SUPPLEMENTAL MATERIALS AND METHODS

Subjects

Young (age 18-41) men and women were recruited to participate in this study. Lean subjects (n = 14) had a body mass index (BMI) ranging from 18-25 kg/m^2. Overweight and obese subjects (n = 28) were recruited across a range of BMI from 28-40 kg/m^2. Subjects were ultimately divided into lean, intermediate (BMI 28-32.5 kg/m^2), and obese (BMI 33-40 kg/m^2) groups. Prior to initiation of the study, subjects were not involved in any physical activity program (> 4 METS for > 30 minutes per day, > 1 day per week) for the previous six months. All subjects were non-smokers with no known history of cardiovascular disease. Subjects with a fasting blood glucose concentration greater than 125 mg/dl were excluded. Subjects were not taking any medications for hypertension, hypercholesterolemia, insulin resistance or non-insulin dependent diabetes mellitus. Subjects abstained from antioxidant supplementation for the duration of the experimental period. All female subjects were on oral contraceptive medication. There were no restrictions with regard to race, gender, or socioeconomic status. All procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University.

Anthropometrics, Metabolic Parameters, and Vascular Injury Markers

Height was measured with a stadiometer to the nearest 0.1 cm. Body mass was measured with a digital electronic scale to the nearest 0.05 kg. BMI was calculated as body mass in kilograms divided by height in meters squared (kg/m^2). Body fat percentage was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI, USA). Following microdialysis probe insertion, approximately 12 ml of blood was collected from an antecubital vein. Blood samples were allowed to clot and then were centrifuged at 3300 rpm for approximately 10 minutes. Serum glucose, insulin, triglycerides (TG), total-cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were assessed by a commercial clinical laboratory (Laboratory Corporation of America). Low density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula[1]: LDL-C (mg/dl) = TC – HDL-C – (TG/5). The homeostatic model of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations as HOMA-IR = [Insulin (mU/l) * Glucose (mmol/l)]/22.5.[2] Serum concentrations of C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), soluble intracellular adhesion molecule-3 (sICAM-3), E-selectin, P-selectin, thrombomodulin, and serum amyloid a (SAA) were measured with human vascular injury panel I and II ELISA kits (Meso Scale Discovery, Rockville, MD).

Microdialysis Protocol

Following an overnight fast, three microdialysis probes with a 20 kDa membrane cut-off (CMA 20 Elite, CMA Microdialysis AB, Solna, Sweden) were inserted at least 3 cm apart into the left vastus lateralis under sterile technique and local anesthesia as previously described,[3,4] while the subject was resting in a hospital bed. Custom CMA 20 Elite microdialysis probes with a 40 mm pre-membrane shaft were used for subjects with a quadriceps skinfold > 10 mm to ensure embedding of the entire 10 mm semi-permeable membrane in the skeletal muscle. A schematic of the microdialysis set-up is illustrated in Supplemental Figure I. Probes were then perfused with a 0.9% saline solution containing 5 mM ethanol (EtOH) with microinfusion pumps (CMA 107, CMA/Microdialysis, Solna, Sweden) at a flow rate of 2.0 µl/min for the remainder of the experiment. The perfusion protocol throughout the experiment is outlined in supplemental table I. One of the three probes had 300 µM apocynin added to the perfusate for the duration of the experiment to test the impact of NADPH oxidase on ROS production and microvascular

endothelial function (Supplemental Table I). Probes were perfused for 60 minutes to allow for recovery from trauma induced by probe insertion. Three-10 minute samples were subsequently collected from each probe in 150 µl polyethylene collection vials, which were capped and stored for ethanol analysis to determine baseline blood flow.

Measurement of in vivo ROS

In vivo skeletal muscle extracellular H_2O_2 and superoxide was measured via microdialysis as described recently.[5] Briefly, microdialysis probes were perfused with saline containing 100 µM Amplex Ultrared (Molecular Probes, Eugene, OR, USA) and 1.0 U/ml horseradish peroxidase (HRP; Sigma Aldrich, St. Louis, MO, USA). Three 20-minute samples were collected during phase 2 of the microdialysis protocol (Supplemental Table I), and fluorescence of the outflowing dialysate was measured with a TD-700 laboratory fluorometer (Turner Designs, Sunnyvale, CA, USA) at Ex 550/Em 570 nm wavelength. 10 U/ml SOD (Sigma Aldrich, St. Louis, MO, USA) was then added to the perfusate (Phase 3, Supplemental Table I) allowing for the conversion of superoxide that crosses over the membrane to H_2O_2, which reacts with Amplex Ultrared/HRP. A schematic of the microdialysis flow circuit is illustrated in Supplemental Figure II. Relative fluorescence units were converted to [H_2O_2], based on an [H_2O_2] standard curve conducted by addition of known concentrations of H_2O_2 to each perfusate following each experiment.[5]

Assessment of Microvascular Endothelial Function

Following assessment of ROS production, the perfusion media was changed on all three probes (Phase 4, Table 1). The new perfusates were composed of a 0.9% saline solution with 5 mM EtOH and one of the following three pharmacological agents: 1) 50 mM sodium nitroprusside (SNP) to test the endothelium-independent vasodilatory response, 2) 50 mM acetylcholine (ACh) to test the endothelium-dependent vasodilatory response, or 3) 50 mM ACh + 300 µM apocynin to test the influence of NADPH oxidase on the endothelium-dependent vasodilatory response. Preliminary data has determined 50 mM concentrations of SNP and ACh to elicit an intermediate vasodilatory response in a dose-response relationship. Probes were perfused for 30 minutes to allow for equilibration, and three-10 minute samples were collected over the subsequent 30 minutes where the pharmacological response is maximal. Samples were stored in capped collection vials at 4°C and analyzed within eight hours. During perfusion, ethanol diffuses from the microdialysis membrane and is carried away from the local area by the microcirculatory blood flow in the immediate vicinity of the microdialysis probe membrane.[3,6-9] Ethanol concentrations of the perfusate and dialysate were analyzed using an enzymatic-flourometric method as described.[8] The ethanol outflow-to-inflow ratio was calculated as $[Ethanol]_{dialysate}/[Ethanol]_{perfusate}$, which is inversely related to local blood flow in a non-linear fashion, and was converted to blood flow units ($ml \cdot min^{-1} \cdot 100\ g^{-1}$) as described.[10,11] Drugs perfused through the microdialysis probe interact with the extracellular environment within an approximate 2 mm radial distance of the semi-permeable membrane,[11,12] effectively creating a 10mm x 2mm x 2mm cylindrical microenvironment within the muscle tissue in which these pharmacologic agents act. This area of action is the same for all subjects, negating the need to normalize responses to tissue volume or to adjust the dose of ACh used as is typically done in investigations of endothelial function involving ACh brachial artery infusion.[13,14]

Aerobic interval training

Following the initial microdialysis session, subjects performed a standardized maximal exercise test to assess maximal aerobic capacity. VO_{2peak} was assessed via open circuit

spirometry (TrueMax 2400; Parvomedics; Salt Lake City, UT, USA) using the Storie protocol, a treadmill ramp protocol where the speed or incline is increased every two minutes until volitional fatigue is reached. Heart rate was recorded every minute throughout the test and immediately upon fatigue to determine the maximum heart rate (HR_{max}). Subjects then performed an aerobic interval training intervention, consisting of exercise three days per week for eight weeks. Exercise consisted of walking/running up an incline (7-12%) on a motor-driven treadmill. Subjects performed a 10-minute warm-up at ~60% HR_{max}, followed by four 4-minute intervals at 88-92% HR_{max} interspersed by 3-minute active recovery periods at ~70% HR_{max}. The intervals were followed by a 4-minute cool down at ~60% HR_{max}, yielding a total exercise time of 42 minutes per session. HR was monitored throughout each training session, and intensity (speed or incline) was increased when HR failed to reach at least 88% HR_{max} during the high-intensity interval. This training protocol was designed after an aerobic interval training protocol which was shown to be superior to continuous moderate exercise at improving brachial artery endothelial function in heart failure[15] and metabolic syndrome[16] patients. The fasting blood draw and microdialysis session were repeated two-days following the final training session to assess the impact of the aerobic interval training intervention on extracellular ROS production and microvascular endothelial function. The maximal exercise test and anthropometric tests were repeated following the final microdialysis session to test the impact the exercise intervention on maximal aerobic capacity and body composition.

Skeletal Muscle Biopsy and Western Blotting

Skeletal muscle biopsy samples were obtained from a subset of lean and obese subjects (n = 5 / group) from the vastus lateralis using a percutaneous muscle biopsy technique.[17] Biopsy samples were obtained immediately prior to microdialysis probe insertion, from the contralateral leg from which the microdialysis probes were inserted. Samples were frozen in liquid nitrogen and stored at -80°C until analysis. Muscle samples were homogenized in 10 times (w/v) TEE buffer containing: 10 mM Tris base, 1 mM EDTA, and 1 mM EGTA, with 0.5% Tween-20, using a glass grinder (Kimble Chase, Rockwood, TN). Homogenates were loaded onto 4-20%, 7.5%, or 10% Tris gels, resolved by SDS-PAGE, and transferred to polyvinylidine difluoride membranes. Membranes were probed with an anti-$gp91^{phox}$ (1:1000, BD Transduction Laboratories #611414, San Diego, CA), anti-$p22^{phox}$ (1:1000, Santa Cruz Biotechnology #sc-20781, Santa Cruz, CA), anti-$p47^{phox}$ (1:2000, Santa Cruz #sc-17844), anti-$p67^{phox}$ (1:1000, Santa Cruz #sc-15342), or xanthine oxidase (1:500, Santa Cruz #sc-20991) primary antibodies at 4°C overnight, washed with TBST, and exposed to anti-mouse or anti-rabbit secondary antibodies at room temperature for 1 hour. Signals were detected by chemiluminescence and quantified by densitometry using Image J software. Membranes were stripped and reprobed with an anti-α-tubulin (1:3000, Cell Signaling Technology #2125, Danvers, MA) antibody for a loading control. Results were expressed normalized to α-tubulin.

Ex vivo Skeletal Muscle Nox Activity Assay

Nox activity was determined from skeletal muscle homogenates as described previously.[18,19] Homogenate samples were added to a mixture containing 10 µM Amplex Red, 1.0 U/ml HRP, and 25 U/ml SOD in PBS. H_2O_2 generation was continuously monitored in real time using a spectrofluorometer (Horiba Jobin Yvon, Ann Arbor, MI) with magnetic stirring and constant temperature control at 37°C. After establishing the baseline rate of H_2O_2 generation, Nox activity was stimulated with the addition of 0.5 mM NADPH, following which the rate of H_2O_2 generation was monitored for five minutes. Nox activity was calculated as change in rate of fluorescence stimulated by NADPH addition, where total NADPH-dependent H_2O_2 generated in the sample was used as an index of Nox activity. Activity was normalized to total protein

content of sample added, as determined by bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

Data and Statistical Analysis

Data are presented as means ± SEM. Statistical analyses were performed with GraphPad Prism (Prism 6.0, GraphPad, San Diego, CA), with an alpha level of 0.05. For microvascular blood flow measurements, changes in blood flow upon ACh or SNP stimulation were expressed as the change from basal blood flow within the same probe. Significant differences between groups or for treatment and/or exercise training effects were determined by two-way repeated measures ANOVA with Tukey's or Sidak's multiple comparisons post hoc analysis as appropriate.

Supplemental References

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