Protocol Summary
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"Vascular and Autonomic Maladaptations in Patients with Vascular Dysfunction".

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BACKGROUND AND INTRODUCTION:

Heart failure (HF) is a serious condition where the heart is unable to fill or pump blood efficiently throughout the body in order to meet the body's needs. Typically, the heart becomes weakened as a result of progressive coronary artery disease or hypertension. There are several different types of HF, including chronic and acute heart failure. Common symptoms seen in patients with HF are fatigue, weakness, rapid or irregular heartbeat, dyspnea, persistent cough or wheezing, lack of appetite, and chronic or acute swelling of feet, ankles, legs, or abdomen. HF most often effects older individuals that have one or more of the following risk factors: high blood pressure, coronary artery disease, myocardial infarction, irregular heartbeats, diabetes, congenital heart defects, sleep apnea, certain viruses, alcohol use, or some kidney conditions. There is no cure for HF and in the most severe stage; heart transplantation (HTx) is usually the best option.

One of the primary criteria in diagnosis and severity of HF is the response to a 6-min walk test, emphasizing the clear detrimental effect of this disease on the capacity for physical activity. Surprisingly, it is now well established that this cannot be attributed to a cardiac limitation, but rather is related to peripheral adaptations characteristic of the disease. Together, several recent studies have advanced the "muscle hypothesis", which proposes that disease-related changes in ergoreceptor (*metaboreceptor* and *mechanoreceptor)* sensitivity in skeletal muscle collectively result in elevated muscle afferent activity that in turn further elevates sympathetic nerve activity (SNA) [1]. While the mechanism responsible for the adaptation is poorly understood, there is new evidence from animal studies that skeletal muscle oxidative stress may contribute to heightened ergoreceptor sensitivity [2]. Nonetheless, relative importance of metabolic versus mechanical receptors to this maladaptive process remains somewhat uncertain.

As HF progresses, poor contractile function and the subsequent reduction in arterial blood pressure is corrected by the carotid and pulmonary baroreflexes. There is evidence both for [3] and against [4] disease-related changes in carotid baroreflex control of heart rate (vagus nerve) and of muscle sympathetic nerve activity (MSNA) in HF, by mechanisms which remain poorly understood. The arterial baroreflex typically buffers the muscle metaboreflex by limiting peripheral vasodilation, but in HF the loss of baroreflex sensitivity could contribute to the apparent metaborefelx sensitivity simply through decreased buffering capacity [5]. To our knowledge, the combined effects of altered baroreflex, chemoreflex, and mechanoreflex responses in HF have not been systematically studied in humans.

Heightened SNA has been identified as a primary predictor of mortality in HF [6]. The consequence of this well described sympathoexcitation during exercise in HF patients may be a level of vasoconstriction in the exercising muscle that cannot be overcome, i.e. an impaired "functional sympatholysis". However, to date, no attempt has been made to evaluate disease-related changes in alpha adrenergic sensitivity in this cohort. Further, if this disease does in fact infer a level of vasoconstriction that is restraining muscle blood flow during exercise, interventions that may limit this maladaptive process are of clear interest. One such intervention is exercise training, which reduces SNA in many experimental models of HF [7, 8]. Recent studies in HF patients have supported earlier animal work, demonstrating the ability of aerobic exercise training to restore SNA to that of young healthy controls [9]. To date, very little is known regarding the mechanism(s) and time course for the well-documented training-induced improvements in SNA

Vascular dysfunction has been documented in patients with HF [10], which includes the inability of vessels to respond to physiological stimuli such as increased blood flow and endothelium-dependent vasodilation [11]. Heart transplantation (HTx)

results in a denervated heart. As a result the changes in cardiac output and heart rate normally triggered by changes in the sympathetic and parasympathetic tone are abolished or blunted.

There has been increasing interest in vascular function as the health and elasticity of major arteries is a risk factor for various types of cardiovascular disease and has been suggested to "naturally" deteriorate with age. In addition, there has also been increasing interest in isolating the physiological and mechanical contributors to the blood flow response, especially during exercise (exercise hyperaemia). The examination of vascular function in patients with HF and following HTx maybe important with regards to the prevalence of cardiovascular disease in these populations. While the abnormal hemodynamic responses common in HF and HTx patients compared with healthy individuals may help to further explain the mechanisms behind vessel vasodilation and changes in blood flow at the onset of exercise.

Oxidative stress is determined by the level of free radicals and the body's antioxidant capacity. High levels of vascular free radicals have been associated with many chronic diseases including heart failure, hypertension, atherosclerosis, diabetes, and the normal aging process [12]. With the presence of heart failure and the invasive nature of a heart transplantation it seems reasonable that ingesting an exogenous antioxidant cocktail (vitamins C, E, and alpha lipoic acid), used with efficacy by our group in the past [13-17], may decrease circulating levels of free radicals and may actually improve vascular function but the exact effects and potential mechanisms of action in this population are unknown.

One mechanism by which the antioxidant cocktail improves vascular function, may be through preventing oxidation of tetrahydrobiopterin (BH_4), a critical co-factor for the endothelial nitric oxide synthase enzyme, allowing greater NO bioavailability and less oxidative stress production [18]. Some suggest that loss of BH_4 may be one of the precipitating factors inducing free radical production [19], and acute administration of BH_4 alone has been demonstrated to improve vascular function in aged [20] and those with HF [21]. Although, evidence from the *in vitro* literature indicates there is a strong potential for synergy between BH_4 and antioxidants [22], this has yet to be tested in humans if BH_4, antioxidants, or BH_4 + antioxidants are capable of improving vascular function in HF patients and age matched controls over placebo.

OBJECTIVES:

The objectives of this study are to provide greater insight into changes in muscle and cardiovascular function and exercise-induced hyperaemia associated with congestive heart failure (HF) and post-heart transplantation (HTx).

Specific Aim 1:

To examine vascular function and the subsequent exercise-induced hyperaemia in both healthy controls and patients having congestive heart failure and post-cardiac transplant.

Hypothesis: There will be progressively reduced vascular function from the time of HTx through 10 years post-HTx.

Hypothesis: HTx patients we will not see an increase in cardiac output with the initiation of passive exercise.

Hypothesis: With the isolated limb passive movement model exercise induced hyperaemia will be significantly attenuated in the HTx patients and little to no hyperaemia will occur in the non-moving control leg.

Hypothesis: The antioxidant cocktail will attenuate circulating levels of free radicals and may improve vascular function, both as assessed by flow-mediated vasodilation (FMD) and the passive movement protocol, but will be further improved with co-ingestion of BH_4, over placebo, antioxidants, or BH_4 alone.

Specific Aim 2:
To determine the extent to which *muscle ergoreceptor* (mechanoreceptor and metaboreceptor) and *arterial baroreflex* dysfunction is present in HF, to evaluate the interaction of these reflexes, and to identify which cardiovascular parameters are most affected by potential ergoreceptor and baroreceptor adaptations.

Hypothesis: HF patients will exhibit greater mechanoreceptor and metaboreceptor sensitivity than age-matched controls, while carotid baroreflex sensitivity will be blunted in HF. This heightened ergoreceptor sensitivity in HF is due, in part, to blunted baroreflex sensitivity.

Hypothesis: A reduction in oxidative stress, induced by either acute or chronic ingestion of the antioxidant cocktail, will reduce mechanoreceptor and metaboreceptor sensitivity in HF patients and not age-matched controls.

Hypothesis: Chronic ingestion of and antioxidant cocktail, combined with the eNOS cofactor tetrahydrobiopterin (BH_4), will reduce mechanoreceptor and metaboreceptor sensitivity in HF patients and not age-matched controls.

Specific Aim 3:
To determine whether *exercise training* can alter muscle ergoreceptor and carotid baroreceptor sensitivity in HF patients, and to characterize the time course for ergoreceptor- and baroreceptor-related changes.

Hypothesis: Exercise training will reduce muscle ergoreceptor sensitivity and increase carotid baroreflex sensitivity if HF but not age-matched controls.

Specific Aim 4:
To determine the degree to which elevated SNS activity in expressed in the exercising muscle at rest and during aerobic exercise.

Hypothesis: HF patients will exhibit a sustained response to infused adrenergic drugs during exercise compared to healthy controls, demonstrating “impaired functional sympatholysis” in the cohort.

PARTICIPANT SELECTION CRITERIA:

Inclusion criteria:
- Patients treated for heart failure (NYHA class I, II, or III)
- Patients who have undergone heart transplantation (HTx)
- Patients with Hypertension (HTN)
- Patients with Chronic Obstructive Pulmonary Disorder (COPD)
- Patients with Scleroderma
- Healthy controls of all ages

Exclusion criteria:

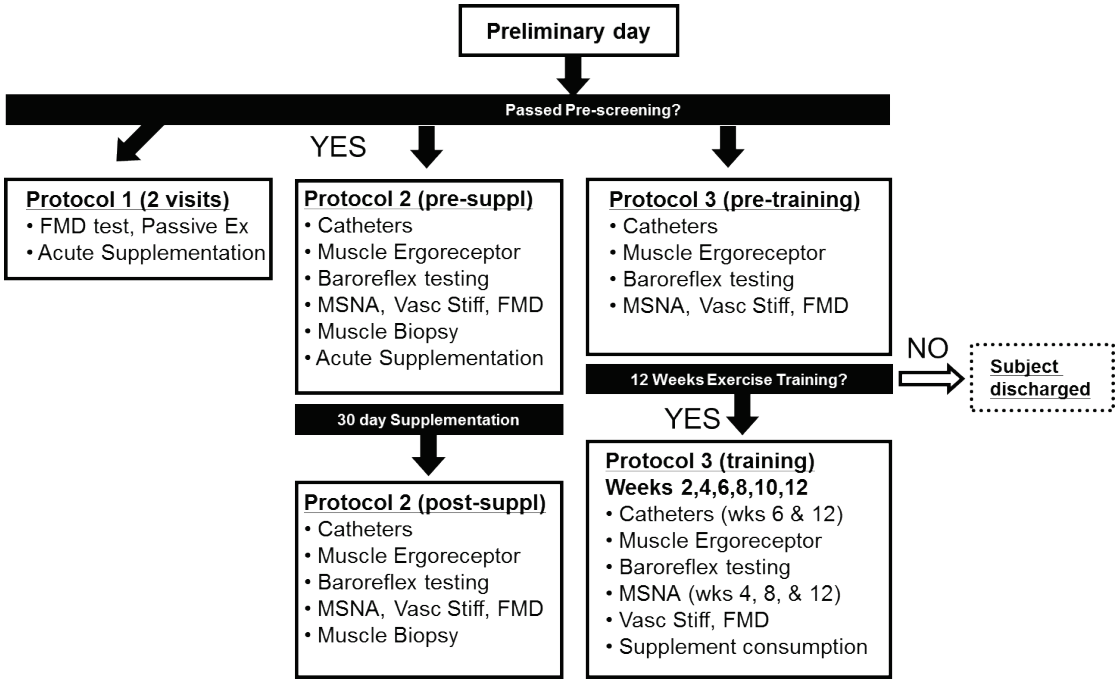
- Age less than 18 years
- Patients unwilling or unable to give consent
- Subjects in whom the study procedures would not be recommended for medical reasons. This determination will be made by Dr. Stehlik, the physician directly responsible for the patient's care.
- It is anticipated that patients who are currently hospitalized would not participate until released to outpatient care.
- NYHA class IV heart failure patients.
- Women who are pregnant.

DESIGN:

Procedures that are part of this study are strictly research-related and are not considered standard of care. This is a cross-sectional and short-term longitudinal study. Patients may be eligible to participate in the study several times (e.g. participation before and after transplant, participation at different time intervals after heart transplantation, participation in a training protocol), but are not required to do so. Patients may enroll in protocols 2, 3, or 4 any time after completion of protocol 1. However, after completing protocol 2, 3, or 4, patients must wait two months before enrolling in an additional protocol. Patients may also participate in protocol 3 (pre-training) or protocol 4 without agreeing to the exercise training portion of the study. The study procedures will include multiple visits to the laboratory in a fasted and rested state, as outlined below. Comparisons will be made between the healthy controls, HF, and HTx patients.

STUDY PROCEDURES:

The protocols that patients may choose to participate are outlined on the diagram below;



STUDY PROCEDURES (continued): 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 that provide details for the underlined techniques and measurements identified in each protocol, listed alphabetically.

Preliminary Day

A signed consent form will be obtained prior to any research procedures. Upon arrival to the lab (VA Bldg. 2, Room 1D21), a screening questionnaire will be filled out that will include questions concerning risks such as: age, family health history, smoking, hypertension, hypercholesterolemia, sleep-related breathing disorders, physical activity and allergies (iodine, latex, drugs, etc). A venous blood sample (4 ml) will be drawn from which a Metabolic Panel, Complete Blood Count (CBC), and a Lipid Panel will be assessed. Women of childbearing age will provide a urine sample for pregnancy testing, and will provide information concerning method of birth control.

Following health history and blood sample, all subjects may be asked to perform several tasks to assess cognitive abilities and fitness levels and to wear a pedometer for a week, which will measure activity levels. All subjects will complete a series of exercise tests. Subjects will complete one or more of the following: a graded exercise test to volitional exhaustion (bike or treadmill, < 15 min), maximal handgrip test (1 min), maximal leg extension test (< 15 min), and maximal plantar flexion test (< 15 min). Sufficient recovery will be allowed between tests, an ECG will be assessed at all times. The PI will be present for all exercise tests, and a physician will be immediately available if needed.

The handgrip, leg extension and plantar flexion graded exercise tests are considered low cardiovascular risk tests due to the recruitment of an isolated small muscle mass that results in minor cardiovascular stress. For all subjects these exercise tests will be conducted in the presence of the PI. In addition, a board certified physician (Dr. Kithas or Supiano) will be immediately available if needed.

For the graded test to volitional exhaustion, exercise will be completed under physician supervision, and the subject 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.

PROTOCOL 1 (Acute Supplementation, 4 visits):

On four separate visits, patients will take one of the following: placebo, antioxidants, BH_4 (10mg/kg), or BH_4 + antioxidants, 2 hours prior to arrival in the lab in a balanced, double-blind fashion. A member of the investigative team will provide these pills to subjects in bags labeled “A”, “B”, “C”, or “D” during the preliminary visit described above. These four visits will take place at least 48 hrs. apart, but are expected to be completed within 2 weeks.**Protocol 1 procedures:** Subjects 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. Upon arriving in the lab a venous blood sample (4 ml) will be drawn from which measures of oxidative stress and antioxidant capacity will be assessed. Following the blood sample, all subjects will have their height, weight, skin fold thickness and resting arterial blood pressure (ABP) recorded. Women of childbearing age will be asked to submit to a urine pregnancy test provided by the research site. Next, arterial stiffness measures will be completed. After lying quietly for an additional 20 minutes, the subject

will undergo the flow mediated vasodilation (FMD) test in their arm (brachial artery). Nitroglycerin (NTG) will then be administered to evaluate endothelium-independent vasodilation. Two oral nitroglycerin tablets (0.8mg) will be administered sublinguilly. Measurements will be made for 5 minutes. Subjects will then rest 20-min to allow drug washout and restoration of steady-state hemodynamics. Following NTG, limb blood flow will be measured during four exercise protocols that utilize a single arm and single leg. First, the arm and leg will be passively moved through a normal range of motion (the participant will stay relaxed and try not to engage their muscles). Qualified personnel of the Vascular Laboratory will be supporting and moving the arm or leg for the participant. Next, an active exercise test will be performed, which requires that the participants give maximum effort for either the arm or leg by moving that appendage through a normal range of motion against some resistance. The arm or leg will be the only part of the body moving or producing any effort.

PROTOCOL 2 (Chronic Supplementation, 7 visits):

For this protocol, patients will be divided into four groups taking one of the following: (1) placebo, (2) antioxidants, (3) BH_4 (10mg/kg), or (4) BH_4 + antioxidants, one time daily for 30 days. A member of the investigative team will provide these pills to subjects in bags labeled “A”, “B”, “C”, or “D” during the preliminary visit described above. Assignment to group will be balanced and both investigator and subject will be blinded to the group. Compliance for pill consumption will be monitored by an online database to which subjects will log in each day or a phone call if internet access is not available.

Day 0: Subjects 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. Upon arrival, women of childbearing age will submit toa urine pregnancy test, provided by the site, as part of the preliminary study process. Catheters will be placed in the arm (brachial artery and vein) or leg (common femoral artery and femoral vein) by a licensed physician using standard sterile technique. After instrumentation, an Arterial Elasticity Measurements/Pulse Contour Analysis (PCA) test will be performed immediately followed by a flow mediated vasodilation (FMD) test in the arm (brachial artery) or leg (popliteal artery). Following the FMD test, a **muscle biopsy** will be taken from the vastus lateralis of one leg. Next, two microelectrodes will be placed in the contralateral leg (popliteal fossa) for direct recording of muscle sympathetic nerve activity (MSNA). Subjects will rest for approximately 10 minutes, followed by resting measurements of arterial blood pressure (ABP), heart rate (HR), limb blood flow (LBF) (ultrasound Doppler), muscle sympathetic nerve activity (MSNA), and collection of resting blood samples.

After resting measurements, baroreflex sensitivity will be assessed using both pharmacologic and direct techniques. Using the modified oxford technique, a series of I.V. pressor and depressor bolus drug infusions will be administered, and changes in ABP, HR, LBF and MSNA will be measured. After a 15-min recovery, carotid baroreflex sensitivity will be assessed directly using a neck chamber that provides localized distension or compression of the carotid artery, provoking small changes in carotid sinus pressure. This neck pressure/neck suction (NP/NS) technique will alter afferent carotid baroreflex activity, and again changes in ABP, HR, LBF, and MSNA will be measured. This will be followed by an additional 15-min recovery for return to a hemodynamic steady-state.

Following baroreflex testing, muscle ergoreceptor sensitivity will be determined. Muscle mechanoreceptor sensitivity will be determined during 3-min of passive leg movement. This will be followed by muscle metaboreceptor sensitivity testing in both the

arm and leg utilizing post-exercise circulatory occlusion (PECO). Three levels of arm (handgrip exercise) and leg (knee-extensor exercise) will be performed at 20, 40, and 60% of maximal effort (3-min bout), with 2-min of PECO at the cessation of each exercise bout. During each of these exercise bouts, ABP, HR, LBF, and MSNA will be measured, and blood samples will be taken at each exercise intensity for blood gas analysis, and to assess the balance between antioxidants and oxidative stress across the leg.

After a 15-min recovery, the PECO protocol described above will again be performed with superimposed carotid baroreflex testing (modified oxford technique) to assess potential metaboreceptor-baroreceptor interaction. 1-2 doses of both PE and SNP will be administered during the post-exercise cuff occlusion depending on the rate of blood pressure change during drug infusion.

Days 10, 20, 30, 33 and 37: Following the baseline visits, subject will Patients will return to the lab five times after enrolling in the 30-day supplementation: days 10, 20, and 30, and two visits following discontinuation of the supplementation (days 33 and 37).

On **Day 30**, subjects will repeat the "Day 0" protocol outlined above.

Days 10, 20, 33, and 37 are non-invasive study days, with the following protocol;
Subjects 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. Upon arriving in the lab a venous blood sample (4 ml) will be drawn from which measures of oxidative stress and antioxidant capacity will be assessed. Following the blood sample, all subjects will have their height, weight, skin fold thickness and resting arterial blood pressure (ABP) recorded. Women of childbearing age will take a urine pregnancy test. Next, arterial stiffness measures will be completed. After lying quietly for an additional 20 minutes, the subject will undergo the flow mediated vasodilation (FMD) test in their arm (brachial artery). Following the FMD test, limb blood flow will be measured during four exercise protocols that utilize a single arm and single leg. First, the arm and leg will be passively moved through a normal range of motion (the participant will stay relaxed and try not to engage their muscles). Qualified personnel of the Vascular Laboratory will be supporting and moving the arm or leg for the participant. Next, an active exercise test will be performed, which requires that the participants give maximum effort for either the arm or leg by moving that appendage through a normal range of motion against some resistance. The arm or leg will be the only part of the body moving or producing any effort.

PROTOCOL 3

Pre-Training Protocol:
Subjects 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, and catheters will be placed in the arm (brachial artery and vein) or leg (common femoral artery and femoral vein) by a licensed physician using standard sterile technique. After instrumentation, an Arterial Elasticity Measurements/Pulse Contour Analysis (PCA) test will be performed, immediately followed by a flow mediated vasodilation (FMD) test in the arm (brachial artery) or leg (popliteal artery). Next, two microelectrodes will be placed in the contralateral leg (popliteal fossa) for direct recording of muscle sympathetic nerve activity (MSNA). Subjects will rest for approximately 10 minutes, followed by resting measurements of arterial blood pressure (ABP), heart rate (HR), limb blood flow (LBF)

(ultrasound Doppler), muscle sympathetic nerve activity (MSNA), and collection of resting blood samples.

After resting measurements, baroreflex sensitivity will be assessed using both pharmacologic and direct techniques. Using the modified oxford technique, a series of I.V. pressor and depressor bolus drug infusions will be administered, and changes in ABP, HR, LBF and MSNA will be measured. After a 15-min recovery, carotid baroreflex sensitivity will be assessed directly using a neck chamber that provides localized distension or compression of the carotid artery, provoking small changes in carotid sinus pressure. This neck pressure/neck suction (NP/NS) technique will alter afferent carotid baroreflex activity, and again changes in ABP, HR, LBF, and MSNA will be measured. This will be followed by an additional 15-min recovery for return to a hemodynamic steady-state.

Following baroreflex testing, muscle ergoreceptor sensitivity will be determined. Muscle mechanoreceptor sensitivity will be determined during 3-min of passive leg movement. This will be followed by muscle metaboreceptor sensitivity testing in both the arm and leg utilizing post-exercise circulatory occlusion (PECO). Three levels of arm (handgrip exercise) and leg (knee-extensor exercise) will be performed at 20, 40, and 60% of maximal effort (3-min bout), with 2-min of PECO at the cessation of each exercise bout. During each of these exercise bouts, ABP, HR, LBF, and MSNA will be measured, and blood samples will be taken (at each exercise intensity) for blood gas analysis and to assess the balance between antioxidants and oxidative stress across the leg.

After a 15-min recovery, the PECO protocol described above will again be performed with superimposed carotid baroreflex testing (modified oxford technique) to assess potential metaboreceptor-baroreceptor interaction. 1-2 doses of both PE and SNP will be administered during the post-exercise cuff occlusion depending on the rate of blood pressure change during drug infusion.

Exercise Training (12 weeks):

Patients who agree to participate in the training portion of the study will report to the laboratory (VA Building 2 room 1D23) for 12 consecutive weeks (2-3 days per week, 1-2 hours per visit) for supervised exercise training. Small muscle mass exercise will be performed so that high intensity training can be carried out without reaching cardiopulmonary limits to O_2 transport, which is of critical importance when studying HF patients. Single-leg training will thus be utilized, using the knee-extensor exercise paradigm described below. Exercise intensity will be customized to the ability of each patient, and will be increased as tolerated every two weeks by re-assessing maximal power output in a conventional incremental test, to gain the greatest effect. A member of the research team will supervise all training sessions, and a physician will be immediately available if needed. Drs. Wray and Richardson have extensive experience with this exercise training protocol, including studies in the elderly, COPD and HF patients [13, 14, 23-33].

Intra-training and Post-training Protocol (weeks 2, 4, 6, 8, 10, and 12):

Patients will undergo bi-weekly assessment arterial elasticity, flow mediated vasodilation (FMD), muscle ergoreceptor sensitivity, and baroreflex sensitivity to track training-related improvements in muscle and cardiovascular parameters. The sequence and instrumentation will be identical to the pre-training protocol described above, with the following two exceptions; MSNA measurements will be made only at weeks 4, 8, and 12, and femoral catheters will be placed only at weeks 6 and 12. For experimental days that

do not include femoral arterial and venous catheters, the antecubital vein will be catheterized.

PROTOCOL 4 (6-8 hours per visit, 2 visits)

Day 1 (Arm Protocol)

Resting measurements (4 hours): Upon arrival to the lab, catheters will be placed in the non-dominant arm (brachial artery and basilica or cephalic vein) by a licensed physician. Following placement of catheters, intra-arterial infusion of Phenylephrine (PE), Dexmedetomidine (DEX), Tyramine (TYR), Phentolamine (PHEN), and Saline (control) will be performed in a randomized fashion. During the PHEN infusion, propranolol will be co-administered (I.V.) to prevent beta adrenergic tachycardia (control subjects only). Each drug infusion will last for approximately 15-min, and 20-min will be allotted between each infusion to allow drug washout and restoration of steady-state hemodynamic values.

Exercising measurements (4 hours): Subjects will perform five bouts of incremental handgrip exercise at three intensities (30%, 45%, and 60% of MVC, four minutes per stage). During each exercise bout, either PE, DEX, TYR, PHEN, or Saline (control) will be administered in a randomized fashion. During the PHEN infusion, propranolol will be co-administered (I.V.) to prevent beta adrenergic tachycardia (control subjects only). For PE, DEX, and TYR, exercise will be performed for 3 min to reach steady-state, followed by 1-min of drug infusion. For the PHEN trial, drug will be administered continuously throughout the exercise protocol and compared to continuous Saline infusion. Each exercise bout will last approximately 12 minutes, with 30 minutes rest between exercise bouts.

During both rest and exercise, arterial blood pressure, venous blood pressure, heart rate, and limb blood flow (ultrasound Doppler) will be measured continuously. An independent observer not involved in data collection will watch the electrocardiogram (EKG) and blood pressure monitors at all times to track the cardiovascular responses to exercise and drug infusions.

Day 2 (Leg Protocol)

Resting measurements (4 hours): Upon arrival to the lab, catheters will be placed in the femoral artery and vein by a licensed physician. Following placement of catheters, intra-arterial infusion of Phenylephrine (PE), Dexmedetomidine (DEX), Tyramine (TYR), Phentolamine (PHEN), and Saline (control) will be performed in a randomized fashion. During the PHEN infusion, propranolol will be co-administered (I.V.) to prevent beta adrenergic tachycardia. Each drug infusion will last for approximately 15-min, and 20-min will be allotted between each infusion to allow drug washout and restoration of steady-state hemodynamic values.

Exercising measurements (4 hours): Subjects will perform five bouts of incremental knee-extensor (KE) exercise at three intensities (25%, 50%, and 75% of KE max, four minutes per stage). During each exercise bout, either PE, DEX, TYR, PHEN, or Saline (control) will be administered in a randomized fashion. During the PHEN infusion, propranolol will be co-administered (I.V.) to prevent beta adrenergic tachycardia. For PE, DEX, and TYR, exercise will be performed for 3 min to reach steady-state, followed by 1-min of drug infusion. For the PHEN trial, drug will be administered continuously throughout the exercise protocol and compared to continuous Saline infusion. Each

exercise bout will last approximately 12 minutes, with 30 minutes rest between exercise bouts.

During both rest and exercise, arterial blood pressure, venous blood pressure, heart rate, and limb blood flow (ultrasound Doppler) will be measured continuously. An independent observer not involved in data collection will watch the electrocardiogram (EKG) and blood pressure monitors at all times to track the cardiovascular responses to exercise and drug infusions.

TECHNIQUES AND MEASUREMENTS

Antioxidant Supplementation: 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 [14-16, 34-36].

Arterial Elasticity Measurements/Pulse Contour Analysis (PCA) test: Arterial elasticity will be measured using the HDI/Pulsewave™CR-2000 CardioVascular Profiling System (Hypertension Diagnostic, Inc., Eagan, Minnesota, USA). The DO-2020 System records and analyzes the blood pressure waveform data from the Arterial PulseWave Sensor. PCA measurements will be obtained in the morning following an overnight fast of at least eight hours and prior to engaging in any strenuous physical activity. Following 10 minutes of supine rest, large and small artery elasticity indices will be obtained. A blood pressure cuff will be wrapped around the subject's upper left arm, and a rigid plastic wrist stabilizer to minimize movement of the right wrist will be used during the PCA test. With the right forearm in a supinated position, an Arterial PulseWave™Sensor will be placed on the skin directly over the radial artery at the point of the strongest pulse. The sensor will be adjusted to the highest relative signal strength, and the large and small arterial elasticity measurements will be obtained during 30 seconds of blood pressure waveform collections. In addition, other cardiovascular parameters will be recorded during this waveform collection, including pulse rate, systolic blood pressure, diastolic blood pressure, systemic vascular resistance, and total vascular impedance. Measurement will be obtained and averaged over three consecutive 30-second trials. This part of the procedures will last approximately 30 minutes.

Blood sampling: *As part of the preliminary screening*, a venous blood sample (4 ml) will be drawn from all subjects for assessment of overall health (metabolic panel, complete blood chemistry, and lipid panel). *On the experimental days*, arterial and venous blood will be collected anaerobically with sterile syringes from the above-mentioned catheters at rest and during each exercise level for assessment of metabolic and aerobic status, and to test for markers of oxidative stress. For protocol 1, 15 ml of venous blood will be obtained. For protocol 2, one sample of arterial (4 ml) and venous (4 ml) blood will be taken at rest and during each level of arm and leg exercise.

Catheter placement: Prior to catheter placement, the subject will first be connected to a 3-lead ECG monitor. 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 by a qualified and privileged physician who will assume responsibility for the placement and removal of these catheters. Following catheter removal, wounds will be dressed and the subjects educated as to proper care. No subject will be released until hemostasis is clearly present. Follow-up contact with the subject will continue over the following two days. It should be noted that the PI and Co-I's of this proposal have significant experience with catheter-related studies [29, 30, 37-46], having coordinated the use and insertion of these exact catheters over a hundred times in the last ten years without incidence of bleeding, infection or tissue damage. In fact, several subjects have performed multiple studies with the PI, indicating the benign nature and positive outcome of the procedures. The PI and Co-I's of this proposal have current University of Utah IRB approval for this procedure in elderly (IRB# 00030810) and COPD patients (IRB# 00032404).

Flow-mediated vasodilation (FMD) test: A blood pressure cuff will be placed on the upper arm and inflated to >250 mmHg for five minutes to partially occlude the forearm. Ultrasound Doppler measurements will be made in the brachial artery to evaluate FMD upon cuff release. Dr. Richardson's laboratory has direct experience with this technique [23].

Limb Blood Flow: An ultrasound Doppler system (Logiq 7, GE Medical Systems, Milwaukee, Wisconsin, USA) equipped with two linear array transducers operating at an imaging frequency of 7-8 MHz and 12-14 MHz will be used for the leg and arm, respectively. Vessel diameter will be determined at a perpendicular angle along the central axis of the scanned area, where the best spatial resolution is achieved. The brachial artery (BA) of the right arm will be insonated approximately midway between the antecubital and axillary regions, medial to the biceps brachii muscle. The blood velocity profile will be obtained simultaneously using the same transducers with a Doppler frequency of 4.0-5.0 MHz, operated in the high-pulsed repetition frequency mode (2-25 kHz) with a sample depth of 1.5-3.5 cm and the probe maintained at an insonation angle of 60° or less. Sample volume will be maximized according to vessel size and centered, verified by real-time ultrasound visualization of the vessel. Using artery diameter and V_{mean}, blood flow is calculated as: blood flow (ml/min) = $V_{mean} \cdot \pi \cdot (\text{Vessel Diameter}/2)^2 \cdot 60$. At all sample points, arterial diameter and angle-corrected, time-averaged, and intensity-weighted mean blood velocity (V_{mean}) values can be calculated using commercially available software (Logiq 7). Ultrasound digital images and velocity spectra segments of five minutes will be recorded and saved to the GE Logiq 7 hard drive for off-line image and waveform analysis. Dr. Richardson's laboratory has extensive experience with this methodology [23, 28, 40, 47, 48].

Modified Oxford Technique: Carotid baroreflex sensitivity will be determined by assessing HR responses to an increase or decrease in arterial blood pressure evoke by I.V. phenylephrine (PE) and sodium nitroprusside (SNP), respectively. Drugs will be administered sequentially intended to provoke a 10-15 mmHg change in blood pressure (PE 100-150 μg; SNP 50-150 μg). These drugs are considered to be investigational by the FDA, and thus an investigational new drug (IND) number has been issued to the PI (IND#108367). This technique has been utilized extensively in a research setting to determine baroreflex sensitivity in HF [49].

Muscle biopsy: All biopsies will be taken from the vastus lateralis approximately 3.5 cm deep, 15 cm proximal to the knee and slightly distal to the ventral mid-line of the muscle. The 5 mm diameter biopsy needle (Bergstrom) is attached to sterile tubing and a syringe to apply a negative pressure to assist in the muscle sample collection [50]. Lidocaine (2%) will be used as local anesthetic. The muscle samples from each biopsy will be immediately frozen in liquid nitrogen and stored at -80^{o}C for future microscopy, EPR spectroscopy, molecular and biochemical assays or immersion-fixed in gluteraldehyde for EM analysis. With our current technique and experience, utilizing 2-3 passes of the needle and double cut, we are able to attain as much as 300-400 mg of muscle which will permit the performance of all proposed analyses. The Principal Investigator has been involved in over 100 of these procedures with no negative outcomes [31-33, 46, 51], and currently holds VA credentialing and IRB approval for this procedure in both COPD patients and control subjects (IRB#00032404, "Mechanisms of Adaptation to Exercise in Health and COPD"). Additionally, in support of this technique, a series of subjects recently underwent 5 biopsies each over a period of time, illustrating that the biopsy experience is acceptable to the subjects.

Muscle sympathetic nerve activity: Postganglionic muscle sympathetic nerve activity (MSNA) will be recorded with standard microneurographic techniques. In the right leg, low-voltage (3-4 Volts) stimuli will be applied to the skin behind the knee using a pencil-shaped electrode. Stimuli will be applied intermittently (i.e. 1 stimulus every 15 seconds) for approximately two minutes to map the path of the nerve. This procedure will cause light muscle twitching of the foot. Once the nerve path is identified, two very small tungsten microelectrodes (1-4 micrometers) will be inserted into the skin. The first electrode will be inserted 2-3 cm distal to the nerve map and acts as a reference, and the second electrode will be inserted near the fibular head and advanced until it reaches the peroneal nerve (1-2 cm). This electrode will be used for recording only. MSNA recordings display a pulse-synchronous burst pattern and an increase in burst frequency with end-expiratory breath hold and Valsalva maneuver, but with no response to startle or skin stroking. These characteristics will be used to determine proper electrode placement for the MSNA recordings. One of the co-investigators (Dr. Wray) is trained in microneurography and routinely performs this procedure under IRB # 00030810, "Vascular Function in Health and Disease".

Neck Pressure/Neck Suction (NP/NS): Graded levels of pressure and suction will be generated by a variable pressure source and delivered through solenoid valves to a custom-made neck chamber that encompasses the anterior 2/3 of the neck, creating an air-tight seal around the neck. The application of neck pressure (NP) increases pressure within the neck chamber that compresses the carotid baroreceptors and causes a decrease in carotid sinus transmural pressure, thereby exposing the carotid baroreceptors to a mild hypotensive stimulus. Neck suction (NS) stretches the carotid baroreceptors and causes an increase in carotid sinus transmural pressure that effectively delivers a mild hypertensive stimulus to the carotid baroreceptors. Baroreflex sensitivity will be assessed by measuring changes in HR, ABP and MSNA during 2–4 trials of random ordered single 5 s pulses of NP (+40 and +20 Torr) and NS (-20, -40, -60, and -80 Torr), or through the rapid pulse train NP/NS protocol (12 consecutive, ECG-triggered 500 ms pulses of NP and NS ranging from +40 to -80 Torr). Dr. Wray has extensive training using the neck collar and NP/NS protocol [52-56].

Non-invasive arterial blood pressure: Systemic arterial blood pressure (ABP) will be measured non-invasively on a continuous basis using finger photoplethysmography (Finometer, Ohmeda, Madison, WI, USA). The finometer cuff is placed on the middle

finger of the right hand and is supported on a modified surgical stand adjusted to position the finger cuff at heart level.

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

Active leg exercise will be performed using a custom-built knee-extensor ergometer with the subject moving the leg between 90°- 180°.

Plantar flexion exercises will be performed using an ergometer specifically designed to isolate the gastrocnemius and the soleus complex.

Arm exercise will involve rhythmic handgrip using a computer-interfaced dynamometer.

Each of these exercise modalities are small muscle mass and therefore only minimally tax the heart and lungs, even at maximal efforts [57]. All exercise testing will be completed in the VA Medical Center Building 2 room 1D23.

Post-Exercise Circulatory Occlusion (PECO): To active afferent muscle fibers that are sensitive to exercise-induced metabolites, supra-systolic (220-240 mmHg) circulatory occlusion of the exercised limb will be applied upon cessation of the 3-min exercise bout. This will be achieved through inflation of a modified blood pressure cuff placed on either the arm (upper arm, immediately proximal to elbow) or leg (upper thigh) for 2-min. This procedure is used routinely in a clinical research setting, including studies involving HF [58].

STUDY DRUGS: The overall objective of all drug infusions for this protocol is to achieve maximal receptor occupancy in the limb while avoiding spillover of the drug into the systemic circulation. All drug doses have been determined using previously published ranges, and are calculated per 100 ml (e.g. dl) limb volume. All drug preparation, dispensing, and disposal will be handled by the VAMC SLC Inpatient Pharmacy. All drugs have been approved by the FDA for use in the manner described herein (IND#113,067).

1. Phenylephrine (PE): At rest, PE will be administered in five doubling doses (0.03-0.06-0.12-0.24-0.48 mcg/dl/min, 2-min per dose) to establish a dose-response for PE-induced vasoconstriction. During exercise, PE will be administered at 0.24 mcg/dl/min, and adjusted for the exercise-induced increase in limb blood flow. The method for determining this dose range is based on previously published studies [40, 59].

2. Dexmedetomidine (DEX): At rest, DEX will be administered in five doubling doses (1.5-3-6-12-24 ng/dl/min, 2-min per dose) to establish a dose-response for DEX-induced vasoconstriction. During exercise, DEX will be administered at 6 ng/dl/min, and adjusted for the exercise-induced increase in limb blood flow. The method for determining this dose range is based on previously published studies [60, 61].

3. Tyramine (TYR): At rest, TYR will be administered in five doubling doses (2-4-8-16-32 mcg/dl/min, 2-min per dose) to establish a dose-response for DEX-induced vasoconstriction. During exercise, TYR will be administered at 8 mcg/dl/min, and

adjusted for the exercise-induced increase in limb blood flow. The method for determining this dose range is based on previously published studies [62-65].

4. Phentolamine (PHEN): At rest, PHEN will be administered in three doubling doses (5-10-20 mcg/dl/min) to establish complete alpha adrenergic blockade. During exercise, PHEN will be administered at 5 mcg/dl/min, and adjusted for the exercise-induced increase in limb blood flow. The method for determining this dose range is based on previously published studies [66-70].

5. Propranalol: At rest and during exercise, propranolol (a non-selective beta adrenergic antagonist) will be administered intravenously for 5-min (0.15 mg/kg, max 10 mg) prior to PHEN infusion to limit the possibility of beta adrenergic tachycardia during PHEN administration. This infusion will be administered only in control subjects, as the HF patients all receive beta-blocking drugs as part of their optimized pharmacotherapy.

6. Kuvan (BH_4): will be administered at a dose of 10 mg/kg body weight.

7. Nitroglycerin (NTG): will be administered to evaluate endothelium-independent vasodilation in the arm and leg. Two oral nitroglycerin tablets (0.8mg) will be administered sublinguilly. Measurements will be made for 5 minutes. Subjects will then rest 20-min to allow drug washout and restoration of steady-state hemodynamics.

POTENTIAL RISKS

Catheter-related risks. There is also the possibility of fainting, dizziness, and possible pain and bruising as a result of catheter insertion. There is a risk of infection, swelling, and discomfort at the insertion sites, and a risk that bleeding may occur after catheters removal. A clot or excessive bleeding at the puncture site could result in a partial blockage of the blood flow to the arm or leg, which in extreme cases could lead to loss of the limb. These risks are minimized by careful patient selection, catheter insertion by a physician with experience using proper equipment and sterile methods, adequate pressure for >30 minutes following catheter removal, and appropriate patient education and follow-up. The PI and several CO-I's named herein have significant cumulative experience in this areas, having been involved in several hundred successful catheter-based studies with no instances of bleeding, infection, femoral vein thrombosis or soft tissue damage as a result of the procedures proposed.

Drug Infusion Risks: At higher intra-arterial doses, the risk exists for significant spillover of the drug into the systemic circulation resulting in acute changes in arterial blood pressure. Thus, during the higher limb blood flow which occurs during exercise, blood pressure will be closely monitored, and infusions will be discontinued if acute hypertension is observed (>15 mmHg rise in systolic or diastolic pressure). The proposed doses for all drugs included in this protocol have been demonstrated to act locally, with minimal systemic effects. The PI and CO-I's on this protocol have extensive experience with intra-arterial drug infusions, and routinely perform infusion studies identical to those described above in a concurrent IRB-approved project (IRB#30810, "Vascular Function in Health & Disease").

Phenylephrine (PE): Systemic doses of PE may cause headache, reflex bradycardia, excitability, restlessness and rarely arrhythmias, and is contraindicated in patients with severe hypertension, ventricular tachycardia, or in patients who are hypersensitive to it or to any of the components. If used in conjunction with oxytocic drugs, the pressor effect of sympathomimetic pressor amines is potentiated. The obstetrician should be warned that some oxytocic drugs may cause severe persistent hypertension and that even a rupture of a cerebral blood vessel may occur during the postpartum period. PE contains sodium metabisulfite, a sulfite that may cause allergic-type reactions including anaphylactic symptoms and life-threatening or less severe asthmatic episodes in certain susceptible people. The overall prevalence of sulfite sensitivity in the general population is unknown and probably low. Sulfite sensitivity is seen more frequently in asthmatic than in nonasthmatic people. Phenylephrine hydrochloride should be employed only with extreme caution in elderly patients or in patients with hyperthyroidism, bradycardia, partial heart block, myocardial disease or severe arteriosclerosis. The pressor effect of sympathomimetic pressor amines is markedly potentiated in patients receiving monoamine oxidase inhibitors (MAOI). Therefore, when initiating pressor therapy in these patients, the initial dose should be small and used with due caution. The pressor response of adrenergic agents may also be potentiated by tricyclic antidepressants.

Dexmedetomidine (DEX): Systemic doses of DEX may cause acute hypotension and bradycardia. Because systemic DEX decreases sympathetic nervous system activity, hypotension and/or bradycardia may be expected to be more pronounced in patients with hypovolemia, diabetes mellitus, or chronic hypertension and in elderly patients. There are no published contraindications.

Tyramine (TYR): Systemic doses of TYR may produce acute hypertension and a reflex bradycardia. There are no published contraindications.

Phentolamine (PHEN): Systemic doses of PHEN may cause acute and prolonged hypotensive episodes, tachycardia, and cardiac arrhythmias. In addition, weakness, dizziness, flushing, orthostatic hypotension, nasal stuffiness, nausea, vomiting, and diarrhea may occur. Myocardial infarction, cerebrovascular spasm, and cerebrovascular occlusion have been reported to occur following the administration of phentolamine, usually in association with marked hypotensive episodes. Systemic PHEN administration is contraindicated in patients with myocardial infarction, coronary insufficiency, angina, or other evidence suggestive of coronary artery disease; hypersensitivity to phentolamine or related compounds.

Propranolol: Contraindicated in patients with cardiogenic shock, sinus bradycardia and greater than first degree block, bronchial asthma, and in patients with known hypersensitivity to the drug. Systemic doses of propranolol may cause bradycardia and hypotension.

Nitroglycerin (NTG): is contraindicated in patients with early myocardial infarction, severe anemia, increased intracranial pressure, and those with a known hypersensitivity to nitroglycerin. Sublingual NTG is also contraindicated in patients who are using a phosphodiesterase-5 (PDE-5) inhibitor (e.g., sildenafil citrate, tadalafil, vardenafil hydrochloride).

Myocardial ischemia, infarction, arrhythmia, and death during exercise. This should be uncovered in the non-invasive initial testing of each subject, and if found the subject will be excluded and referred to his/her physician. The responses to absolute and relative

indications for the termination of an exercise test are variable depending upon the finding. However, an incident of ischemia presented as chest pain, shortness of breath, acute hypotension (<80/40 mmHg), or abnormal ECG findings (>2-3 mm ST depression or appearance of S_3 or S_4 heart sounds) would result in the immediate termination of exercise. The subject would be placed in a supine position, administered oxygen and assessed by the on site physician and research staff. If the condition did not improve or worsened either 911 or the Code Team would be called dependent upon the location. In all cases this occurrence would be documented and the subject would ultimately be referred to their physician. Cardiac function will be monitored during all exercise studies with an ECG monitor.

Microneurography: The nerve recording procedure occasionally may result in the leg muscles feeling tired one or two days after the experiment, a pins-and-needles feeling (paresthesia), or a greater sensitivity to touch in the leg. However, since 1979, microneurography has been performed on several thousand subjects in the U.S. and abroad without any lasting complications. Dr. Wray has performed microneurography in many subjects without persistent complications. Many subjects volunteer repeatedly for microneurographic studies, indicative of the benign nature of this technique.

Muscle biospy risks: Using sterile technique and a minimal skin incision (about 1/8") over the anterolateral mid-thigh, the standard needle aspiration method for muscle biopsy has not produced bleeding nor infectious complications to date. There is the possibility of a small scar the size of the incision (1/8"). No subject is released until hemostasis has been secured, and the skin site dressed. Subjects are always carefully instructed in the routine prevention of infection and bleeding of biopsy sites**.** As lidocaine is used as the anesthetic there is the risk of toxicity, however the low concentration 0.5% and the use of less than 10-15 ml greatly reduces this concern.

Neck Pressure/Neck Suction (NP/NS): There are no reports in the medical literature evaluating the risks of NP/NS in either patients or healthy control subjects. However, there are over 100 studies indexed in the NLM PubMed database (keyword search "neck"; "suction"; "heart rate") that have used the NP/NS technique safely and effectively, with no report of catastrophic events such aortic dissection or stroke. Included among these are eight published studies from four independent labs which have utilized the technique in heart failure patients [71-78]. The nature of the NP/NS technique is somewhat similar to carotid sinus massage, providing some framework for risk assessment. Contraindications for carotid sinus massage are the presence of a carotid bruit, a known carotid stenosis >50%, and a history of myocardial infarction or stroke within the preceding 3 months. These contraindications will serve as exclusion criteria for participation in the NP/NS portion of the study, and in these patients, only the modified oxford technique will be used to assess baroreflex sensitivity. Further, ultrasound Doppler of the carotid artery and physical exam will be performed in all patients during the preliminary visit to the laboratory to identify as-yet undiagnosed carotid stenoses and bruits, respectively. If any significant plaque is identified during these exams, only the modified Oxford test will be performed for assessment of baroreflex function.

Pregnancy Risks: The risks for some of the procedures included in protocols 2 and 3 to an unborn fetus are not known. Thus, if participant is pregnant, they may not participate in these protocols. If participant is not pregnant, but of childbearing age, they must use appropriate contraceptives to prevent pregnancy while participating in these protocols. Participants will be asked to submit to a urine pregnancy test provided by the research

site. Should participants become pregnant while enrolled, they will be withdrawn from the study.

Participation in multiple protocols: Each protocol is associated with the risks outlined above. However, there is no known accumulative risk associated with participation in multiple protocols.

STATISTICAL METHODS, DATA ANALYSIS AND INTERPRETATION:

Protocol 1: The data collected in this study will examine the effects of cardiac transplantation on vascular function and exercise hyperaemia. The cross-sectional data will be analyzed using student's t-tests and analysis of variance (ANOVA). A repeated-measure ANOVA will be performed on all longitudinal data collected. Correlations will be performed on all the data. A priori power analysis will be performed, but previous studies suggest that approximately 20 subjects per study group will be necessary to achieve adequate power with the expected effect size.

Protocols 2, 3 and 4: A total of 80 subjects (40 with heart failure NYHA class I-III and 40 age-matched controls) will be recruited for the protocols outlined above. With the stringent restrictions and pre-screening processes outlined above, we estimate a 6:1 ratio of those interested in participating to those who are ultimately deemed eligible. Further, we estimate a dropout rate of 10%, which may increase for subjects who agree to participate in multiple protocols. We have taken these issues of subject recruitment and adherence into account in the overall experimental design, such that the protocols with the proposed sample size are attainable within the timeline of the proposed project.

The experimental design and statistical analyses have been developed with an emphasis upon the focused and well powered testing of the primary hypotheses, but guided by the parallel intent to minimize subject burden and study costs. The proposed sample size (n = 80) is based upon power analyses of preliminary data for primary measurements such as HR, ABP, MSNA, and blood flow. To ensure the recognition of even smaller differences than those observed in preliminary data, we will study 40 subjects per group, resulting in >90% power. Statistical analyses will include independent statistics (ANOVA and non-paired t-tests) to examine the differences in the cross-sectional comparisons (i.e. HF vs. age-matched controls). Two or three-way repeated measures ANOVA will be utilized to identify between group differences in these outcome measurements during multiple levels of exercise (i.e. PECO test) and during multiple levels of baroreflex stimulation. In addition, each specific aim will routinely utilize regression analyses to assist in the interpretation of the data collected. Although some multivariate measurements are being made and such an approach will likely require the characterization of covariation patterns, our initial approach will be predominantly univariate. This design will be aided by the detailed subject screening and subject selection which minimizes the number of covariates by, where possible, equally distributing variables across groups (e.g. gender, activity level, etc.). However, secondary covariate analyses will be performed that determine the role of measured variables that are not driven by age or disease state, but do exert an influence upon the major dependent variables.

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.

The following procedures are in place in the Vascular Laboratory to assure safety of research participants:

1. Properly trained personnel will monitor all exercises to ensure they are performed correctly.
2. The blood draws will be performed by a certified phlebotomist/registered nurse, who is familiar with all disease transmission safeguards and emergency procedures, should a problem arise.
3. Blood pressure cuffs will only be inflated to obtain the necessary measurements, and will be deflated immediately afterwards.
4. Immediate emergency – call 911, as typically employed at the Salt Lake City VA Medical Center that are not covered by the code team from the hospital.
5. A crash cart and defibrillator are maintained in the laboratory.

The following procedures are in place in the Vascular Laboratory to protect sensitive subject information:

1. The laboratory is kept locked when there are no lab personnel present.
2. The Logic 7 is turned off when not in use for vascular analyses. The computer is password protected and only the investigators related to this project know the password.
3. Hard copies of subject information will be kept in a locked file cabinet in Dr. Richardson's office. Any electronic copies will be kept on a password secured computer.
4. Subjects are given copies of their measurements upon request.
5. Each subject will be identified by code numbers. These numbers will be used in a statistical spreadsheet while all statistical analyses are performed. The maximal length of storing this information is 5 years, upon which it will be destroyed.

ADMINISTRATIVE RESPONSIBILITIES:

Facilities and Equipment: All protocols will be conducted in the state-of-the-art Human Vascular Laboratory at the VA Medical Center, Building B, room 1D23. The laboratory measures 600 sq ft in size, and is more than adequate to house these studies. This laboratory contains all equipment necessary to complete the proposed work, including: Three adjustable hydraulic gurneys; Three ultrasound Doppler system (Logiq 7 and Logiq I, GE Medical Systems, Milwaukee, Wisconsin, USA) for determination of limb blood flow and vasodilation; A Finger photoplethysmograph (Finometer, Ohmeda, Madison, WI, USA) for measurements of arterial blood pressure; A metabolic cart (O_2 consumption) with spirometer (pulmonary function testing); (A 12-lead ECG; A "crash-cart" and defibrillator; Several custom-built ergometers specifically designed for knee-extensor, plantar flexion, handgrip, and cycling exercise; and several PC-based work stations for data acquisition and analysis. All research records and collected samples will be housed in a locked structure in this laboratory.

Recruitment: All cardiac transplant subjects in this study will be volunteers recruited through the VA hospital and University of Utah Cardiac Transplant Program, though printed advertisement or physician referral. In the case of physician referral, patients who are deemed eligible for the study will be referred to the study coordinator to gather further information – the physician will not provide patients with a detailed description of the study. The healthy control subjects will be recruited by printed advertisement, physician referral, and word of mouth around the VA hospital and the University of Utah.

Subject payment will be as follows:

Preliminary Day: Subjects will be paid $20 upon completion.

Protocol 1: Subjects will be paid $100 upon completion of the four visits, $15/hr pro-rate.

Protocol 2: Subjects will be paid $300 ($15/hr pro-rate) upon completion of the chronic supplementation regimen.

Protocol 3: Subjects may choose to participate only in the pre-training portion of the study, or enroll in the exercise training program.

- **Subjects who do not enroll** in the exercise training portion of the protocol will receive a total of $100 upon completion of the "pre-training" visit, $15/hr pro-rate.
- **Subjects who enroll** in the exercise training portion of the protocol will be paid $500 ($15/hr. pro-rate) upon completion of the exercise training

Protocol 4: Subjects will be paid $150 per visit, two visits required.

Data Safety & Monitoring:

Although there are risks associated with the proposed studies, these risks are considered minimal. Nonetheless, in the interest of participant safety and to ensure compliance with IRB and FDA regulations, we propose the implementation of a data safety and monitoring group. Four members of the Utah Vascular Research Lab faculty (Drs. Richardson, Amann, Trinity and Wray), Mark Supiano, M.D., Micah Drummond, Ph.D., Alan Light, Ph.D., Jacob Jessop, D.O. and Dorothea Rosenberger, M.D. will make up the monitoring group, all of whom are affiliated with research at the University of Utah and the SLC VA. These members will meet on a quarterly basis, and, in the interim, the board will convene in response to any adverse events or unanticipated problems associated with the proposed studies.

In order to meet the regulatory requirements of 21 CFR 312.56 regarding monitoring for compliance with federal regulations, thereby ensuring data quality and that the rights, safety and welfare of study subjects are adequately protected, this study will include a provision for internal monitoring by an independent qualified reviewer. The PI will identify a qualified reviewer and present the name of the proposed reviewer to the DSMB for approval. The reviewer will monitor all aspects of the study for compliance with federal regulations and Good Clinical Practices after the first three (3) subjects have been enrolled.

The reviewer will provide a written summary of findings which will be presented to the DSMB at the next meeting. Corrective actions indicated will be followed by the DSMB at subsequent meetings and documented in the minutes. If indicated, the DSMB will require additional monitoring. Also, if indicated, any protocol deviations or violations will be reported the IRB with appropriate corrective action described and a preventative action plan outlined to avoid recurrence.

If follow-up monitoring is not indicated, there will be a final monitoring visit after the last subject has been enrolled but prior to study close-out. As above, a written summary of findings will be provided to the DSMB for any appropriate action prior to DSMB authorization of study closure. This final monitoring report will be provided to the IRB as part of the Final Report in ERICA.

All subjects participate on a strictly voluntary basis, and thus any protocol may be stopped at any time and for any reason at the subject's request. Additional criteria to

discontinue a protocol will be any evidence that the subject's health is at risk, as identified by a member of the investigative team. Subject safety is at the forefront of all studies, and for this reason all investigators are trained to identify overt (i.e. abnormal ECG patterns, acute change in blood pressure, etc.) and more subtle (i.e. skin pallor or redness, changes in subject demeanor, etc.) signs that a subject may be adversely affected by the experimental interventions. Adverse events and unanticipated problems are broadly defined as any instance of a medical complication arising from catheter insertion (i.e. excessive bleeding and swelling at insertion site) or drug infusion (i.e. acute hypertension, bradycardia, etc.) as listed under the "potential risks" section below.

In the instance of an adverse event, the protocol will be discontinued and the study will be temporarily suspended while DSMB members will review the cause of the event. When appropriate, unanticipated adverse events will be reported within 10 working days, in accordance with the University of Utah IRB Reporting Policy.

IND Regulations for Data Safety & Monitoring:

As a sponsor of an IND, we are additionally responsible for reporting protocol deviations, violations, and adverse events to the FDA, which will be implemented in compliance with the Code of Federal Regulation (21CFR312.32, b – e), as follows;

(b) Review of safety information. The sponsor shall promptly review all information relevant to the safety of the drug obtained or otherwise received by the sponsor from any source, foreign or domestic, including information derived from any clinical or epidemiological investigations, animal investigations, commercial marketing experience, reports in the scientific literature, and unpublished scientific papers, as well as reports from foreign regulatory authorities that have not already been previously reported to the agency by the sponsor.

(c) IND safety reports --(1)Written reports --(i) The sponsor shall notify FDA and all participating investigators in a written IND safety report of:

> *(A) Any adverse experience associated with the use of the drug that is both serious and unexpected; or*
>
> *(B) Any finding from tests in laboratory animals that suggests a significant risk for human subjects including reports of mutagenicity, teratogenicity, or carcinogenicity. Each notification shall be made as soon as possible and in no event later than 15 calendar days after the sponsor's initial receipt of the information. Each written notification may be submitted on FDA Form 3500A or in a narrative format (foreign events may be submitted either on an FDA Form 3500A or, if preferred, on a CIOMS I form; reports from animal or epidemiological studies shall be submitted in a narrative format) and shall bear prominent identification of its contents, i.e. , "IND Safety Report." Each written notification to FDA shall be transmitted to the FDA new drug review division in the Center for Drug Evaluation and Research or the product review division in the Center for Biologics Evaluation and Research that has responsibility for review of the IND. If FDA determines that additional data are needed, the agency may require further data to be submitted.*
>
> > *(ii) In each written IND safety report, the sponsor shall identify all safety reports previously filed with the IND concerning a similar adverse*

experience, and shall analyze the significance of the adverse experience in light of the previous, similar reports.

(2)Telephone and facsimile transmission safety reports. The sponsor shall also notify FDA by telephone or by facsimile transmission of any unexpected fatal or life-threatening experience associated with the use of the drug as soon as possible but in no event later than 7 calendar days after the sponsor's initial receipt of the information. Each telephone call or facsimile transmission to FDA shall be transmitted to the FDA new drug review division in the Center for Drug Evaluation and Research or the product review division in the Center for Biologics Evaluation and Research that has responsibility for review of the IND.

(3)Reporting format or frequency. FDA may request a sponsor to submit IND safety reports in a format or at a frequency different than that required under this paragraph. The sponsor may also propose and adopt a different reporting format or frequency if the change is agreed to in advance by the director of the new drug review division in the Center for Drug Evaluation and Research or the director of the products review division in the Center for Biologics Evaluation and Research which is responsible for review of the IND.

(4) A sponsor of a clinical study of a marketed drug is not required to make a safety report for any adverse experience associated with use of the drug that is not from the clinical study itself.

(d) Followup.

(1) The sponsor shall promptly investigate all safety information received by it.

(2) Followup information to a safety report shall be submitted as soon as the relevant information is available.

(3) If the results of a sponsor's investigation show that an adverse drug experience not initially determined to be reportable under paragraph (c) of this section is so reportable, the sponsor shall report such experience in a written safety report as soon as possible, but in no event later than 15 calendar days after the determination is made.

(4) Results of a sponsor's investigation of other safety information shall be submitted, as appropriate, in an information amendment or annual report.

(e) Disclaimer. A safety report or other information submitted by a sponsor under this part (and any release by FDA of that report or information) does not necessarily reflect a conclusion by the sponsor or FDA that the report or information constitutes an admission that the drug caused or contributed to an adverse experience. A sponsor need not admit, and may deny, that the report or information submitted by the sponsor constitutes an admission that the drug caused or contributed to an adverse experience.

We are also required by the FDA to provide annual reports, in accordance with 21CFR312.33, as follows;

A sponsor shall within 60 days of the anniversary date that the IND went into effect, submit a brief report of the progress of the investigation that includes:

(a)Individual study information. A brief summary of the status of each study in progress and each study completed during the previous year. The summary is required to include the following information for each study:

(1) The title of the study (with any appropriate study identifiers such as protocol number), its purpose, a brief statement identifying the patient population, and a statement as to whether the study is completed.

(2) The total number of subjects initially planned for inclusion in the study; the number entered into the study to date, tabulated by age group, gender, and race; the number whose participation in the study was completed as planned; and the number who dropped out of the study for any reason.

(3) If the study has been completed, or if interim results are known, a brief description of any available study results.

(b)Summary information. Information obtained during the previous year's clinical and nonclinical investigations, including:

(1) A narrative or tabular summary showing the most frequent and most serious adverse experiences by body system.

(2) A summary of all IND safety reports submitted during the past year.

(3) A list of subjects who died during participation in the investigation, with the cause of death for each subject.

(4) A list of subjects who dropped out during the course of the investigation in association with any adverse experience, whether or not thought to be drug related.

(5) A brief description of what, if anything, was obtained that is pertinent to an understanding of the drug's actions, including, for example, information about dose response, information from controlled trials, and information about bioavailability.

(6) A list of the preclinical studies (including animal studies) completed or in progress during the past year and a summary of the major preclinical findings.

(7) A summary of any significant manufacturing or microbiological changes made during the past year.

(c) A description of the general investigational plan for the coming year to replace that submitted 1 year earlier. The general investigational plan shall contain the information required under 312.23(a)(3)(iv).

(d) If the investigator brochure has been revised, a description of the revision and a copy of the new brochure.

(e) A description of any significant Phase 1 protocol modifications made during the previous year and not previously reported to the IND in a protocol amendment.

(f) A brief summary of significant foreign marketing developments with the drug during the past year, such as approval of marketing in any country or withdrawal or suspension from marketing in any country.

(g) If desired by the sponsor, a log of any outstanding business with respect to the IND for which the sponsor requests or expects a reply, comment, or meeting

Control of Investigational Drugs: The VA Pharmacy will order, store, and dispense Phenylephrine Hydrochloride USP and Sodium Nitroprusside USP. The investigative team will administer and dispose of the drugs, which will be documented according to FDA guidelines.

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