"Understanding the Exercise Hypertension Paradox: Implications for Rehabilitation and Mechanisms Involved in the Exaggerated Exercise Pressor Reflex"

NCT02034422

Protocol Summary and Statistical Analysis Plan

11/4/2022

Please note that this summary proposal and statistical analysis plan includes trials, assessment, and protocols included in, but not limited to, NCT02034422.

Background and Introduction

Background and Introduction:

Hypertension is a major risk factor for the development of stroke, heart failure, coronary heart disease, peripheral artery disease, and renal disease. Currently, 67 million (~30%) Americans are diagnosed with hypertension and less than half of these individuals are receiving effective antihypertensive therapy. When left untreated hypertension often develops into these more severe diseases thereby increasing the financial burden and detrimental impact of hypertension exponentially. Regular physical activity should be considered the cornerstone of preventing and managing hypertension, however, individuals with hypertension exhibit marked exercise intolerance characterized by an exaggerated increase in blood pressure during physical activity (i.e. exercise pressor reflex or EPR) and impairments in skeletal muscle blood flow. This excessive EPR and concomitant reduction in muscle blood flow, increase the risk of adverse cardiovascular events such as myocardial infarction, cardiac arrest, or stroke during or immediately after exercise and is an established risk factor for cardiovascular morbidity and mortality. Identifying the mechanisms associated with this augmented EPR and impaired blood flow response to exercise will lead to the development of novel strategies aimed at improving exercise tolerance and reducing risks associated with exercise in hypertension. In order for exercise to be a viable and safe treatment for hypertension the causes and consequences of the exaggerated EPR must be clearly understood.

The goal of the proposed research is to understand the causes and consequences of the exaggerated increase in blood pressure during exercise in hypertension ultimately leading to the improved exercise tolerance and reduced risk of exercise in hypertension. The cause of this exaggerated blood pressure response is complex and likely involves multiple factors working simultaneously. First, skeletal muscle afferent feedback is hypothesized to be overactive in hypertension leading to the excessive EPR. Afferent feedback works directly through the autonomic nervous system and the cardiovascular control center to withdraw parasympathetic restraint and increase sympathetic activity. In hypertension, the sympathetic nervous system is chronically elevated even during antihypertensive therapy; therefore, any input that further stimulates this pathway is likely to result in an exaggerated EPR. Second, elevated sympathetic nervous system activity elicits profound and sustained peripheral vasoconstriction resulting in augmented vascular resistance. It is hypothesized that α-adrenergic-induced peripheral vasoconstriction remains intact during exercise leading to impaired skeletal muscle perfusion and reduced oxygen delivery. Third, vascular function is impaired in individuals with hypertension. The mechanisms contributing to this vascular dysfunction are not clear but may be due to an imbalance between vasodilatory capacity and vasoconstrictor action. Finally, hypertension is characterized by elevated oxidative stress, the negative consequences of which include overstimulation of skeletal muscle afferents and impaired skeletal muscle blood flow. Clearly the integration of afferent feedback, the sympathetic nervous system, vascular dysfunction, and oxidative stress is important in understanding exercise responses in

hypertension. Using a reductionist approach, the mechanisms involved in the exaggerated EPR and impaired blood flow can be identified and information gained to safely and effectively treat hypertension, thereby reducing the risk of exercise in this population.

To determine whether the amendment for dietary nitrate requires an IND we have been following the guidelines put forth by the FDA in the document entitled:**Guidance for Clinical Investigators, Sponsors, and IRBs: Investigational New Drug Applications (INDs) - Determining Whether Human Research Studies Can Be Conducted Without an IND"**- page 15 of the document states the following "If the clinical investigation is intended only to evaluate the dietary supplement's effect on the structure or function of the body, an IND is not required. Our investigation is not intended to mitigate, cure, treat, or prevent a disease. Based on these guidelines the dietary supplement proposed is 1) not considered a drug, 2) will not be used to diagnose, cure, mitigate, treat, or prevent a disease, and 3) will be used to characterize the mechanism by which the supplement acts to maintain structure or function (specifically, blood flow). Therefore, based on these guidelines, as explicitly stated by the FDA, we believe an IND is not required.

Purpose and Objectives

Objectives: A series of experimental protocols have been designed to specifically evaluate the role of afferent feedback, sympathetic nervous system activation, vascular function, oxidative stress, and exercise training on the exercise pressor and blood flow response to exercise in healthy and hypertensive populations.

Specific aim - Protocol 1: Determine the contribution of afferent feedback to the exaggerated exercise pressor reflex and blood flow response in hypertension. Hypothesis: Patients with hypertension will exhibit heightened afferent feedback arising from oversensitive group III (mechanosensitive) and group IV (metabosensitive) fibers resulting in the exaggerated EPR and impaired skeletal muscle blood flow. Inhibition of afferent feedback will attenuate the EPR, increase muscle blood flow, and improve exercise tolerance in individuals with hypertension.

Specific aim - Protocol 2: Determine the contribution of sympathetically-induced vasoconstriction to the exercise pressor reflex and blood flow response in hypertension. Hypothesis: Patients with hypertension will exhibit robust and sustained α-adrenergic-mediated vasoconstriction during exercise indicating impaired functional sympatholysis. Inhibition of α- adrenergic vasoconstriction will improve skeletal muscle blood flow and attenuate the exaggerated EPR in hypertension. Dietary nitrate supplementation will improve blood flow by improving the body's natural ability to inhibit a-adrenergic-mediated vasoconstriction during exercise.

Specific aim – Protocol 3: Determine how endothelial-dependent and endothelial independent mechanisms contribute to impaired vascular function in hypertension. Hypothesis: Patients with hypertension will exhibit vascular dysfunction arising primarily

from endothelial-dependent mechanisms including impaired nitric oxide (NO), prostaglandin (PG), and endothelial derived hyperpolarizing factor (EDHF) mediated vasodilation.

Specific aim - Protocol 4: Determine the contribution of elevated oxidative stress to the exercise pressor reflex and blood flow response in hypertension. Hypothesis: Hypertensive subjects will exhibit elevated free radical production during exercise as directly assessed by electron paramagnetic resonance spectroscopy. Novel evidence using isolated endothelial cells will directly link the vascular endothelium to elevated oxidative stress in hypertension. Reductions in oxidative stress by oral antioxidant therapy will improve skeletal muscle blood flow and reduce afferent fiber sensitivity leading to an attenuated EPR in hypertensive subjects.

Specific aim - Protocol 5: Determine the remediable effect of combined antioxidant treatment and exercise rehabilitation in the treatment of hypertension. Hypothesis: Acute antioxidant treatment administered prior to exercise in hypertensive patients will ameliorate the exaggerated EPR resulting in a normal and safe blood pressure response to exercise-based rehabilitation. This two-pronged approach (antioxidants and exercise training) will result in a safely achieved reduction in skeletal muscle afferent feedback facilitating improved exercise tolerance, improved muscle blood flow and ultimately reduced cardiovascular risk in this population.

Study Population

Age of Participants: 18-99

Sample Size:

At Utah:	72
All Centers:	72

Inclusion Criteria:

Inclusion Criteria:

- Healthy individuals 18 years of age or older
- Hypertensive individuals (either diagnosed or presenting with high blood pressure)

Exclusion Criteria:

Exclusion Criteria:

- Age of less than 18 years

- Individuals unwilling or unable to give consent
- Participants in whom the study procedures would not be recommended for medical reasons
- Resting BP > 180/110
- Women who are pregnant

Design

Placebo Controlled Trial
Phase I Clinical Trial

Cross-sectional (comparing normotensive to hypertensive), longitudinal (comparing pre and post an intervention)

Study Procedures

Recruitment/Participant Identification Process:

Recruitment & Participant Compensation

Participants will be recruited by staff at the Utah Vascular Research Laboratory in conjunction with staff in the Department of Family and Preventative Medicine. Recruitment strategies will include recruitment fliers (VAMC SLC and University of Utah), VA and University patient databases, newspaper advertisements, clinic visits for recruitment with the approval of the candidate's MD, and through word of mouth. A formal analysis of electronic medical records using appropriate ICD-9 codes and drug indices may be used to identify eligible participants. Electronic medical records from both the VAMC SLC and University of Utah are currently available to the investigative team and will be used in this analysis. Following identification of eligible participants, a direct mail campaign will take place in which eligible participants may receive a letter from the research team stating the purpose of the study, the requirements of the study, study compensation, contact information, and information to opt-out from further contact and participation. Eligible participants that expressed interest in the study or choose not to opt-out from further communication will be contacted by a member of the research recruitment team. Staff from the Department of Family and Preventative Medicine have used such techniques for over 20 years to successfully recruit participants for large scale clinically based research investigations at the University of Utah and have the infrastructure and knowledge to assist the Principal Investigator in this process.

Recruitment will also be done in person, handing out fliers around Salt Lake City, at Farmers Market, Libraries, Shopping centers. Ads and fliers may also be put on classifield websites such as KSL and Pulse. Also in newspaper ads, e.g. the deseret news and local city newspapers. Additionally through ResearchMatch or websites (social media) postings.

Pregnancy testing will be performed on all women of child bearing age and potential. Any pregnant participant will be excluded from the study. Pregnancy testing will be performed on all women of child bearing age and potential. Any pregnant participants will be excluded from the study.

Informed Consent:

Description of location(s) where consent will be obtained:
VA Bldg. Room 1D10 or 1D21

Description of the consent process(es), including the timing of consent:
Participants may consent either the day they visit the lab (to consent), or if they wish, take the time to think about whether or not they want to participate. Participants are also welcome to discuss their potential participation with family/friends, and make an appointment at a later time to actually consent if they so desire.

Requested Waivers/Alterations of Consent:

Waiver of Informed Consent	VA Waiver of Consent for Recruitment Purposes Only

Procedures:

STUDY PROCEDURES

An overview of each visit to the laboratory, including chronology of each protocol, is listed on the following pages. This is followed by separate headings which provide details for participant instrumentation, infused drug information, and the techniques and measurements identified in each protocol.

PRELIMINARY SCREENING AND EXERCISE TESTING DAY (all participants, 1-2 hours)

After obtaining informed consent, participants will report to the laboratory (VA Bldg 2, Room 1D21) for orientation, completion of medical history and activity questionnaires, and may have a medical examination performed by one of the study physicians. A screening questionnaire will be filled out that will include questions concerning risks such as: age, family health history, smoking, hypertension, hypercholesterolemia, and physical activity and allergies (iodine, latex, drugs, etc). A venous blood sample (4 ml) may be drawn from which a Metabolic Panel, Complete Blood Count (CBC), and a Lipid Panel will be assessed.

Next, eligible participants may be asked to complete one *or* more of the following: a graded exercise test to volitional exhaustion (bike or treadmill), with or without a mouthpiece to determine oxygen consumption, maximal and sub-maximal handgrip test, maximal and sub-maximal leg extension test, and maximal and sub-maximal plantar flexion test. Sufficient recovery will be allowed between tests, and ECG will be assessed at all times. The hand grip, leg extension, and plantar flexion graded exercise tests are considered low cardiovascular risk test due to the recruitment of an isolated small muscle mass that results in minor

cardiovascular stress. The graded whole-body exercise tests to volitional exhaustion (bike, treadmill), when performed in participants over the age of 45 or presenting with hypertension (> 140/90) will be completed under physician supervision, and the participant will be monitored by an ECG throughout the exercise test. Any abnormal ECG responses during the exercise test (overt arrhythmia, severe [3 mm] ST segment depression, elevated [1mm] ST segment in non-Q wave lead, frequent extra-ventricular systoles) or adverse responses such as chest pain, leg pain, or unsteadiness will serve as criteria to stop the test, in accordance with American Heart Association guidelines. Questionable findings will be referred to a cardiologist for inspection/diagnosis prior to further involvement in the protocol.Additionally cognitive assessment such as measuring the participant's ability to follow basic instruction regarding exercise training and physical assessments such as a 6-minute walk test may be performed. Participants may be asked to wear an accelerometer to measure activity levels

If the participant is unable to complete all the testing listed above a second visit may be scheduled to complete the testing.

Following the exercise, testing participants may be given instruction and equipment for assessment of ambulatory blood pressure and physical activity monitoring. At this time hypertensive participants receiving antihypertensive treatment may begin a 2-week non-diuretic withdrawal period based on the recommendation and supervision of study physicians. During this washout period participants will perform twice daily assessment of BP and will keep a log of all BP measures. Prior to the start of the 2-week washout and at day 14 ambulatory blood pressure may be assessed over a 24 hour period.

Due to the nature of the testing performed under this protocol, we will not enroll pregnant women. Additionally, women of child-bearing age will be asked to undergo a urine pregnancy test provided by the research site to confirm that these female participants are not pregnant. Additionally, these participants will be asked not to become pregnant while participating in this protocol. If a female participant becomes pregnant she will be asked to inform the PI or other designee immediately and will be withdrawn from any part of this protocol.

PROTOCOL 1: Determine the contribution of afferent feedback to the exaggerated exercise pressor reflex and blood flow response in hypertension. (1 day, 5-7 hours)

Participants will report to the laboratory (VA bldg. 2 room 1D21) in a fasted state. Catheters may be placed in the leg (common femoral artery and femoral vein) or arm (brachial artery and antecubital vein) by a licensed physician using standard sterile technique. During the catheterization process endothelial cells may be collected using the sterile J-wire (see Participant **Instrumentation** for more detailed description of this procedure). It should be noted that the insertion of the J-wire is common practice for catheter placement. Following placement of catheters, resting measurements of arterial blood pressure, heart rate, and limb blood flow (ultrasound Doppler) will be taken, and resting blood samples (arterial and venous) may be collected. Measures may be made at rest, during passive limb movement (isolation of group III mechanosensitive afferents[18,19]), exercise, or immediately following exercise during post exercise circulatory occlusion (PECO - isolation of group IV metabosensitive afferents[18,20]) according to the general timeline presented below. KE, plantar flexion, or handgrip exercise may be performed at several exercise intensities including, but not limited to, 25, 50, and 75% maximal work rate (WR_{max}) or maximal voluntary contraction (MVC) as determined during preliminary testing. A sodium bicarbonate lotion (or placebo lotion) may be

applied topically prior to exercise. Each exercise intensity may be continued for up to 5 min to achieve steady-state pulmonary oxygen consumption. During each work bout the following sequence of events may be conducted (as indicated by the arrows in the timeline below): (1) sampling of femoral arterial and venous blood for the direct measurement of free radicals and blood gases, (2) continuous measurement of central hemodynamics (heart rate, stroke volume, cardiac output) and arterial and venous pressures (indwelling catheter pressure), and (3) leg or arm blood flow via ultrasound Doppler. Immediately following each exercise stage PECO may be performed. After a 90 min recovery, the protocol may be repeated in the fentanyl/afferent blocked condition. The efficacy and extent of the block may be tested by neurological exam including cutaneous hypoaesthesia to pinprick and cold perception on the torso, upper, and lower limbs.

PROTOCOL 2: Determine the contribution of sympathetically-mediated vasoconstriction to the exercise pressor reflex and blood flow response in hypertension. (1 day, 6 - 8 hours)

Participants will report to the laboratory (VA bldg. 2 room 1D21) in a fasted state. Catheters may be placed in the leg (common femoral artery and femoral vein) or arm (brachial artery and antecubital vein) by a licensed physician using standard sterile technique. During the catheterization process endothelial cells may be collected using the sterile J-wire (see **Participant Instrumentation** for more detailed description of this procedure). It should be noted that the insertion of the J-wire is common practice for catheter placement. Following placement of catheters, resting measurements of arterial blood pressure, heart rate, and limb blood flow (ultrasound Doppler) may be taken, and resting blood samples (arterial and venous) may be collected. Phenylephrine (PE, α1 selective) and dexmedetomidine (DEX, α2-selective) dose-responses may be performed at rest to establish maximal α-adrenergic mediated vasoconstriction (Timeline A). Dose responses may consist of upto 7 doubling doses of PE and DEX starting at approximately 0.05 mcg/dl/min and 1.5 ng/dl/min, respectively. Each dose may be infused for 1 to 5 min. Following ~30 min of drug washout and the return of basal blood flow KE, plantar flexion, *or* handgrip exercise may be performed with PE or DEX infusion. Several bouts of exercise may be performed at exercise intensities including, but not limited to, 25, 50, and 75% of WR_{max}. Each exercise intensity may be continued for up to 10. During each work bout the following sequence of events may be conducted: pre-infusion (control) recording of blood flow and central hemodynamics; pre-infusion (control) sampling of arterial and venous blood for measurement of blood gases and norepinephrine; and infusion of PE or DEX with continuous monitoring of blood flow and central hemodynamics. The order of the PE or DEX trials may be counterbalanced and infusion rates will be matched and adjusted to account for increases in blood flow during exercise[21]. After ~60 min of recovery and the return of basal blood flow and central hemodynamics, phentolamine (PHEN, non-selective α-adrenergic antagonist) may be infused at rest at three doses (5, 10, and 20 mcg/dl/min) followed by a norepinephrine (NE) challenge (~20 ng/dl/min) to test the efficacy of the phentolamine-induced inhibition of α-adrenergic vasoconstriction (Timeline B). Immediately following the NE challenge exercise may commence with continuous PHEN infusion. Several exercise bouts including, but not limited to, 25, 50, and 75% of WRmax may be performed. Each exercise bout may be performed for up to 6 min and periods of recovery

may be included between exercise bouts. Identical measures as previously described for the pre-infusion portion of the agonist trials may be repeated.

Prior to reporting to the laboratory participants may undergo an acute (1 dose) dietary supplementation by consuming two nitrate rich beetroot juice (70 ml per drink, 13 mM/day, 0.9g of nitrates) or nitrate-depleted placebos (70 ml per drink, 0mM/day, 0.0g of nitrates). Participants will be instructed to drink two 70 ml containers of placebo on the day of the experiment approximately 1 to 2 hours before the start of the study. Following the first half of the trial (corresponding to the "A" timeline below), the participant will drink two 70ml nitrate rich beetroot juice supplements and repeat the series of exercise bouts 2 hours following consumption. During this supplementation period participants will be asked to refrain eating abnormal amounts (>300g/day) of leafy green vegetables (e.g. spinach), consumption of alcohol, and the use of mouthwash products containing alcohol.

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PROTOCOL 3: Determine how endothelial-dependent and endothelial independent mechanisms contribute to impaired vascular function in hypertension. (1 day, 5 - 7 hours).

Participants will report to the laboratory (VA bldg. 2 room 1D21) in a fasted state. Catheters may be placed in the leg (common femoral artery and femoral vein) or arm (brachial artery and antecubital vein) by a licensed physician using standard sterile technique. During the catheterization process endothelial cells may be collected using the sterile J-wire (see **Participant Instrumentation** for more detailed description of this procedure). It should be noted that the insertion of the J-wire is common practice for catheter placement. Following placement of catheters, resting measurements of arterial blood pressure, heart rate, and limb blood flow (ultrasound Doppler) will be taken, and resting blood samples (arterial and venous) may be collected. Next a series of experimental trials involving intra-arterial infusions of various drugs may be performed to systematically identify the contribution of vasodilatory mechanisms to movement and/or contraction-induced hyperemia. The infusion protocol, if performed, will examine the independent and combined role PG, EDHF, and/or NO by pharmacologically inhibiting these vasodilators via ketorolac (KET), fluconazole (FLU), and/or L-NMMA, respectively. A general protocol is presented below approximately 30 minutes will be allotted between the single, double, and triple trials. It should be noted that in certain trials not all blockade protocols will be performed. For instance some individuals may

perform only the single blockade while others will perform the single and double blockade, while others may perform all three.

PROTOCOL 4: Determine the contribution of elevated oxidative stress to the exercise pressor reflex and blood flow response in hypertension. (1 day, 5 - 7 hours).

Participants will report to the laboratory (VA bldg. 2 room 1D21) at ~ 0800 in a fasted state, having abstained from caffeine and exercise for the past 12 hours. Catheters may be placed in the leg (common femoral artery and femoral vein) or arm (brachial artery and antecubital vein) by a licensed physician using standard sterile technique. During the catheterization process endothelial cells may be collected using the sterile J-wire (see Participants Instrumentation for more detailed description of this procedure). It should be noted that the insertion of the J-wire is common practice for catheter placement. Following placement of catheters, resting measurements of arterial blood pressure, heart rate, and limb blood flow (ultrasound Doppler) will be taken, and resting blood samples (arterial and venous) may be collected. Control measures may be made at rest, during passive limb movement, exercise, and immediately following exercise during post exercise circulatory occlusion (PECO). Protocol 4 is nearly identical to protocol 1 with the potential addition of flow mediated dilation (FMD) to assess vascular function in the popliteal, femoral or brachial artery. The FMD will be performed in accordance with recently published guidelines [22] employing distal cuff placement for 5 min at 250 mmHg and continuous assessment of arterial diameter for 2 min following cuff release using edge detection software. All measures will first be performed in the control condition. During each work bout the following sequence of events may be conducted (as indicated by the arrows in the timeline below): (1) sampling of arterial and venous blood for the direct measurement of free radicals, blood gases, and markers of oxidative stress (ascorbic acid, glutathione, lipid hydroperoxide, 8-isoprostanes, nitrite/nitrate, ferric reducing ability of plasma). (2) continuous measurement of central hemodynamics (heart rate, stroke volume, cardiac output) and arterial and venous pressures, and (3) femoral blood flow via ultrasound Doppler. After a 120 min recovery, the protocol may be repeated following administration of an oral antioxidant cocktail (AOC, consisting of 1000 mg vitamin C, 600 IU vitamin E, and 600 mg alpha lipoic acid) or infusion of vitamin C (range 1 to 5 grams). We have previously reported reduced oxidative stress, diminished free radical levels, and improved vascular function with this AOC [23-27]. Moreover, this 120 min period is of sufficient duration to allow for vitamin absorption into the circulation. Endothelial cells (if collected using the J-wire technique during catheterization for Specific Aims 1, 2, and 3), may be measured for nitrotyrosine, NAD(P)H oxidase, xanthine oxidase, superoxide dismutase, catalase, nuclear factor-KB (NF-KB).

PROTOCOL 5: Determine the remediable effect of combined antioxidant treatment and exercise rehabilitation in the treatment of hypertension.

Protocol 5a (Acute experiment, 1 day, 5 - 7 hours), this visit will also serve as the pre-exercise rehabilitation visit described in protocol 5b.

Participants will report to the laboratory (VA bldg. 2 room 1D21) in a fasted state. Upon arrival at the laboratory Participants will be instrumented and an antecubital venous catheter may be placed in the forearm. This relatively benign procedure confers minimal risk and is

similar to a venipuncture. During the placement of the venous catheter endothelial cells (EC) may be collected as previously described [28,29] and subsequently analyzed for markers of oxidative stress. The venous catheter will also allow for the sampling of blood at rest and during exercise. All blood samples may be analyzed for free radicals (direct marker of oxidative stress using electron paramagnetic resonance spectroscopy), indirect markers of oxidative stress (lipid hydroperoxides, 8-isoprostanes), and markers of global antioxidant capacity [ferric reducing ability of plasma (FRAP), reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione]. Blood samples may be collected at rest and during the final minute of exercise at each work rate.

Following instrumentation the participant will be transferred to the knee extensor ergometer and passive leg movement may be performed as presented in the general experimental timeline below. Passive leg movement allows for the assessment of group III mechanoreceptor sensitivity. Following passive leg movement participants may perform incremental knee extension or handgrip exercise at 25, 50, and 75% of their previously established WRmax. Each work rate may be continued for up to 6 minutes to achieve steady-state pulmonary oxygen consumption and the following sequence of events may be conducted: (1) sampling of venous blood for the direct measurement of free radicals and markers of oxidative stress, (2) continuous measurement of mean arterial blood pressure, (3) femoral blood flow via Doppler ultrasonography, and (4) evaluation of group IV metaboreceptor sensitivity via post exercise circulatory occlusion (PECO). After the final workrate and PECO maneuver have been completed in the control condition participants may ingest the oral antioxidant cocktail (Vitamins C, E and alpha lipoic acid) and rest for 2 hours before repeating the protocol. Alternatively, participants may receive an infusion of vitamin c prior to or during exercise. A subset of participants may perform a time-control experiment to ensure that the impact of the antioxidant cocktail is not confounded by time.

On a separate visit to the laboratory a muscle biopsy may be obtained from either the gastrocnemius or the vastus lateralis. Biomechanical and structural properties of the muscle will be made. Biopsy samples will be taken at rest following the application of local anesthetic (1% Lidocaine) and incision of the skin and superficial tissue at the local site of the muscle biopsy. The biopsy samples will be immersed in liquid N2 within 15-20 seconds. EPR spectroscopy at 77K may be performed on these muscle biopsies and then two portions will be thawed, one in the PBN spin trap and again analyzed by EPR spectroscopy and the other analyzed for antioxidant content. The remainder of the muscle sample will be analyzed for structure and allow the normalization of findings for mitochondrial volume. A portion of the muscle tissue collected during the resting muscle biopsy will be allocated to morphometric and mitochondrial measurements.

Protocol 5b (Interventional experiment - exercise rehabilitation, 8 weeks, 3 days/week, 1 hour per day).

Data collected from the experiment in protocol 5a will be used to determine pre-intervention status of the hypertensive participants. The exercise rehabilitation intervention will be based upon the cardiac rehabilitation program currently used by the VA SLC Cardiac Rehabilitation Center with the important exception that 2-hrs prior to each exercise session participants will ingest the oral antioxidant cocktail (Please refer to S Participant Instrumentation for the full description of the exercise rehabilitation program). Participants enrolled in the antioxidant

only portion of this aim will ingest the antioxidant 3 times per week (Monday, Wednesday, and Friday). Following the 6-week intervention the effectiveness of the treatment (antioxidant or combined antioxidant plus exercise rehabilitation) will be reassessed according the methods described in protocol 5a. Additionally, the general/preliminary protocols described above will be reassessed to determine changes in exercise tolerance and aerobic exercise capacity.

PARTICIPANT INSTRUMENTATION (alphabetical order)

Antioxidant and blood measures: Ascorbic acid will be measured in the plasma by the vitamin C assay kit (CosmoBio, Carlsbad, CA). **Ferric Reducing Ability of Plasma** will be determined according the spectrophotometric technique previously described [30]. The FRAP method uses the ability of antioxidants to reduce a FE^{3+}-TPTZ complex to its blue colored Fe^{2+} form. **GSH, GSSG and Total Glutathione** will be measured using BioVision Glutathione Assay Kit (#K264-100) (San Francisco, CA). **Lipid hydroperoxides** will be assessed by the lipid hydroperoxide (LPO) assay kit (Caymen, Ann Arbor, MI). **Nitrite/Nitrate** levels will be measured in the plasma using a standard fluorometric assay kit (Caymen, AnnArbor, MI). **8-isoprostane**, a marker of lipid perodixation and oxidative stress, will be analyzed in the plasma using Cell Biolabs 8-iso-PGF2α kit (#STA-337) (San Diego, CA).

Antioxidant Cocktail: Antioxidant or placebo will be consumed in two doses approximately 120 minutes and 90 minutes prior to the other procedures. The first antioxidant dose at 120 minutes prior will include: Vitamin E 200 IU (1 tablet), Vitamin C 500 mg (1 tablet), and Alpha-lipoic Acid 300 mg (3 tablets). The second antioxidant dose approximately 90 minutes prior will include: Vitamin E 400 IU (water dispersable) (1 tablet), Vitamin C 500 mg (1 tablet), and Alpha-lipoic Acid 300 mg (3 tablets). The PI's and Co-I's have previously utilized this supplementation protocol in studies of both aging and disease and a very similar protocol when testing the ability of antioxidant supplementation to prevent free radical-mediated damage [26,31-35].

Blood Draw: Blood draws will be performed by Dr. Trinity or one of the other trained investigators in phlebotomy in our group. In the rare case that this procedure is particularly difficult, as this is a standard procedure performed in our laboratory, the participant will be escorted to the VA Medical Center's blood laboratory in an adjacent building where full-time phlebotomists will perform the blood draw.

Catheterization: At each catheter insertion site, local anesthesia with lidocaine will be applied (1% lidocaine; 1.33mg/kg assuming 75 kg body weight, with an upper limit of 10 ml). Sterile technique will be applied to minimize risk of infection. 1.0 mg of atropine will be available in case of sudden vagal reactions. The leg (femoral artery and vein, 18G Cook Catheter or equivalent) will be cannulated. A qualified and privileged physician will be responsible for the placement and removal of these catheters. Following catheter removal, wounds will be dressed and the participants educated as to proper care. Participants will not be released until hemostasis is clearly present. Follow-up contact with the participant will continue over the following two days. It should be noted that the Drs. Trinity, Richardson, Supiano and Wray have significant experience with catheter-related studies [36-45], having

coordinated the use and insertion of these exact catheters hundreds of times in the last twenty years without incidence of bleeding, infection or tissue damage. In fact, several participants have performed multiple studies with our group, indicating the benign nature and positive outcome of the procedures.

Central hemodynamics: Heart rate, stroke volume, cardiac output, and mean arterial pressure will be determined non-invasively with a finometer (Finapres Medical Systems, Amsterdam, The Netherlands) positioned at heart level. The finometer uses beat by beat arterial pressure waveform analysis as assessed by photoplethysmography using the Modelflow method (Beatscope, version 1.1; Finapres Medical Systems), which in combination with heart rate has been documented to accurately estimate cardiac output during a variety of experimental protocols [46,47]. This technique requires a blood pressure cuff be placed on the upper arm (biceps) and on the finger of the participant. Mean arterial blood pressure will also be measured by an automatic sphygmometer (Tango+, Suntech, North Carolina) at rest and during exercise.

Dietary Nitrate Supplementation: Nitrate-rich (70 ml per drink, 5 mM/day, 0.45g of nitrates) or nitrate-depleted (placebo; 70 ml per drink, 0mM/day, 0.0g of nitrates) beetroot juice will be consumed for 1 to 5 consecutive days prior to reporting to laboratory. This supplement is commercially available (Beet It, James White Drinks, Ipswich, UK) and has been reported to reduce blood pressure and improve exercise performance by increasing nitric oxide bioavailability [48-53].

Electron paramagnetic resonance spectroscopy (EPRS) Ex-vivo spin-trapping with PBN (190 mM/L in 0.9% NaCl) will be utilized for the downstream detection of lipid-derived radical species in blood, as previously described [23,48,49]. We will use the spin-trap concentration of 3:1 ratio of whole blood-trap that has proved optimal in our previous studies. We will also use a series of ferrous iron-dithiocarbamate ligand spin-trap complexes to directly assess NO concentration in blood. Briefly, spin-trap adducts will be extracted from serum (or solute in the case of tissue) into toluene, vacuum degassed for 2 cycles and analyzed at 21°C using an EMX X-band EPR spectrometer fitted with an ER TM110 cavity (Bruker, Karlsruhe, Germany). Spectrometer conditions will be as follows: 20mW experimentally validated non-saturating incident microwave power, 0.5 G modulation, 1 x 105 receiver gain, 82 msec time constant, 3450G magnetic field centre and ± 50G scan width, for 15 incremental scans. EPR spectral parameters will be obtained using commercially available software (Bruker Win EPR System, Version 2.11) and filtered identically. The average spectral peak-to-trough line height will be considered a measure.

Endothelial Cell (EC) collection and protein expression: The procedures for the collection of EC and measurement of endothelial protein expression have been previously described [28]. Briefly, 2 to 4 sterile J-wires are advanced into the femoral or brachial artery and/or vein (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter, then transferred to a dissociation buffer solution, where ECs are recovered by washing and centrifugation. EC protein expression will be determined according the method described by Donato et al 2007 [28]. Following EC collection, collected cells will be fixed with 3.7% formaldehyde and plated on poly-L-lysine coated slides (Sigma Chemical, St Louis, Mo). For immunofluorescence

staining ECs will be rehydrated with PBS and nonspecific binding sites blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pa). Afterward cells will be incubated with monoclonal antibodies for one of the following: nitrotyrosine (Abcam; Cambridge, Mass), xanthine oxidase (US Biological; Swampscott, Mass), NAD(P)H oxidase-p47phox (Abcam; Cambridge, Mass), CuZn SOD (Upstate; Lake Placid, NY), Mn SOD (Research Diagnostics; Concord, Mass), catalase (Abcam; Cambridge, Mass) or NF-KB p65 (Novus; Littleton, Colo). Cells will next be incubated with CY3-conjugated secondary antibodies (Research Diagnostics; Concord, Mass). For analysis, slides will be viewed using a fluorescence microscope (Eclipse 600, Nikon, Melville, NY) and EC images will be digitally captured by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Inc., Tucson, Ariz). ECs will be documented by cell staining of von Willebrand factor and nuclear integrity will be confirmed using DAPI (4',6'-diamidino-2-phenylindole hydrochloride) staining. Once endothelial cells with intact nuclei are identified, they will be analyzed using Metamorph Software (Universal Imaging Corp, Downingtown, Pa) to quantify the intensity of CY3 staining (i.e. average pixel intensity). Values will be reported as ratios of EC protein expression/human umbilical vein EC (HUVEC); this minimizes the possible confounding effects of differences in intensity of staining among different staining sessions.

Exercise Testing: Passive leg movement will be performed by a member of the research team moving the participant's leg through a normal range of motion (90°- 180°).

Single contraction exercise protocols will be performed with either the upper (handgrip) or lower (knee extension) extremity. A single maximal voluntary contraction (MVC) will be established for each participant using a handgrip or knee extension dynamometer with an analog output. This MVC value will be used to calculate 10%, 20%, 30%, 40% and 50% of MVC for the single contraction. Following a 2 min baseline period the participant will perform the rapid (< 1 sec) single contraction and central and peripheral hemodynamics will be assessed for 1 min post contraction.

Active leg exercise will be performed using a custom-built knee-extensor ergometer with the participant moving the leg between 90°- 180° at a rate of approximately 60 rpm.

Plantar flexion exercises will be performed using an ergometer specifically designed to isolate the gastrocnemius and the soleus complex at a rate of approximately 60 rpm.

Handgrip exercise will be performed with the participant laying supine on the laboratory bed with handgrip dynamometer in their right hand. The right arm will be extended perpendicular to the participant's torso and supported by a height-adjustable table. Doppler ultrasound will be performed by a trained sonographer in order to obtain brachial artery blood flow measurements. Exercise will be performed for 4 stages each lasting 3 - 5 minutes and separated by at least 1 min of rest. Intensity will range from 10 to 60% of the participant's maximal handgrip strength. During exercise ECG and blood pressure will be continuously monitored. Arm exercise will involve rhythmic handgrip using a computer-interfaced dynamometer.

The graded exercise test will be performed on a cycle ergometer or treadmill using a conventional ramp test to exhaustion. Work rate/exercise intensity will be increased by an

appropriately tailored level from 2.5-25 watts in each step, depending upon capabilities. Pulmonary ventilation and gas exchange will be determined continuously using a low-resistance two-way breathing valve (Hans-Rudolph 2700, dead space 90 ml) and a pneumotachometer (Fleisch no. 3) on the expiratory side. Signals will be assimilated by a PC and O_2 and CO_2 analysis will be performed in real time (Parvo Medics, UT). All of this equipment is currently in place and has been used routinely in our laboratory with both healthy volunteers and patients. Throughout all our exercise testing, safety is ensured through careful echocardiogram (ECG) monitoring, availability of a defibrillator, an oxygen source, emergency drugs, and attendance of qualified personnel.

Flow Mediated Dilation (FMD): After 15-20 minutes in the supine position a blood pressure cuff will be placed on the lower leg or forearm. Ultrasound images of the artery and Doppler waveforms will be obtained prior to cuff inflation (baseline). The cuff will then be inflated to 250mmHg for 5 minutes. Following the cuff release, ultrasound images of the popliteal artery and Doppler waveforms will be recorded continuously for 2 minutes. During this time, arterial images during diastole will be captured by image analysis software (Medical Imaging Applications). All recorded ultrasound images and velocity spectra segments will be saved to the GE Logiq 7 hard drive for off-line image and waveform analysis. The time averaged, and intensity-weighted mean blood velocity (Vmean) will be averaged across 5 second intervals for the entire 2 minute clip following cuff release. In addition, edge detecting software will be utilized to measure arterial diameter frame by frame, which will also be averaged into 5 second intervals. Summed shear rate throughout the 2 minutes will be calculated based on vessel diameter and blood velocity. The maximum change in vessel diameter, relative to baseline, will be determined and expressed as %FMD and %FMD/summed shear rate. A greater FMD response is an indicator of healthier vessels. A typical response for a young healthy participants would be 8 - 12% or greater dilation, whereas a poor response would be <5%. In addition, it is also important to be able to express the change in diameter relative to shear stress, as the shear stress is the stimulus for the NO-induced dilation. Again, greater FMD/shear values are indicative of healthier vessels.

Limb blood flow: Blood flow will be measured with an ultrasound Doppler (Logiq 7, GE) equipped with linear array mechanical sector transducers operating at an imaging frequency of 10-14 MHz. Vessel diameter will be determined at a perpendicular angle along the central axis of the scanned area, where the best spatial resolution can be achieved. The blood velocity profile will be obtained using the same transducers with Doppler frequency of 4.0-5.0 MHz, operated at high-pulsed repetition frequency mode (2-25 kHz) with a depth of 1.5-3.5cm. Special care will be taken to avoid aliasing, to ensure that probe position is stable, the insonation angle does not vary, and that the sample volume is positioned in the center of the vessel and adjusted to cover the width of the diameter and the blood velocity distribution. Blood velocity measurements will be obtained with the probe at an appropriate angle to maintain an insonation angle of 60º or less and the sample volume centered. Using arterial diameter and mean velocity (Vmean), blood flow will be calculated as: *Blood Flow (mL/min) = Vmean · π · (Vessel Diameter/2)2 · 60.*

Statistical Methods, Data Analysis and Interpretation

A total of 72 participants will be recruited for the protocols outlined above. While participation in more than one study will not be compulsory, we will invite all participants to participate in multiple protocols to strengthen the statistical comparisons. The data will be collected in a series of integrated protocols performed at different visits for each specific aim. Power analysis (discussed in detail below) based on preliminary work in our laboratory and previous findings in the literature reveals that 20 participants per group will result in adequate power to determine significant differences between hypertensive and normotensive participants for each of the specific aims. At each of the visits involving catheterization, the femoral artery and vein in the exercising limb will be catheterized allowing continuous blood pressure measurement, repeated measures of arterial blood flow, and arterial-venous metabolite determinants (such as O_2 and free radical concentrations to measure delivery or production rates). Measurements will be made at specific time points before, during and after knee extension exercise. The exercise stages of the protocol include baseline (stage 0), passive movement (stage 1), and 3 exercise levels (25% = stage 2, 50% = stage 3, and 75% = stage 4 of previously determined maximum single-leg work load). A key variable to be measured will be the exercise pressor reflex (EPR), which for the purpose of data analysis is strictly defined as the steady state blood pressure at a given stage of exercise minus the baseline (stage 0) blood pressure. Analogous responses will be calculated for changes in blood flow and oxygen delivery. Measurements during passive movement (stage 1) and PECO are used to address separate hypotheses regarding the contribution of mechanoreceptor and metaboreceptor afferent feedback. The approach for analysis of EPR is given below, comparable statistical methods will be applied to the other variables and are not discussed in detail here.

The magnitude of the EPR is expected to be influenced by afferent feedback (Specific Aim #1), sympathetic activity (Specific Aim #2), and oxidative stress (Specific Aim #3). The main hypothesis of each specific aim posits that the tested component will be greater in hypertensive than normotensive participants. The outcome variable to be compared is the difference in EPR before and after a given treatment. Thus, if $uEPR_4$ is the untreated EPR at stage 4 and $fEPR_4$ is the EPR measured after fentanyl administration, then ($uEPR_4$ - $fEPR_4$) may be considered the afferent-mediated portion of the EPR and is compared in hypertensive versus normotensive. Since untreated and fentanyl-treated EPRs will be generated at 3 graded exercise levels, a repeated measures ANCOVA using a mixed general linear model (with participant as the repeated measure) will be tested with the dependent variable as ($uEPR_i$ – $fEPR_i$) (where i = 2 to 4 for the respective exercise stages) and independent variables will include age, gender, hypertension status, and i (exercise stage).

Power analyses are summarized in the tables below. The strategy for these analyses was to conservatively test power for outcomes at stage 4 (e.g., $uEPR_4$ - $fEPR_4$), comparing hypertensive to normotensive, recognizing that the inclusion of the supporting measures at exercise stages 2 and 3 will only serve to increase power.

Clinical Records: Patient demographics (DOB, gender, height, weight), past medical history, medication use, and results of cardiovascular tests (echocardiogram, catheterization, stress test) will be collected