NADPH Oxidase Activity and Muscle Microvascular Dysfunction in Obesity
September 5th, 2019

4. Experimental Design and Methodology

Subjects: Twenty-five sedentary class 2 and above obese [BMI ≥35 kg/m^2, waist circumference >40 inches (men) or >35 inches (women)] men and women (18-45 yrs) will participate in these experiments. Individuals under 18 years of age will not be investigated due to the invasive nature of the protocol. Individuals greater than 45 years of age will not be investigated due to the age-related decline in endothelial function. There will be no restrictions with regard to race, sex, or socioeconomic status. Women will be premenopausal, will be on combined estrogen/progestin hormonal contraceptive therapy (oral pill, transdermal patch or vaginal ring) and will be studied within the seven day inactive (placebo) phase of hormonal contraceptive therapy (comparable to the early follicular phase in the normal menstrual cycle; verified by blood estradiol analyses). Ensuring consistency in studying the women during the phase of hormonal contraceptive therapy with lowest plasma estradiol concentrations is important due to the known positive effects of estradiol on endothelial function.

Sedentary obese individuals will have been weight stable for the preceding 6 months, and will have the Metabolic Syndrome. The Metabolic Syndrome will be defined according to the most widely used criteria for the Metabolic Syndrome from the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Individuals with the Metabolic Syndrome have at least three of the following:1) Central obesity as measured by waist circumference (men ≥40 inches; women ≥35 inches); 2) Fasting blood triglycerides ≥ 150 mg/dL; 3) Blood HDL cholesterol in men<40 mg/dL and women <50 mg/dL; 4) Blood pressure > 130/85 mmHg; 5) Fasting blood glucose ≥ 110 mg/dL, and (not per ATP III).

Before participating in this study, subjects will undergo a medical history, physical examination, and screening for cardiovascular disease and type 2 diabetes. Evaluations consist of a graded treadmill stress test, a blood chemistry, body composition analysis by Dual X-ray Absorptiometry (DXA) and a minimum waist circumference (between the xiphoid process and the umbilicus) measure.

Exclusions Subjects participating in purposeful endurance exercise training (>20 min/day, >1 day/week) will be excluded. Pre-menopausal female subjects must not be pregnant or lactating, and must have had regular menstrual cycles for the past year. All subjects will be screened for cardiovascular and peripheral vascular disease. Individuals taking medications that may affect central or peripheral circulation, are on nonsteroidal anti-inflammatory agents or serotonin reuptake inhibitors, who smoke or chew tobacco, have diabetes (fasting blood glucose >125 mg/dL), hypertension >160/95 mmHg (individuals with diabetes and high degree of hypertension will be excluded to keep a more uniform group of individuals with the metabolic syndrome), congestive heart failure, angina, or peripheral vascular disease. Individuals with ECG evidence of serious arrhythmias and/or acute myocardial ischemia reflected in ST-segment depression of 1 mm or greater at rest or during exercise will be excluded. Individuals with chronic infections, paralysis due to stroke, advanced Parkinson's Disease, severe rheumatoid arthritis or other serious orthopedic problems that would prevent performance of the exercise training tasks will be excluded. Subjects will not be allowed to take antioxidant, herbal or vitamin supplementation for at least 2 weeks prior to investigation since some of these agents have been shown to improve endothelium-dependent vasodilatation. Subjects will not be allowed to ingest caffeine the day of the experiment. Subjects will be excluded if weight changes by more than 5% during the training program or if their exercise adherence is below 90% of the exercise sessions or total exercise time.

Timeframe: Subjects will be recruited, screened, enrolled, and tested during months 3-28 of this grant. Subject recruitment procedures and procurement of needed supplies will occur during the first 2 months of the project. Additional recruitment, as well as screening and testing of subjects will occur in 1-month cycles over the ensuing 25 months at a rate of approximately 1 per cycle. Twenty-five microdialysis experiments will therefore be performed on participants both before and after 8 weeks of exercise training, for a total of 50 microdialysis experiments. This plan will therefore require 2 microdialysis tests per month on average during the testing months. Testing of these subjects includes six hours for the microdialysis procedure. Biochemical analysis of samples requires approximately six hours per experiment, and data reduction another five hours per experiment. Additional time (five hours per week) is required for subject recruitment, preliminary testing and follow-up. The recruitment and testing goals are therefore realistic in light of the personnel and percentage efforts allocated, coupled with personnel and resources not charged to the grant.

Table 1: Protocol (Example timeline for one person)

Day -21	Days -3 to -1	Day 0	Days 1-56	Days 57-59	Day 60	Day 61
Subject recruited, screened*,	3-day diet and activity record***	Pre-training microdialysis (muscle biopsy)	Aerobic interval exercise,	3-day diet and activity record***	Post-training microdialysis (muscle biopsy)	Post 8-week testing**

enrolled and tested**			3 days per week			

*Screened for: Cardiovascular disease, diabetes, and medications.
**Testing: Resting blood pressure, DEXA, a VO_{2peak} test and anthropometric measures will be performed.
***__Diet and activity records__ will be kept by subjects for three days prior to the microdialysis experiment to control for the short-term effects of activity and diet. Diets will be analyzed with Nutritionist Pro software.

Microdialysis Study Days Subjects will report to the laboratory following an overnight fast. While subjects are quietly resting, an overnight fasted blood draw will be obtained from an antecubital vein to be analyzed for traditional (insulin, glucose, lipids, c-reactive protein) and non-traditional [(cytokines TNF-alpha, IL-6, IL-13, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and P-selectin] cardio-metabolic disease risk/inflammatory markers in blood using ELISA's from EMD Millipore, or our clinical analyzer (Beckman Access systems). In addition, plasma glutathione (GSH/GSSG) will be analyzed as a marker of systemic oxidative stress using a kit from Percipio Biosciences (Bioxytech GSH/GSSG). While not the focus of this study, this will allow correlational analysis of these disease risk markers with endothelial function and ROS data from the microdialysis studies.

Microdialysis Procedure Seven microdialysis probes (CMA 20: 10mm * 0.5 mm 20kilodalton cutoff membrane, 5 cm probe shaft length, inlet tubing and outlet tubing 20cm; CMA/Microdialysis, Stockholm, Sweden) will be percutaneously inserted, 3 cm apart, into the vastus lateralis of the quadriceps femoris muscle group (3-4 probes per leg) using sterile technique under local anesthesia (1% lidocaine). The probes will be perfused with the appropriate solution at a rate of 2.0 µL/min using CMA/102 microinfusion pumps (CMA/Microdialysis, Stockholm, Sweden) with agents to test for endothelium-dependent and endothelium independent vasodilation as well as ROS production from specific NOX sources. For each sample where blood flow is measured, dialysate will be stored at 4 °C and analyzed for ethanol (for blood flow/microvascular exchange) within 24 hours. All perfusates and dialysates will be protected from light exposure.

Microdialysis ethanol outflow/inflow ratio Ethanol (5 mM) will be included in the perfusion medium to monitor nutritive blood flow/microvascular exchange in the area of the microdialysis probe as described in previous publications [30-32]. During perfusion, ethanol diffuses from the microdialysis membrane and away from the local area by the microcirculatory blood flow/microvasculature exchange in the immediate vicinity of the microdialysis probe membrane. Ethanol concentrations will be analyzed in dialysate and perfusate samples using an enzymatic-fluorometric method [30]. Each sample will be analyzed in triplicate, with each triplicate requiring 2 µL of dialysate or perfusate. Results will be expressed as the ratio of [ethanol]dialysate to [ethanol]perfusate (ethanol outflow-to-inflow ratio), a qualitative indicator of blood flow [30]. The ethanol outflow-to-inflow ratio is inversely related to blood flow in a nonlinear fashion [32] and can be converted to units of blood flow ($ml \cdot min^{-1} \cdot 100\ g^{-1}$) using the equations of Wallgren et al [33].

Fluorescence analysis of H_2O_2 concentrations Fluorescence spectrometry of skeletal muscle extracellular ROS will be performed using Amplex UltraRed (Molecular Probes, Eugene, OR). Amplex UltraRed is a fluorogenic substrate with very low background fluorescence which reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin. 100 µM Amplex UltraRed reagent and 1.0 U/mL horseradish peroxidase (HRP) will be included in the perfusion medium, with and without the addition of 10 U/mL SOD, allowing ROS produced *in vivo* to react with the fluorogenic substrate within the microdialysis probe. HRP and SOD are too large to cross the membrane, thus enzymatic reactions will occur within the microdialysis probe. Dialysate will be collected in a 150 µL polyethylene tube wrapped in tin foil, and fluorescence intensity will be measured immediately upon collection. Fluorescence intensity will be measured using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) at an excitation wavelength of 550 nm and an emission wavelength of 570 nm. H_2O_2 concentrations of dialysate will be calculated on the basis of an H_2O_2 standard curve generated using H_2O_2 and Amplex UltraRed after subtraction of background fluorescence in the perfusate from each microdialysis stage (Figure 3 of pilot data).

Determination of peak aerobic capacity: All subjects will perform a standardized maximal exercise test on a treadmill upon enrollment in the study and again upon completion of the training program. Subjects will individually adjust speed and incline (3-6 $km \cdot hr^{-1}$, 0-5%) for a 5-minute warm-up period. After the warm-up period, the subject's expired O_2 and CO_2 will be continuously monitored via open circuit spirometry (TrueOne 2400; Parvomedics; Salt Lake City, UT). The VO_{2peak} test will be performed using a ramp protocol where the speed will be constant and the incline will be increased 2% every two-minutes until VO_{2peak} is reached. The starting speed will be individually adjusted based on the warm-up speeds and heart rates, with the intention of obtaining VO_{2peak} within 8-12 minutes. During the test, 12-lead ECG will be monitored continuously, and blood

pressure will be monitored every two minutes. In asymptomatic subjects who do not develop ECG abnormalities, the end point for the exercise test will be severe fatigue. Achievement of VO_2peak will be determined by the following criteria: 1) plateau in oxygen consumption with increased exercise intensity, 2) respiratory exchange ratio (RER) >1.1, and 3) heart rate greater than 85% of age-predicted maximal heart rate. The test will be repeated on a second day if two of the three criteria are not met.

Training Protocol: All participants will perform aerobic interval exercise training three times per week on non-consecutive days for eight weeks. Exercise will consist of walking/running up an incline on a treadmill. Subjects will warm up for 10 minutes at 70% of maximal heart rate (HRmax as determined during the VO_{2peak} test) before performing four 4-minute intervals at 90% of HRmax with a 3-minute active recovery at 70% of HRmax between intervals and a 5-minute cool-down period, yielding a total exercise time of 40 minutes. The investigators have used this protocol in previous R15 8-week training studies, and this training protocol has been used successfully to improve aerobic capacity and flow-mediated dilation in CAD patients and patients with the metabolic syndrome [34,35].

Determination of body composition:
Fat-free mass, fat mass, and percent body fat will be determined using dual-energy x-ray absorptiometry (DXA). Body mass and height will be measured for the calculation of body mass index (BMI: kg/m^2). Minimum waist and maximum hip circumferences will be also measured to potentially be used as a covariate in statistical analyses due to the relationship of w/h ratio and waist circumference with cardio-metabolic disease risk.

Muscle markers of oxidative stress: The *in vivo* NADPH oxidase activity (ROS measures) and the in-vivo endothelial dysfunction measures in the absence and presence of mitochondrial ROS inhibition and NADPH oxidase inhibition are the important focus of this study. However, the vascular adhesion proteins ICAM-1 and VCAM-1 could also be measured as indicators of potential endothelial damage because their expression is upregulated by ROS. 3-Nitrotyrosine will be measured as an indicator of nitric oxide-dependent reactive nitrogen species-related stress. Oxidized and reduced forms of glutathione (GSH to GSSG) may be measured as a measure of oxidative stress, with 4-hydroxynonenal protein adducts (HNE-adducts) measured as an additional marker of oxidative damage in biopsy homogenates as skeletal muscle oxidative stress markers. Biopsies will therefore be taken in the resting state before and after training in those participants agreeing to the biopsy procedure. Muscle eNOS, xanthine oxidase, NOX isoforms, ICAM-1 and VCAM-1 may be quantified by western blot and localized by immunohistochemistry using antibodies from Upstate/Millipore,. Western blot analysis will also be run for 3-nitrotyrosine using antibodies from Upstate Cell Signaling Solutions, Lake Placid, NY. Muscle glutathione (GSH/GSSG) will be measured using a kit from Percipio Biosciences. 4-hydroxynonenal protein adducts (HNE-adducts) will be quantified by western blot and localized by immunohistochemistry using antibodies from Oxis International, Inc. (Foster City, CA). It is anticipated that endothelial and skeletal muscle markers of oxidative stress will be lower, and antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, thioredoxin reductase, and catalase will be higher, after than before training. Biopsy samples will allow for correlational analysis of markers of oxidative stress with endothelial functional data from microdialysis.

Sample size and Power Analyses: Aim 1 considerations: Group sample size of 22 achieves >80% power to detect a difference of 0.18 uM H_2O_2 (ROS: from mean of 0.6 uM) with estimated group standard deviation (sigma) of .30 and with a significance level (alpha) of 0.05. These means and anticipated differences between treatment and control probes are reasonable estimates based on the presented data in figure 4 above and estimates of meaningful physiological differences between treatment and control probes. Aim 2 considerations: Aim 2 is a prospective design of ROS response to perfused agents before as compared to after training. Group sample size of 22 achieves >80% power to detect a difference of 0.15 between the null hypothesis that probe ROS mean percent change is 0.2 (20% mean percent change between pre and post) and the alternative hypothesis that the mean is 0.35 (35% mean percent change between pre and post) with estimated group standard deviations of percent change of 0.136 and with a significance level (alpha) of 0.05 using a two-sided two-sample t-test. This will answer Aim 2 of the study for determining any pre-training/post-training differences. Thus, there is confidence that 25 subjects per group will be large enough to detect differences in ROS while allowing for the potential 10% dropout experienced in past training studies.
Statistical Analyses and Interpretation of Results: Treatment versus control probe comparison will be performed at the appropriate phases for Aims 1a-1d via student t-tests or ANOVAs specific for the sub-aim. Quantification will occur of the change in the outcome variables (ethanol outlow/inflow ratio or H_2O_2) with training and compare the training response via sub-aim specific (Aims 2a-2b) two-way repeated measures ANOVAs [(probe type (treatment vs control) by time (pre vs post)]. Student Neuman Keuls' post-hoc tests will

be used when significant main effects are observed. Pearson-Product Moment correlations will be run to compare the ROS data and endothelial-dependent responses, as it is anticipated that there will be an inverse relationship between dialysate ROS and response to acetylcholine. Exploratory analyses will be run comparing subgroups African American and Caucasian participants, as well as male and female participants due to the potential differences in oxidative stress, inflammation, endothelial function and CVD risk in these groups. The funding level will not allow enough participants to be studied to provide adequate statistical power to address these issues. Statistical analysis will be run using SPSS /PASW Statistics version 18, with an alpha level of 0.05.