of these procedures, the muscle was removed and cleared of all nonmuscular tissue, and the mass was determined. Finally, the muscle was prepared for the determination of microvessel density, the aortic and other arterial segments were removed for assessment of NO bioavailability and NOS activity, and the contralateral gastrocnemius muscle was removed for the determination of MMP-2 and MMP-9 expression/activity, and citrate synthase activity, as described above.

Data and Statistical Analyses

Arterial reactivity. Dilator responses of isolated arterioles after challenge with ACh or sodium nitroprusside were fit with the three-parameter logistic equation:

\[ y = \min + \left( \max - \min \right) \left( 1 + 10^{\log \beta + \log \alpha x - y} \right) \]

where \( y \) represents the change in arteriolar diameter, “\( \min \)” and “\( \max \)” represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing agonist concentration, \( x \) is the logarithm of the agonist concentration, and logED50 represents the logarithm of the agonist concentration (\( x \)) at which the response (\( y \)) is halfway between the lower and upper bounds.

Vascular mechanics. The calculations for determining the passive mechanical characteristics of the microvessel wall were as follows.

Incremental arteriolar distensibility (DISTINC; % change in arteriolar diameter/mmHg) was calculated as:

\[ \text{DIST}\text{INC} = \frac{\Delta \text{ID} / (\text{ID} \times \Delta \text{P}_{\text{int}})}{100} \]

where \( \Delta \text{ID} \) represents the change in internal arteriolar diameter for each incremental change in intraluminal pressure (\( \Delta \text{P}_{\text{int}} \)).

For the calculation of circumferential stress, intraluminal pressure was converted from mmHg to N/m², where 1 mmHg = 1.334 × 10⁻⁵ N/m². Circumferential stress (\( \sigma \)) was then calculated as:

\[ \sigma = \frac{(\text{P}_{\text{int}} \times \text{ID})}{(2\pi T)} \]

where WT represents wall thickness (µm; calculated as one-half of the difference between arteriolar outer diameter and inner diameter).

Circumferential strain (\( \varepsilon \)) was calculated as:

\[ \varepsilon = \frac{(\text{ID} - \text{ID}_0)}{\text{ID}_0} \]

where \( \text{ID}_0 \) represents the internal arteriolar diameter at the lowest intraluminal pressure (i.e., 5 mmHg). Circumferential stress vs. strain curves were fit with an exponential regression equation: \( y = \alpha e^{\beta x} \), where \( y \) represents circumferential wall stress at a given wall strain \( x \), \( \alpha_0 \) represents an intercept term, and \( \beta \) represents a constant related to the rate of increase of the stress-vs.-strain curve. All fitting of regression equations employed ordinary least-squares analysis with \( r^2 > 0.91 \).

Correlations between vascular NO bioavailability (estimated using the upper bound for the ACh concentration-response curves), plasma cytokine levels, and microvessel density were estimated using a
weighted-average methodology where the average value at neighboring points was estimated using an inverse distance computation.

All data are presented as means ± SE. For arteriolar reactivity, statistically significant differences in upper bound or logED50 were determined using ANOVA. Similarly, differences in individual characteristics describing the rat groups in the present study, microvessel density within gastrocnemius muscle, slope coefficients describing the circumferential stress vs. strain relationship, NO production, NOS activity, and gastrocnemius muscle perfusion were determined using ANOVA. In all cases, Student-Newman-Keuls post hoc test was used when appropriate, and \( P < 0.05 \) was taken to reflect statistical significance.

**RESULTS**

Data describing the baseline characteristics of rats under the conditions of the present study are summarized in Table 1. At the time of experimentation, sedentary OZR (OZR-SED) rats were significantly heavier than their lean counterparts (LZR-SED), and demonstrated impaired glycemic control and severe hypertriglyceridemia. The imposition of the chronic exercise (EX) regimen did not significantly impact the measured variables in lean rats (LZR-EX) but lowered body mass, plasma insulin levels, and plasma triglyceride levels in obese animals (OZR-EX). In contrast, chronic treadmill running did not impact either mean arterial pressure or plasma cholesterol in OZR. Gastrocnemius muscle citrate synthase activity was not different between LZR and OZR under either the SED or EX condition. However, citrate synthase activity was significantly increased in both groups from levels under SED conditions in response to imposition of the chronic exercise training regimen.

Data describing vascular NO bioavailability are summarized in Fig. 1. In response to challenge with increasing concentrations of ACh, arterioles from LZR-SED exhibited substantial dilator reactivity, whereas, in contrast, vessels from OZR-SED demonstrated an attenuated dilation. Although chronic exercise had no significant impact on dilator responses to ACh in LZR, resistance arterioles from OZR-EX demonstrated an improved reactivity in response to agonist application (Fig. 1A). In all animal groups, acute administration of t-NAME severely attenuated arteriolar dilator reactivity in response to ACh (data not shown). These data were consistent with agonist-induced NO bioavailability, determined using the amperometric NO sensors and the isolated aortic strips, since the low NO release in response to increasing concentrations of methacholine in OZR-SED was improved with exercise in OZR-EX toward levels determined in both sedentary and trained LZR (Fig. 1B). Interestingly, NOS activity was not different between LZR-SED and OZR-SED, and imposition of a chronic exercise regimen had no impact on these relationships (Fig. 1C). Arteriolar dilator reactivity did not differ between the four animal groups in response to application of the NO donor sodium nitroprusside (Fig. 1D).

Figure 2 presents the circumferential stress vs. strain relationship of arteriolar wall (Fig. 2A) and the gastrocnemius muscle microvessel density (Fig. 2B) from the four rat groups in the present study. Compared with responses in LZR-SED, arterioles from OZR-SED exhibited increased arteriolar wall stiffness, manifested as a left shift in the stress vs. strain relationship (Fig. 2A). However, this increased stiffness was not altered in response to chronic exercise, since neither LZR-EX nor OZR-EX demonstrated alterations in this response compared with that determined in their SED controls. In contrast, skeletal muscle microvessel density in OZR-SED, reduced compared with that in LZR-SED, was improved as a result of chronic exercise training (Fig. 2B), although this remained significantly lower than that in the LZR-SED animals.

Active hyperemia (Fig. 3A) and minimum vascular resistance (Fig. 3B) within the gastrocnemius muscle of rats under the conditions of the current study are summarized in Fig. 3. Although the increase in skeletal muscle blood flow after 3 min of 5 Hz isometric twitch stimulation was attenuated in OZR-SED rats compared with that in LZR-SED, chronic exercise training improved this response in OZR-EX such that it was not significantly different from that in LZR (Fig. 3A). As shown in Fig. 3B, the minimum vascular resistance across the maximally dilated gastrocnemius muscle of OZR-SED was elevated compared with that in LZR-SED, although chronic exercise training lowered this relationship to an intermediate level between that determined for OZR-SED and LZR-SED.

Protein analysis of MMP-2 expression and activity within skeletal muscle homogenates from rats under the conditions of the present study are summarized in Fig. 4. Neither MMP-2 expression (Fig. 4A) nor activity (Fig. 4B) was altered in skeletal muscle homogenates in the four groups of rats in the current study. Neither MMP-9 expression nor activity was consistently demonstrated in the skeletal muscle homogenates of these animals, either under sedentary conditions or in response to chronic exercise.

Figure 5 presents data describing alterations in the levels of proangiogenic cytokines in the plasma of sedentary and exercised LZR and OZR. As shown in Fig. 5A, plasma IL-13 levels were significantly reduced in OZR-SED compared with that determined in LZR. However, after 10 wk of treadmill exercise, plasma levels of IL-13 were increased in OZR-EX compared with that determined in sedentary animals. Plasma levels of monocyte chemotactic protein (MCP)-1 (Fig. 5B) and IL-1β (Fig. 5C) were significantly elevated in OZR-SED compared with LZR-EX, and chronic exercise training reduced the levels of these markers of inflammation in OZR-EX.
although these remained significantly greater than that in LZR-SED. Although additional cytokines demonstrated alterations with either the development of the metabolic syndrome or the imposition of a chronic exercise training regimen in OZR, under the conditions of the present current study, only these three cytokines demonstrated both consistent and statistically significant changes.

Figure 6 presents correlations between vascular NO bioavailability, proangiogenic cytokines, and microvessel density in all rats in the current study. Vascular NO bioavailability, estimated using the upper bound of the arteriolar dilator reac-

Fig. 1. Data (means ± SE) describing skeletal muscle arteriolar dilation in response to increasing concentrations of ACh (A) in both sedentary (SED) and exercise (EX)-trained lean Zucker rat (LZR) and obese Zucker rat (OZR). Also presented are data describing vascular nitric oxide bioavailability after challenge with increasing concentrations of methacholine, determined using amperometric nitric oxide sensors (B), and endothelial nitric oxide synthase activity, determined using a commercially available kit determining the production of labeled citrulline from arginine (C). In D, arteriolar dilation in response to challenge with the nitric oxide donor sodium nitroprusside is presented for sedentary and exercise-trained rats; n = 6–8 rats for each group; P < 0.05 vs. LZR-SED (*) and vs. OZR-SED (†).
activity to ACh, was positively correlated with the plasma levels of the proangiogenic cytokine IL-13 (Fig. 6A), and both were positively correlated with microvessel density. In contrast, vascular NO bioavailability was negatively correlated with angiogenic cytokines MCP-1 (Fig. 6B) and IL-1β (Fig. 6C), and these cytokines were also negatively associated with gastrocnemius muscle microvessel density.

**DISCUSSION**

Previous studies have clearly demonstrated a critical role for vascular NO bioavailability in regulating angiogenic responses (31, 39, 44). Furthermore, our recent studies have strongly suggested that a chronic reduction in vascular NO bioavailability may represent a major contributing mechanism underlying the progressive skeletal muscle microvascular rarefaction in the metabolic syndrome (13). As such, the purpose of the present study was to determine if a chronic improvement in vascular NO bioavailability in the OZR would blunt the severity of the evolving reduction in skeletal muscle microvessel density. Because previous studies have repeatedly demonstrated that chronic exercise training will improve eNOS expression and/or activity, and thus vascular NO bioavailability (4, 19, 29, 52), we employed a chronic treadmill running protocol for OZR as a means for increasing NO bioavailability. Additionally, building from recent studies from Matsunaga et al. (35, 36), the present study also tested the hypothesis that an improvement in skeletal muscle microvessel density determined in OZR as a result of the exercise training regimen...

---

**Fig. 3.** Data (means ± SE) describing perfusion of the in situ gastrocnemius muscle of rats at rest and in response to 3 min of 5-Hz isometric twitch contraction, delivered through the sciatic nerve (A) and the pressure vs. flow relationship determined during pump perfusion of the maximally dilated in situ gastrocnemius muscle (B) in sedentary and exercise-trained LZR and OZR; n = 6–8 rats for each group. P < 0.05 vs. LZR-SED (*) and vs. OZR-SED (†).

---

**Fig. 4.** Western blot (A) and gelatin zymography (B) for matrix metalloproteinase (MMP)-2 in skeletal muscle homogenates from sedentary and exercise-trained LZR and OZR. Also presented are the results of these analyses presented as semiquantitative densitometric units (means ± SE; n = tissue from 6–8 animals for each), normalized to the band intensity in LZR-SED. Neither expression nor activity of MMP-9 could consistently be demonstrated in skeletal muscle homogenates of rats under the conditions of the present study.
would be associated with an elevated vascular NO bioavailability-based reduction in the expression and/or activity of MMP-2 and MMP-9 within the skeletal muscle compared with levels determined in sedentary animals. The results from the present study suggest that chronic treadmill running exercise can increase vascular NO bioavailability, and thus blunt the severity of the rarefaction of the skeletal muscle microcirculation of OZR. However, these results also suggest that this effect was independent of any alterations in skeletal muscle MMP-2 and/or MMP-9 expression or activity, since these parameters were unaltered by either evolution of the metabolic syndrome or imposition of the exercise regimen. Finally, the beneficial impact of the chronic exercise training regimen on vascular NO bioavailability and skeletal muscle microvessel density was associated with an alteration in the levels of specific markers of chronic inflammation, including an increased plasma level of IL-13 and a reduction in the levels of MCP-1 and IL-1β.

The initial observation of the present study was that imposition of a chronic exercise training regimen reduced the severity of several indexes of the metabolic syndrome, independent of diet. This observation was expected in that regular exercise has been shown previously to be highly effective in reducing the severity of several parameters associated with the metabolic syndrome, including insulin resistance, hypertriglyceridemia, and severe obesity (3, 7, 23, 40, 43). Interestingly, the effectiveness of exercise training on the moderate hypertension that develops in OZR is somewhat unclear, since both antihypertensive effects of exercise (1) and no effect (53 and the present study) have been demonstrated. It is possible that this may reflect the duration of the training regimen, since Arvola et al. (1) employed a 22-wk protocol compared with the shorter-duration protocols used in the present study and by Xiang et al. (53).

Numerous previous studies have convincingly demonstrated that a chronic exercise regimen is effective in increasing vascular NO bioavailability (4, 19, 29, 52), although the specific mechanisms underlying this are less clearly elucidated. Although some investigators have demonstrated increases in eNOS expression or activity, other recent observations are heterogeneous with regard to the longitudinal location within the vascular networks (38). Furthermore, other studies have been unable to demonstrate consistent differences in NOS expression or activity and have concluded that different elements of the NOS signaling cascade may be involved in the exercise training-induced elevation in NO bioavailability (46). Results from the present study support these previous observations, since imposition of the chronic exercise regimen increased agonist-induced mechanical responses (ACh; Fig. 1A) or NO release (methacholine; Fig. 1B), although eNOS expression (Fig. 1C) did not differ between any of the groups within the present study. These results strongly suggest that chronic exercise training was able to prevent a portion of the chronic reduction in vascular NO bioavailability in OZR and that this was correlated with a reduction in obesity and an improved glycemic control (Table 1).

Consistent with our previous results (12–15), microvascular remodeling associated with progression of the metabolic syndrome in OZR falls into the following two major categories: reduced distensibility of the arteriolar wall and rarefaction of the microvessel networks. With regard to arteriolar distensibility, the increased microvessel wall stiffness that develops with the metabolic syndrome in OZR, also evident in humans (10), was not ameliorated to any discernible extent by the exercise regimen. This lack of an effect on reduced vascular distensibility as a result of chronic exercise in the metabolic syndrome has also been identified in humans through the Atherosclerosis Risk in Communities study (45) and suggests that this process may be divorced from vascular NO bioavailability. In contrast,
imposition of an exercise regimen blunted rarefaction of the microvessel network in OZR such that vessel density within skeletal muscle of these animals reached an intermediate level between control (sedentary) LZR and OZR.

The functional implications of these alterations in vascular network structure are presented in Fig. 3, where a blunted hyperemic response to near-maximum metabolic demand, for isometric twitch contractions, and an elevated minimum vascular resistance were partially improved as a result of the chronic exercise training. Given that the employed degree of metabolic demand results in a near-maximal level of physiological dilation and that the conditions under which minimum vascular resistance was determined represent the maximum vascular dilation and recruitment, these data primarily address issues of vascular structure rather than reactivity. Furthermore, because arteriolar distensibility was not improved with exercise and vascular NO bioavailability, alterations in hyperemic responses and minimum vascular resistance may predominantly reflect alterations in microvessel density rather than structural narrowing. When integrated with results from our previous studies (12, 13), these observations suggest that prevention of the chronic reduction in vascular NO bioavailability in OZR may be beneficial in terms of preventing degradation of microvessel density, which can contribute to the elevated vascular resistance (14), impaired active hyperemia (15), and premature skeletal muscle fatigue (15) in these animals.

Previous studies have determined that the evolution of the metabolic syndrome is closely associated with the genesis of a chronic subacute inflammatory state (8, 22, 30), including elevated markers such as MCP-1 (50, 51) and IL-1β (26). Furthermore, ongoing studies have also determined that imposition of a chronic exercise regimen can have the beneficial impact of blunting the severity of this inflammatory condition and can include reductions in the levels of C-reactive peptide, IL-1β, tumor necrosis factor-α, and IL-6 (18, 42, 48). To investigate the correlation between the genesis of the metabolic syndrome and the regulation of skeletal muscle microvessel density in response to chronic exercise, we determined levels of inflammatory cytokines associated with angiogenesis within the setting of cancerous tumor progression [IL-13 (16, 33, 34), MCP-1 (25, 27, 28, 33), and IL-1β (9, 41, 54)]. As presented in Fig. 6, where data from all rats in the present study were included, inflammatory markers associated with proangiogenesis in cancer demonstrated significant alterations in the current study. IL-13 levels, reduced in the metabolic syndrome in sedentary OZR, were increased as a result of the exercise regimen and were positively correlated with vascular NO bioavailability and skeletal muscle microvessel density. In contrast, MCP-1 and IL-1β, proangiogenic cytokines in cancer, were strongly elevated in sedentary OZR, conditions where vascular NO bioavailability was very low and microvessel density was reduced. However, with imposition of the chronic

Fig. 6. Correlations between vascular nitric oxide bioavailability, plasma cytokine levels, and skeletal muscle microvessel density in rats. Data are presented for specific proangiogenic cytokines IL-13 (A), MCP-1 (B), and IL-1β (C) and their correlations with microvessel density and vascular NO bioavailability (estimated using the upper bound of the ACh concentration-response curves from isolated skeletal muscle resistance arterioles). The presented data represent the surface of best fit through the data from all rats; individual data points and error terms are omitted for increased clarity and ease of interpretation.
exercise regimen, which caused an increased NO bioavailability and skeletal muscle vascular density, the levels of MCP-1 and IL-1β were substantially attenuated. It may be the case that markers of angiogenic potential in cancer are less predictive of microvessel density in the setting of the metabolic syndrome where microvascular networks are rarefied. Furthermore, these results suggest that the imposition of a chronic proangiogenic stimulus (e.g., exercise) may further complicate the predictive power of these inflammatory markers in terms of angiogenic potential. Clearly, further investigation into the role of inflammation as a contributor to the regulation of skeletal muscle microvessel density in the metabolic syndrome and the role of chronic vascular NO bioavailability is warranted.

Previous studies by Matsunaga et al. (35, 36) indicated that ischemic collateralization of the canine myocardium was strongly dependent on a sufficient bioavailability of NO, which in part would constrain activation of MMP-2 and MMP-9 and the subsequent generation of angiotatin. Although our recent work suggests that the skeletal muscle microvessel rarefaction that develops in normal rats under conditions of chronic L-NAME treatment may not be dependent on MMP activity and angiotatin production (J. C. Frisbee, J. Balch Samora, and D. P. Basile, unpublished observation), the concept of MMP activation contributing to microvascular rarefaction under the conditions of the metabolic syndrome has not been addressed. The results from the present study suggest that neither MMP-2 (which was unchanged) nor MMP-9 (which was not consistently demonstrated) expression and/or activity contributed to a significant extent to either the reduction in skeletal muscle microvessel density under conditions of the metabolic syndrome or the blunting of this effect as a result of chronic exercise in OZR.

In summary, with evolution of the metabolic syndrome, chronic reduction in vascular NO availability is associated with a progressive rarefaction of peripheral microvascular networks. Although this rarefactive response appears to be independent of activity of MMP-2 and MMP-9, it is associated with considerable alterations in the inflammatory profile. Chronic exercise training blunts the severity of the metabolic syndrome in OZR and is associated with increased vascular NO bioavailability vs. sedentary animals. This improved NO bioavailability is correlated with an increased skeletal muscle microvessel density in OZR and an altered profile of inflammatory markers associated with angiogenesis, such that levels of select proangiogenic cytokines are returned toward normal (in LZR). These alterations in NO levels, profiles of inflammation, and microvessel density are associated with an improved active hyperemia and a reduced minimum vascular resistance of in situ skeletal muscle in trained OZR vs. that determined in sedentary control animals.

ACKNOWLEDGMENTS

We thank Milinda E. James and Adam G. Goodwill at the West Virginia University Health Sciences Center for expert technical assistance. Portions of this work were presented at the American Heart Association Atherosclerosis, Thrombosis and Vascular Biology Council Meeting, Denver, CO, April 2006.

GRANTS

This work was funded by Grant 0330194N from the American Heart Association and National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-64668.


26. Hristova MA. *H2492 EXERCISE, NITRIC OXIDE, AND MICROVESSEL RAREFACTION*.


