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COMIRB Protocol

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Project Title: Role of vascular function: oxygen delivery vs oxygen utilization in the exercise impairment in type 2 diabetes
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I. Hypotheses and Specific Aims: It is well established that functional exercise capacity and peak oxygen uptake (VO_2) are reduced in patients with type 2 diabetes mellitus (T2DM) compared with healthy counterparts (31, 44, 45). The mechanisms underlying the exercise deficit in T2DM remain largely unknown, but previous work has suggested that reduced exercise blood flow (30) and impaired submaximal VO_2 (44) may be contributing factors. Both of these findings (30, 44) are consistent with a peripheral impairment of skeletal muscle oxygen delivery, oxygen utilization, or both. Indeed, dysfunction of skeletal muscle metabolism plays a key role in the pathophysiology of T2DM, and considerable work has described abnormalities of oxidative function in the skeletal muscle of people with T2DM (26, 28, 42, 46). Given this, it is likely that the causes of exercise intolerance in T2DM may relate to specific defects at the level of the skeletal muscle, particularly given that skeletal muscle blood flow and oxidative capacity are impaired in diabetes. However, to our knowledge, no one has related these peripheral muscle abnormalities to the diminished exercise function in this patient group.

The overarching hypothesis for the proposed research is that both a failure to adequately increase muscle oxygen delivery following the onset of exercise and reduced oxidative function of skeletal muscle contribute to the acute oxygen deficit and diminished exercise tolerance that has been observed in patients with T2DM. Accordingly, **the specific aim of this research proposal is:**

Specific Aim 1. Is the oxygen deficit in diabetes revealed by exercise due to limitation in muscle oxygen delivery, muscle utilization or both?

Rationale. Previous work has demonstrated reduced peak VO_2 and slowed VO_2 kinetics in people with T2DM compared with control subjects (43). However, the relative contributions of leg blood flow versus oxygen utilization limitations could not be evaluated. Thus, on a systems level, this specific aim will evaluate the dynamics of leg blood flow (and thus oxygen delivery) using Doppler ultrasound, muscle deoxygenation (O_2 extraction) using near infrared spectroscopy (NIRS), and pulmonary VO_2 during single leg knee extension exercise to identify the predominant mechanisms of oxygen delivery versus oxygen utilization abnormalities in the muscle of T2DM during the transition from rest to exercise. These studies will generate new information on the determinants of muscle oxygen uptake kinetics in diabetes and provide a mechanistic foundation for interpretation of improved exercise responses with future interventions such as exercise training.

Hypothesis 1: Leg blood flow kinetics following the onset of exercise are slowed during exercise in people T2DM compared with healthy controls.

Hypothesis 2: Skeletal muscle deoxygenation (a reflection of oxygen extraction) is more rapid in T2DM muscle compared with healthy controls.

II. Background and Significance: Type 2 diabetes (T2DM) is the most prevalent form of diabetes, affecting nearly 22 million people in the United States. Chronic exposure to the dysmetabolic environment of diabetes and insulin resistance (hyperglycemia, hyperlipidemia, elevated oxidative stress, and inflammation) is a primary factor in the development of diabetic complications. However, there is an unexplained impairment in exercise capacity and exercise tolerance in T2DM. Despite this gap in our understanding, very little research has focused on the potential mechanisms underlying exercise intolerance in T2DM, particularly as it relates to the abnormalities of muscle oxidative metabolism and blood flow described in T2DM. This proposal will address the mechanisms of exercise dysfunction at the level of the peripheral skeletal muscle (**Figure 1**).

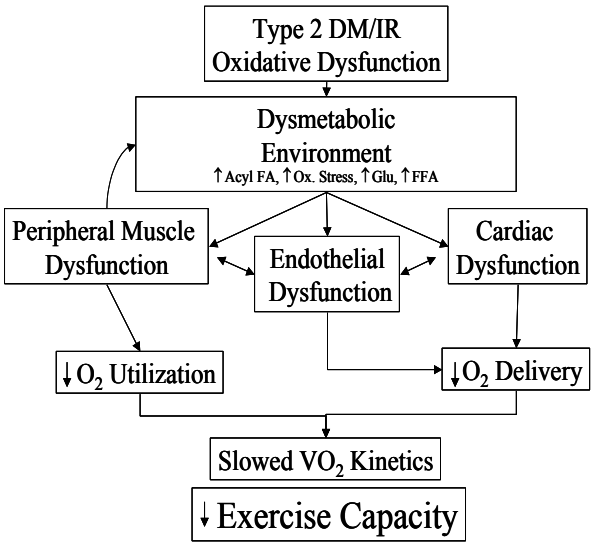
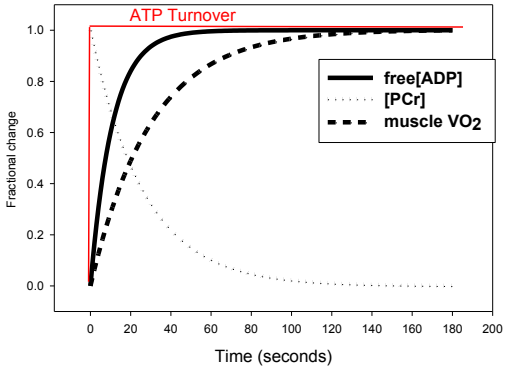


Figure 1. The dysmetabolic environment resulting from T2Dm is related to endothelial, cardiac, and peripheral skeletal muscle dysfunction. How these abnormalities affect exercise capacity and exercise tolerance in T2DM remains unclear.

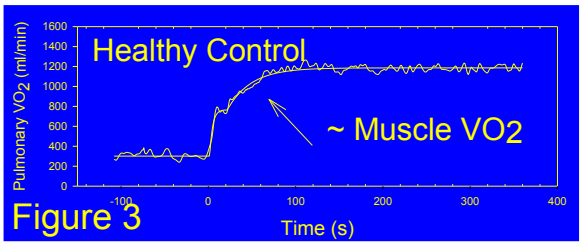
Skeletal muscle pathophysiology in T2DM. The pathophysiology of T2DM is complex and not completely understood, but early changes in insulin sensitivity of skeletal muscle and liver appear to play a major role. The severity of insulin resistance in T2DM skeletal muscle is related to a diminished activity of oxidative enzymes (26), the accumulation of muscle triglycerides (27), as well as to reduced electron transport activity of intact mitochondria (28). There is also evidence of capillary basement membrane thickening (55) and a tendency for decreased capillary density in T2DM skeletal muscle (24). Whether these structural changes are related to altered muscle fiber type composition (i.e. greater proportion of low oxidative type IIb fibers relative to highly oxidative type I fibers) (35) and reduced muscle glycogen content in T2DM (19) is unclear. However, it appears that both the general ability to deliver oxygen to the skeletal muscle as well as the ability of the muscle to utilize oxygen during exercise may be compromised in T2DM.

Exercise response dynamics. One method to investigate the potential contributions of muscle abnormalities lies in studying the dynamic adaptation of the system in response to exercise stress. The onset of exercise is associated with an immediate demand for energy, which is initially met by pre-formed high-energy phosphates (creatine phosphate and ATP). However, sustained exercise requires the production of ATP via oxidative phosphorylation (and hence oxygen consumption)(**Figure 2**) (1, 48, 54). The time course of increasing aerobic ATP production following exercise onset is defined by a time constant that is determined by the inherent activation, sensitivity, and capacitance of the muscle mitochondria (18, 38, 54). However, once initiated, maintaining optimal rates of oxidative phosphorylation with continued exercise is dependent on: 1) adequate O_2 delivery via enhanced exercise blood flow and 2) the ability to increase O_2 extraction as described by the Fick principle: $VO_2 = Q * (a\text{-}vO_2 \text{ difference})$. Thus, the time course of increase in muscle VO_2 following exercise onset (e.g. time constant of VO_2 kinetics) is dependent upon both the dynamics blood flow and inherent control of mitochondrial respiration.

Figure 2



Determinants of VO_2 in the exercise transient. We can assess the systemic integration of pulmonary gas exchange and cardiovascular function (e.g. O_2 delivery) to meet the O_2 utilization demands of the exercising muscle through measuring the kinetics of pulmonary VO_2 during the transition from rest to constant work rate exercise (3, 44). In healthy subjects, the time constant of pulmonary VO_2 kinetics following exercise onset exercise is rapid and follows a near finite time course, considered to reflect the inherent control of mitochondrial oxidative metabolism (**Figure 3**)(12, 14). However, the VO_2 kinetic time constant may become slowed when O_2 transport and/or O_2 utilization of the working skeletal muscle is impaired (e.g. hypoxemia (12, 25) and cardiopulmonary diseases (41, 51). Thus, in the presence of impaired O_2 delivery, abnormal muscle metabolic control, or reduced muscle oxidative capacity, a slowed time constant of VO_2 kinetics reflects the cumulative effects of these abnormalities. In this manner, the measure of VO_2 kinetics provides an effective measure of the integration of O_2 supply and utilization with exercise, but also describes the functionally relevant ability to adjust to the repeated demands of submaximal activities encountered in daily life.



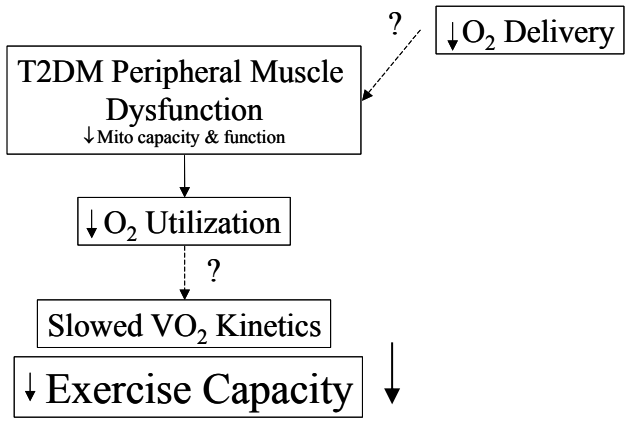


Figure 4. Impairments in muscle oxygen delivery secondary to abnormal cardiovascular control and/or muscle oxygen utilization due to muscle oxidative dysfunction may contribute to the observed exercise defect in T2DM.

Oxygen uptake kinetics in T2DM. Previously, we have demonstrated slowed pulmonary VO_2 kinetic time constants in uncomplicated T2DM compared with controls (5, 32). The magnitude of slowed VO_2 kinetics in T2DM was correlated with the functional impairment in peak exercise VO_2, suggesting the mechanisms of submaximal and peak exercise limitation may be linked. We have also shown that exercise training significantly increases VO_{2peak} and improves VO_2 kinetics (e.g. faster time constant) in women with T2DM, but this does not appear to fully normalize the exercise defect in relation to healthy subjects (6). However, what is not known is how peripheral oxygen delivery and/or oxygen utilization of the skeletal muscle may influence the exercise response and subsequent limitation in T2DM (**Figure 4**). A necessary approach to address these issues would require measurements be made at the level of the exercising muscle. Previous work in animal models of diabetes have described reduced capillary red blood cell flux and a rapid and precipitous fall of microvascular PO_2 ($_{mv}PO_2$) in skeletal muscle (e.g. muscle oxygenation) following the onset of contractions compared with healthy animals (4, 29). Similar observations have recently been made in the skeletal muscle of Goto-Kakazaki rats (model of T2DM) (D. Padilla, KSU, personal communication 2006). These data suggest an initial O_2 delivery impairment (reflected by the rapid O_2 extraction) but also of a significant impairment of oxidative metabolism in diabetic skeletal muscle (as later in exercise, $_{MV}PO_2$ rose towards resting levels despite continued contractions). Thus, at least in diabetic animals, there appears to be an unexpected dysregulation of O_2 delivery to O_2 utilization in exercising skeletal muscle. Similar experiments are needed in humans to address these abnormalities in human T2DM. In this proposal, we will utilize an integrated approach of pulmonary VO_2, leg blood flow, and measures of local muscle oxygenation following the onset of exercise to address the mechanisms of muscle oxygen delivery and oxygen utilization in T2DM. This combination of non-invasive, in vivo measurements highlights a novel systems level aspect of the current proposal.

Tools to assess skeletal muscle de-oxygenation kinetics. Our approach will include a relatively new method that offers functional insight into the balance of oxygen delivery to utilization of local muscle, near infrared spectroscopy (NIRS). Similar to measures of $mvPO_2$, changes in NIR indices of muscle oxygenation reflect the local balance between O_2 delivery and O_2 utilization as described by the Fick principle where changes in muscle O_2 saturation (StO_2) and the concentration of deoxyhemoglobin/myoglobin ([HHb]) may be considered to reflect changes in muscle O_2 extraction. From this principle, the factors that determine VO_2 kinetics (e.g. the kinetics of blood flow (Q) and muscle O_2 extraction (a-v O_2 difference)) may be evaluated (11). Modeling studies have elucidated the expected profiles of muscle deoxygenation under varying conditions of O_2 delivery (13). For example, a primary impairment of muscle O_2 delivery relative to O_2 utilization following the onset of exercise yields predictably faster muscle deoxygenation kinetics (e.g. rapid O_2 extraction), a result of the poor matching between O_2 delivery and O_2 demand. Conversely, an inherent impairment of muscle O_2 utilization relative to O_2 delivery following exercise onset results in slowed muscle deoxygenation kinetics (e.g. impaired O_2 extraction). Thus, a conceptual paradigm for interpretation of muscle deoxygenation kinetics in health and disease has been established and can provide directional evidence of the potential contributing mechanisms limiting the exercise response. We will utilize this powerful set of non-invasive measurements to evaluate the relative contributions of muscle oxygen delivery and oxygen utilization in relation to the altered muscle oxygen uptake responses observed in T2DM.

Background Summary. Peak and submaximal exercise responses are impaired in T2DM, the mechanisms of which may be related to abnormalities in exercise blood flow (oxygen delivery) and/or skeletal muscle oxidative dysfunction (i.e. impaired oxygen utilization). The relative importance of these physiologic system abnormalities in the exercise impairment in T2DM has not been resolved. One factor that may be related to the observed mitochondrial dysfunction and potentially the exercise anomaly in T2DM is the reduced skeletal muscle oxidative capacity and function. The purpose of this study is to address the relative importance of these mechanisms and establish a background from which the efficacy of interventions such as exercise therapy may improve the diabetic condition.

Innovation. The proposed study will be the first to examine the dynamics of oxygen delivery and oxygen utilization in T2DM skeletal muscle and relate these observations to the described impairment in T2DM exercise performance. We will be applying a new non-invasive technology to this important issue in diabetes. Further, this study will be the first to evaluate parameters of T2DM skeletal muscle oxidative function in men and women as it relates to functional exercise outcome parameters. This unique combination of techniques has strong potential to reveal the underlying mechanism disrupting exercise capacity in diabetes and moves us forward from the descriptive studies in this area to date. This innovative set of experiments will be the beginning of an important link between metabolic abnormalities of T2DM and exercise capacity.

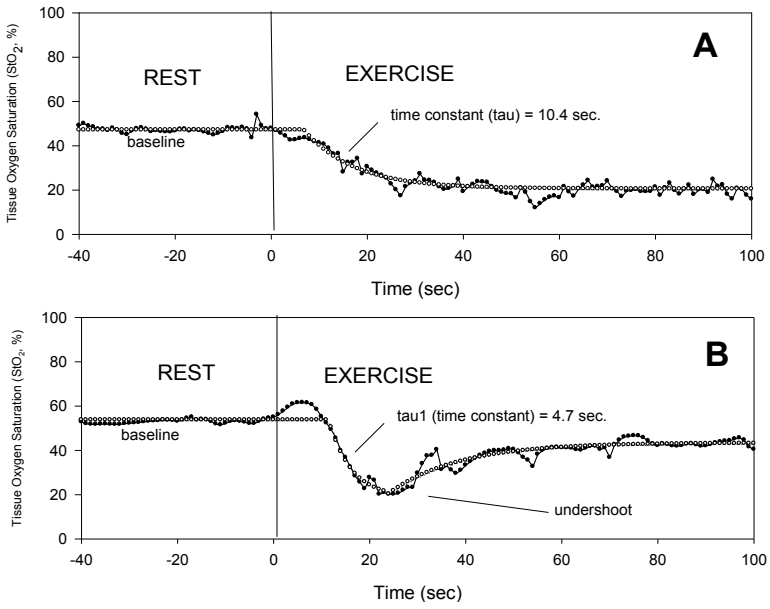


Figure 5. Muscle deoxygenation kinetics in the rest-exercise transition in a Healthy control (A) and subject with T2DM (B). Exercise begins at time=0.

Significance. It is likely that the causes of exercise intolerance in T2DM may relate to specific defects at the level of the skeletal muscle, particularly given that this target organ plays an

important role in the pathophysiology of insulin resistance and T2DM. The significance of slowed muscle VO_2 kinetics following exercise onset relates physical activity encountered in daily life. As a result, slowed VO_2 kinetics confer a dependence on non-oxidative energy sources during the early portion of exercise causing greater phosphocreatine degradation, a dependence upon non-oxidative ATP synthesis, and accumulation of hydrogen ion ([H+]) in skeletal muscle that may, in part, contribute to muscle contractile dysfunction, early sensations of muscular fatigue, and a reduction in exercise tolerance observed in T2DM patients (49). It has been suggested that this mechanism could predispose persons with T2DM to "feel worse' when performing exercise, a distinct problem given that exercise is a cornerstone of treatment in this population and compliance with this treatment is poor (6). In this context, the characterization of skeletal muscle oxygen utilization and hemodynamic responses during exercise will provide meaningful insight into the diabetes-specific determinants of functional exercise capacity. More broadly and from a practical standpoint, these data will advance our understanding of the interaction between skeletal muscle metabolism and muscle blood flow in the exercise responses in health and T2DM and provide a platform upon which to understand skeletal muscle response to different forms of exercise training interventions.

III. **Preliminary Studies/Progress Report:**

Data supporting hypotheses of Specific Aim1: Muscle deoxygenation kinetics are abnormal in T2DM skeletal muscle (Bauer preliminary observations, 2006). Muscle de-oxygenation kinetics were followed during the transition to moderate constant work rate exercise in a sedentary healthy control (**Figure 5A**) and a subject with T2DM (**Figure 5B**). Both subjects demonstrated similar resting muscle oxygen saturation (StO_2) prior to exercise onset. Following the onset of contractions and a short time delay, muscle StO_2 followed an exponential decline with a time constant of 10.4 sec. in the healthy subject and 4.7 sec. in the T2DM subject. (The T2DM subject was associated with slowed VO_2 kinetics; e.g. time constant of 55.4 seconds). The subject with T2DM demonstrated an 'undershoot' of StO_2 and subsequent StO_2 increase towards resting values that was not observed in the healthy muscle. **Interpretation:** In the subject with T2DM, StO_2 decreased more rapidly (i.e. more rapid O_2 extraction) consistent with an initial mismatch of muscle O_2 delivery to O_2 utilization (i.e. muscle O_2 delivery was slowed). In accordance with Fick's Law of diffusion ($VO_2 = DO_2 (PO_{2capillary} – PO_{2Mitochondria})$), the rapid fall and "undershoot" of muscle StO_2 in the subject with T2DM could impair capillary-myocyte PO_2 gradient as previously suggested in diabetic animals (4). However, as exercise continued, muscle StO_2 rebounded towards resting levels in T2DM muscle (again similar to diabetic animals), indicating that later in exercise muscle O_2 delivery continued to increase at a time when O_2 utilization was slowing. This latter response was unexpected given that steady state exercise blood flow to the legs in T2DM has previously been shown to be reduced (30); and thus, we would have predicted oxygen extraction and muscle deoxygenation to be greater than in control (i.e. lower StO_2). Indeed, this latter response during the exercise transition could

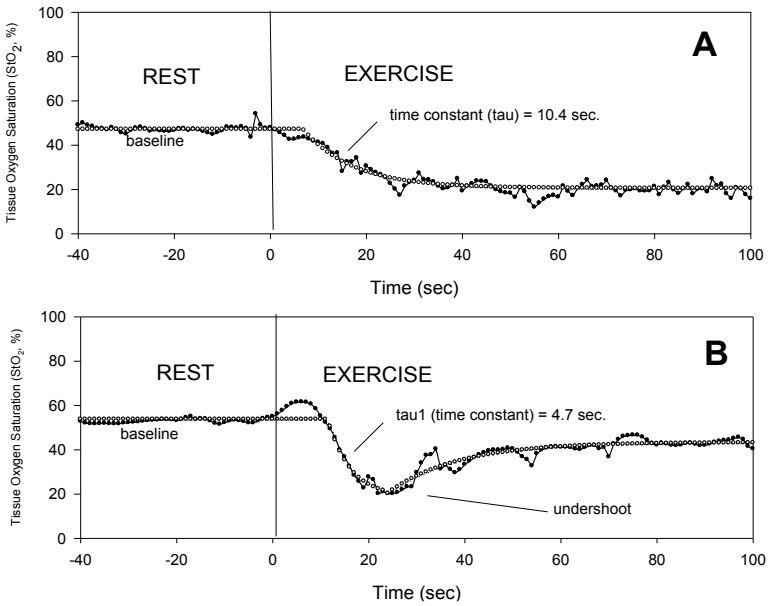


Figure 5. Muscle deoxygenation kinetics in the rest-exercise transition in a Healthy control (A) and subject with T2DM (B). Exercise begins at time=0.

reflect an inherent impairment of muscle oxygen utilization due to oxidative dysfunction.

IV. Research Methods

A. Outcome Measure(s):

Rationale. Previous work has demonstrated reduced peak VO_2 and slowed VO_2 kinetics in people with T2DM compared with control subjects (43). However, the relative contributions of leg blood flow versus oxygen utilization limitations could not be evaluated. Thus, on a systems level, this specific aim will evaluate the dynamics of leg blood flow (and thus oxygen delivery) using Doppler ultrasound, muscle deoxygenation (O_2 extraction) using near infrared spectroscopy (NIRS), and pulmonary VO_2 during single leg knee extension exercise to identify the predominant mechanisms of oxygen delivery versus oxygen utilization abnormalities in the muscle of T2DM during the transition from rest to exercise.

Experimental plan. The proposed study will determine the leg blood flow kinetics using Doppler ultrasound, muscle deoxygenation kinetics using NIRS, and pulmonary VO_2 kinetics by metabolic cart during plantar flexion exercise. By combining echo measurements with noninvasive estimates of pressure, the hydraulic impedance and local stiffness of the aorta (total systemic), brachial (arm) and femoral (leg) will be measured using techniques previously developed for pulmonary vessels [1-4]. Further, 2-dimesional echo will be used to image the heart, allowing the calculation of ventricular strains through speckle tracking methods; strain noninvasively characterizes heart function. Insulin sensitivity will be determined using the one stage hyperinsulinemic-euglycemic clamp technique (8). Rates of mitochondrial phosphorylation will be assessed by magnetic resonance spectroscopy. Muscle biopsies of the gastrocnemeus will be obtained to measure muscle histology and mitochondrial respiration. Twenty healthy, overweight sedentary subjects, 15 lean controls, and 20 subjects with T2DM (specific methods are described below) will be recruited for this study. Mathematical modeling of the measured physiological responses will be used to determine the kinetic time constants of each parameter.

Analysis. The primary endpoints for *Specific Aim 1* are the respective time constants of leg blood flow kinetics, vascular impedances and local stiffness, muscle deoxygenation kinetics, and VO_2 kinetics as measured during the transition from rest to constant work rate exercise. The time constant of each parameter, the first three harmonics of each impedance, and elastic modulus for each vessel will be used to compare the healthy and T2DM groups using a two sample independent t-test with equal variances. Given the small planned sample sizes, should the assumption of normality or equal variance of the measures appear unreasonable based on group plots, non-parametric tests will be utilized. To examine the planned correlations between measured exercise parameters, correlations (Pearson's r) and scatter plots will be utilized.

B. Description of Population to be Enrolled: Study Design and Research Methods

Recruiting methods. Subjects will be recruited by advertisements approved by COMIRB for this purpose. All ads will be approved by COMIRB. Ads will be posted on line, in flyers, in newspaper ads, university internet research sites, and radio ads in the Denver area. Appropriate subjects will be informed of the research project by the research coordinator and offered to read through the consent form prior to study participation. *Process:* Individuals will be screened initially by phone and all study procedures will be thoroughly explained. If an individual meets minimum eligibility criteria and expresses interest in receiving further information, the consent form will then be mailed/delivered for his/her review. Scheduling for the screening visit will occur after subjects have had ample time to review the consent form (at least one week). When subjects arrive for the screening visit, the consent is thoroughly reviewed by the investigator in a quiet setting without distractions. Participants are prompted to ask questions and to give their interpretation of the study in order to assess their understanding. Participants will be given a final copy of the signed and dated consent form and encouraged to review it often and ask questions as they progress through the study. The Investigators have completed training in the proper methods of obtaining informed consent (COMIRB 101).

Inclusion and Exclusion criteria. Subjects with T2DM, health lean, and healthy overweight sedentary subjects will be enrolled who are between the ages of 30 and 70 years. Presence of

T2DM will be confirmed by chart review. Healthy, sedentary subjects will be defined by normal history and physical exam. Sedentary behavior in all subjects will be objectively defined as not participating in a regular exercise program (<one bout of exercise/week) and will be further established by use of a questionnaire (Low level Physical Activity Recall, LOPAR). Subjects will be excluded if they have: 1. documented cardiovascular disease in medical history (CAD or PAD), ECG findings of cardiac ischemia or conduction system abnormalities with GXT, or other symptoms limiting exercise function; 2. uncontrolled hypertension: SBP > 150, DBP> 110. ; 3. Obstructive pulmonary disease or asthma, (FeV1 < 0.7); 4. peripheral neuropathy; 5. Subjects taking beta blockers, insulin, or TZD; 6. BMI between 25 and 40 for those with T2DM and overweight controls. BMI less than 23 for lean controls; 7. current or past smoking within the last 2 years; ; 8. Anemia, (tHb < 10mg/dl); 9. Autonomic dysfunction (e.g. fall in BP >20mmHg on standing without change in HR); 10. Control HbA1c > 5.7; T2DM HbA1c > 10; 11. Type 1 DM; 12. Any implanted metal in their body.

Selection of study population. Based on local demographic information (Table 1), the expected demographics of subjects selected for this study are described in Table 2.

Table 1. Local demographics in percentages.

	American Indian or Alaskan native	Asian or Pacific Islander	Black, not of Hispanic origin	Hispanic	White, not of Hispanic origin	Other or unknown	Total
Female							
Male	1	1	16	12	70		100
Unknown							
Total	1	1	16	12	70		100

Table 2. Expected distribution for this study in numbers of subjects.

	American Indian or Alaskan native	Asian or Pacific Islander	Black, not of Hispanic origin	Hispanic	White, not of Hispanic origin	Other or unknown	Total
Female			4	3	19	2	28
Male			4	3	19	1	27
Unknown							
Total			8	6	38	3	55

Patient accrual. A total of 55 subjects from the Denver area (15 lean controls, 20 people with T2DM, and 20 overweight control subjects) will be enrolled in this study.

Estimated duration of the study. The estimated duration of participation for each subject in this study is 30 hours. The estimated duration to accrue and evaluate subjects for this study is 2 years.

C. Description, Risks and Justification of Procedures and Data Collection Tools:

Treatment, intervention, or observation. This study is designed to observe the exercise responses of subjects during moderate intensity exercise. The study will evaluate leg VO2 kinetics in patients with T2DM compared with healthy subjects. Measurements of gases consumed and produced, blood flow to the legs at rest and during calf exercise, and muscle tissue saturation by non-invasive monitoring will be evaluated. The use of a similarly-aged control group is to ensure that any differences observed between groups were due to the disease of T2DM and not simply a result of aging per se.

Examinations, laboratory tests, procedures, and follow-up visits.

All patient visits and tests/procedures will take place at the University of Colorado Hospital Clinical Translational Research Center. Subjects will come to the GCRC for a total of 8 study visits.
Visit one: Screening visit. Following informed consenting procedures, the visit will include a Low-level Physical Activity Recall (LoPAR) Questionnaire, a history and physical exam, blood draw and urine sample taken for clinical tests, autonomic nervous system testing, dietary survey, and a dual x-ray absorptomitrey (DXA).
Visit two:. Femoral artery flow mediated dilation (FMD), calf reactive hyperemia, femoral artery diameter, and calf blood flow at rest and after cuff occlusion will be measured via standardized techniques. Response to sublingual nitroglycerin will serve as a measure of endothelium independent vasodilation. MVC will also be assessed. Cardiac echocardiographic measurements and echo measurements. Input impedance and stiffness will be assessed at three systemic vascular locations by combining non-invasive measurements of blood flow (PW Doppler) and lumen diameter (tissue Doppler) with non-invasive approximations of pressure from arterial tonometry. Further, echo images will be taken to obtain right and left ventricular (RV & LV) longitudinal strain, LV circumferential strain, and LV torsion. Subjects will perform a graded exercise test (GXT) on a stationary bicycle for determination of maximal workrate and VO_2max. Muscle deoxygenation by NIRS and arterial oxygen saturation by oximeter, will be monitored at this visit. Positioning of the subjects on the precision isokinetic dynamometer (CSMI, Stoughton, MA), the use of a metronome, and practice of plantar flexion at a low resistance will also occur in order to familiarize the subjects with the equipment..
Visit three: A muscle biopsy of the dominant leg gastrocnemius will be performed at this visit. A hyperinsulinemic euglycemic clamp, and sphygmocor will be performed in the CTRC during this visit .
Visit four: Five bouts of single leg isotonic plantar flexion exercise will be performed by dynamometry on both the dominant and non-dominant leg (total of 10 bouts per subject). Each plantar flexion exercise test will be performed for 5 minutes using the dominant leg followed by 6 minutes of recovery. Resistance will be set as a % of MVC. Two of the four bouts will be at 10% MVC, and two bouts will be performed at 20% MVC. Non-invasive measures of common femoral artery blood flow by Doppler ultrasound, arterial oxygen saturation by oximeter, and muscle deoxygenation by NIRS will be monitored at this visit. Calf blood flow by plethysmography and femoral artery FMD will also be assessed. Additionally, one bout of submaximal testing at 20% MVC on both the dominant and non-dominant leg will occur while breathing 100% O_2. Following completion of the isotonic exercises and a 20 minute rest period, three bouts of single leg isometric plantar flexion exercises will be performed by the dominant leg only. Contraction during the exercise will be at a % of MVC. The first contraction will be for 20% MVC for 120 seconds and the second will be for70% MVC for 90 seconds. The third bout will be for 70% MVC for 90 seconds. The three bouts will be separated by a rest period. Additionally, two bouts of single leg isometric plantar flexion exercise (one at 20% MVC and the other at 70% MVC) will be performed by the dominant leg while breathing 100% O_2.
Visit five: During this visit, three bouts of single leg isometric plantar flexion exercise will be performed on the dominant leg. Contraction during the exercise will be at a % of MVC. The first contraction will be for 70% MVC for 90 seconds, the second will be for 70% MVC for 90 seconds and the third will be at 70% MVC for 90 seconds. The three bouts will be separated by a rest period. The second bout of single leg isometric plantar flexion exercise (70% MVC) will be performed by the dominant leg while breathing 100% O_2. The third bout of single leg isometric plantar flexion exercise (70% MVC) will be performed 5 minutes after the subject ingests a sublingual 300 µg nitroglycerin tablet. During this visit, muscle oxidative function during exercise will be assessed via P MRS at rest, during, and in recovery from all plantar flexion exercise tests.
Exercise Training Period: Single leg calf muscle endurance training will be performed 5 days per week for two consecutive weeks and until visits 6, 7, and 8 are complete.
Visit six: Femoral artery flow mediated dilation (FMD), calf reactive hyperemia, femoral artery diameter, and calf blood flow at rest and after cuff occlusion will be measured via standardized techniques. MVC will also be assessed. Non-invasive measures of common femoral artery blood flow by Doppler ultrasound, muscle deoxygenation by NIRS and arterial oxygen saturation by

oximeter will be monitored at this visit. Calf blood flow by plethysmography and arterial stiffness/endothelial function will be non-invasively measured by the Sphygmocor system will also be assessed. During this visit, seven bouts of single leg isotonic plantar flexion exercise will be performed by dynamometry the dominant and five bouts will be performed on the non-dominant leg). On the dominant leg two bouts will be performed at 10% pre exercise training MVC. On both the dominant and non-dominant leg two bouts will be performed at 10% post exercise training MVC, and two bouts will be performed at 20% post exercise training MVC. Each bout will be performed for 5 minutes followed by 6 minutes of rest. Additionally, one bout of single leg isotonic plantar flexion exercise will be performed on each leg at 20% post exercise training MVC while breathing 100% O_2. Following completion of the isotonic exercises and a 20 minute rest period, subjects will perform seven bouts of single leg isometric plantar flexion exercises on the dominant leg and five bouts of single leg isometric plantar flexion exercises on the non-dominant leg. One bout at 20% pre exercise training MVC for 120 seconds will be performed by the dominant leg only. One bout at 70% pre exercise training MVC for 90 seconds will be performed by the dominant leg only and one bout at 70% post exercise training MVC for 90 seconds will be performed by dominant. Each bout will be separated by a rest period. Additionally, a single bout of single leg isometric plantar flexion exercise (one at 70% post exercise training MVC) will be performed by the dominant leg while breathing 100% O_2.

Visit seven: During this visit, four bouts of single leg isometric plantar flexion exercise will be performed on the dominant leg. Contraction during the exercise will be at a % of MVC. The first contraction will be for 70% MVC for 90 seconds, the second will be for 70% MVC for 90 seconds and the third will be at 70% MVC for 90 seconds. The three bouts will be separated by a rest period. The second bout of single leg isometric plantar flexion exercise (70% MVC) will be performed by the dominant leg while breathing 100% O_2. The third bout of single leg isometric plantar flexion exercise (70% MVC) will be performed 5 minutes after the subject ingests a sublingual 300 µg nitroglycerin tablet. During this visit, muscle oxidative function during exercise will be assessed via P MRS at rest, during, and in recovery from all plantar flexion exercise tests. A standardized incremental bicycle exercise test and plethysmography will be performed for the determination of systemic exercise capacity.

Visit eight: Calf arterial stiffness/endothelial function will be non-invasively measured by the Sphygmocor system. A gastrocnemius muscle biopsy will be obtained from untrained and trained calf muscles of subjects.

Optional Visit Nine: Submaximal exercise tests and VO_2max test will be performed for O_2 uptake and heart rate kinetics with NIRS for muscle oxygen extraction. A resting and exercise EKG and vital signs will be performed during the visit.

Optional Visit Ten: Submaximal exercise tests and VO_2max test will be performed while breathing 60% O_2 for O_2 uptake and heart rate kinetics with NIRS for muscle oxygen extraction. A resting and exercise EKG and vital signs will be performed during the visit.

Specific Methods.

Aortic Impedance and Stiffness ultrasound pulse-wave (PW) Doppler images characterizing transient blood flow velocity will be obtained in the Aorta from through a parasternal short axis approach and in the femoral and brachial vessels using windows described previously. Color M-mode tissue Doppler images (CMM-TDI) will be used to obtain diameter time-histories of the Aorta from the suprasternal notch view and again of the femoral and brachial vessels using standard windows. Both image sets will be obtained using a GE Vivid Seven ultrasound scanner. For the PW Doppler images of the Aorta, two dimensional echo and color Doppler will be used to align the ultrasound beamline parallel to the main flow direction within the Aorta to minimize errors due to ultrasound beam angulation. In the two peripheral vessels, beamline correction will be utilized due to necessity, but will be reduced as much as possible. The CMM-TDI view of all three vessels will provide a long axis representation with the vessel walls perpendicular to the ultrasound beam; during acquisition, the beam will be swept through the long axis of the vessel in question to

determine maximal diameter. Brachial and femoral pressures will be simultaneously measured via arterial tonometry (SphygmoCor), while Aortic pressure will be approximated [REF]; each will be digitized into the Vivid Seven scanner through its physio port to insure image-pressure synchrony. From PW Doppler and pressure, impedance will be obtained, and from CMM-TDI of a vascular wall and pressure, stiffness will be obtained; the analysis process for these measurements has been well studied and validated [1-5].

Arterial Oxygen Saturation. Oxygen saturation will be assessed in all subjects during all exercise tests using an Ohmed oximeter placed on the index finger (Ohmed Corp., Louisville, Colorado).

Blood collection and preparation: Up to 4 teaspoons of blood will be drawn for clinical laboratory measurements that include markers of insulin resistance (fasting insulin/glucose, c-peptide) and standard screening labs (CBC, Cr, lipid panel, urine protein). Although secondary in nature, the requested clinical laboratory markers are necessary to document T2DM disease severity (IR) and identify exclusion criteria.

Biopsy Procedures: Extensive prior work from our lab using muscle biopsies has focused on metabolic abnormalities in patients with peripheral arterial disease (47;21). To measure muscle histology and mitochondrial respiration in our patients in the proposed study, we will perform skeletal muscle biopsies from the medial aspect of the gastrocnemius as previously described (Robbins et al, 2011). A modified Bergstrom needle technique will be utilized to obtain 40-50 milligrams of skeletal muscle following local anesthesia with 2% lidocaine and a 1cm skin incision.(5) Samples will be embedded in cross-section using optical cutting temperature (OCT) tissue freezing medium (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA), snap frozen in liquid nitrogen, and stored at -80°C.). Histological analysis will be done using published methods (Robbins 2011). The capillary density of each section will be determined by the number of endothelial cells/mm2, calculated by dividing the total number of CD31-positive (fluoroscein-stained) cells by the area (mm2 of tissue) which will be measured with a stage micrometer and Image–Pro Plus (Robbins 2011). Dr. Irene Schauer, Investigator on this study, will perform the muscle biopsies and also make the mitochondrial respiration measures. Dr. Schauer acquired this latter technique at an international OROBOROS workshop in Schroeken, Austria and has a local collaborator to assist in optimization of the assay.

Cardiac Echocardiographic Measurements. Standard two-dimensional and Doppler echocardiography will be performed pre and post treatment [85;86] with a commercially available ultrasound system by Dr. Jennifer Dorosz, Investigator and Cardiologist on this protocol (GE Vivid 7 Dimension, Milwaukee, WI). Chamber sizes, LV end-systolic and diastolic chamber dimensions and wall thickness, fractional shortening and left ventricular mass will be quantitated by standard techniques for all individuals. Ejection fraction will be calculated using the Method of Disks. In addition to these standard measurements, cardiac function will be assessed by tissue Doppler, strain, and strain rate imaging using speckle tracking software (Echopak, GE Dimension, Milwaukee, WI). Tissue Doppler of both the septal and lateral mitral annuli will measure E' and A' velocities, E':A' ratio, and E:E' ratio. Flow propagation will be determined by color M-mode. Global systolic strain (S), systolic strain rate (SrS),and diastolic strain rate (SrE and SrA) will be obtained from strain and strain rate curves using speckle tracking software[19;104;105].

Consenting procedures. Prior to the screening visit, a copy of the consent form will be mailed to the potential subject. Scheduling for the screening visit will occur after the subject has had the opportunity to review the consent form (at least one week). At the screening visit, informed consent will be obtained by the study coordinator/Principal Investigator after a detailed explanation of all study procedures/risks and after the subject has had the opportunity to fully review and sign the consent form. The study coordinator/Principal Investigator has completed the COMIRB 101.

Constant work rate (CWR) exercise. Subjects will perform five-seven bouts of single leg plantar flexion exercise performed by dynamometer (CSMI, Stoughton, MA). During visits 4 and 6 each plantar flexion exercise test will be performed for 5 minutes using the dominant leg followed by 6 minutes of recovery. The dynamometer resistance will be set as a % of the individual's MVC, and the frequency of voluntary muscle contractiosn will be 0.5Hz. A rest period will separate each bout. During visit 4 two bouts of plantar flexion exercise will be performed at 10% MVC, and three bouts will be performed at 20% MVC. All bouts will be performed on both dominant and non-dominant

legs. During visit 6 two bouts of plantar flexion exercise will be performed at 10% pre exercise training MVC, two about at 10% post exercise training MVC, and three bouts will be performed at 20% post exercise training MVC. The bout at 10% pre exercise training MVC will be performed by dominant leg only, all other bouts will be performed by both legs.

DEXA (Dual-energy X-Ray Absorptiometry): Body composition and bone mineral density will be measured using the DEXA technique(53).This technique relies on the absorption of dual electron wavelengths for the assessment of body fat, lean tissue, and bone mineral density. During the procedure, the subject will be supine on the measurement table, and the arm of the machine will slowly pass over their body. Body fat distribution will be determined using the waist-to-hip ratio where the waist circumference is measured 1/2 the distance from the lowest point of the ribs to the iliac crest and the hip circumference is measured at the level of the greater trochanter.

Dietary interviews: Customary macronutrient pattern will be ascertained by diet interview on the CTRC (94). A three day diet provided by the CTRC will be used prior to certain visits as stipulated in the Visit schedule. The CTRC dietician will prescribe a standardized nutrient breakdown including 15% protein, 35% fat and 50% carbohydrate for the three days prior to visits 2, 3, 4, and 5. The range of sodium in the CTRC diet will be about 1.5 to 1.75 mg/per calorie. In addition, sodium intake guidelines will be followed throughout the study to assure standard hydration and especially with visits 2, 3, 4, and 5 where the CTRC diet will be utilized for 3 days prior to the visits.

Endothelial Function: Drs. Regensteiner and Bauer have extensive experience in using FMD to assess endothelial function [5;44]. FMD will be measured following the protocol of Celermajer [44;106-108] that measures dilation of the femoral artery by ultrasound in response to hyperemia. The femoral artery diameter will be measured using B-mode ultrasound images, with the use of a GE Vivid 7 ultrasound system and a 10.0-mHz linear-array transducer as previously reported in our lab (GE vivid 7 Dimension, Milwaukee WI.). Scans will be obtained with subjects at rest and following reactive hyperemia for assessment of FMD. After measuring responses to cuff occlusion, subjects lie quietly for 15 minutes and then sublingual nitroglycerin (300 μg) is administered to assess endothelium independent vasodilation.

Equipment Familiarization. Proper positioning of each subject on the precision isokinetic dynamometer (CSMI, Stoughton, MA) will be determined. Several single-leg plantar flexion at a low resistance along with the use of a metronome will be performed on the precision isokinetic dynamometer (CSMI, Stoughton, MA) to familiarize subjects with the equipment and procedure.

Estimation of microvascular blood flow kinetics. The estimation of microvascular blood flow kinetics will be determined as the time course of Q(capillary), estimated from the rearrangement of the Fick equation [Q(capillary) = VO(2)(muscle)/(a-v)O(2)] using the time course of pulmonary VO(2) and NIRS derived [HHb] as proxies for VO(2)(muscle) and (a-v)O(2), respectively as previously described (14).

Exercise training. It has been previously shown that muscle oxidative function, enzymes, and microvascular structure can be increased following an intensive exercise training protocol [122]. Following the completion of Visit 5, subjects will undergo supervised single leg, exercise training of the index (dominant) calf muscle 5 days per week for two weeks – alternating weight-bearing single leg calf raises and single leg calf extensions by endurance resistance training (weight machine apparatus). A prescribed exercise protocol will be completed and supervised by an exercise specialist (Dr. Bauer) with close supervision by the Principal Investigator and the study physicians. Each session lasts a total of 60 minutes in the Energy Balance Lab at the UC School of Medicine. A 14 day time period was chosen because improvements in all exercise performance parameters are seen in T2D within this period. For each session, subjects will have a 5 minute warm-up, a 45 minute period of moderately intense endurance and resistance exercise (following a prescription set with the MVC test conducted before training begins), and a 10 minute cool-down. Subjects will utilize standing single leg calf raises repeated to fatigue as well as resistance exercise (single leg calf extensions) performed on a weight-stack machine. Typically, subjects will spend 10-15 minutes on each piece of equipment before switching to a different piece of equipment. Of the 45 minute exercise period, at least 30 min of the time will be spent performing the actual single leg exercise training protocol. The intensity/duration of each set of calf exercises will be increased on a weekly basis as tolerated, by increasing work load and number of single leg calf raises, as applicable. Based on our previous experience in this patient population, this program is both tolerable and safe.

Hyperinsulinemic euglycemic clamp: In order to determine insulin sensitivity, subjects will be admitted to the CTRC and fasted for ≥12 hr during visit 1. Two intravenous cannulae will be established: one for infusions, and the second in retrograde fashion in a dorsal vein of the opposite arm for blood sampling. To obtain arterialized venous blood samples, this hand will be kept warm with a heating pad. A descending primed continuous infusion of insulin (final 40 $mU/m^2/min$) will be initiated at T=0 (about 8AM) and continued until T=180 min for determination of insulin sensitivity. Plasma glucose will be maintained at 90 mg/dl by variable infusion of 20% dextrose. An infusion of potassium will also be given to maintain normal potassium concentration. The rate of glucose infusion will be adjusted based on arterialized blood specimens drawn every 5 minutes, with plasma glucose quantified using a ™Analox glucose analyzer at the bedside. Blood samples will also be obtained for determinations of free fatty acids, triglycerides, insulin, and glycerol at 60 min. intervals. After infusion is stopped, the subject will be given a standard meal, and plasma glucose levels measured every 15-30 min. for an hour to check for possible hypoglycemia. The subject will then be discharged.

Kinetic Analysis. Kinetic analysis of pulmonary VO_2 and NIRS will be performed as previously described (2, 3). Breath-by-breath VO_2, muscle deoxygenation (HHb) will be time averaged to one-second intervals. Leg blood flow will be evaluated across each contraction. Mathematical models will be employed to fit to the average response curves for VO_2, leg blood flow, and muscle O_2 desaturation (StO_2 and HHb) using non-linear, least squares regression techniques (Sigmaplot, 2002) using the following formulas as appropriate for each parameter: $X(t)= X(b) + A1 [1- e -(-t-td/\tau)]$, $X(t)= X(b) + A1 [1- e -(-t-td/\tau1)]+ A2 [1-e-(-t-td2/\tau2)]$, $X(t)= X(b) + A1 [1- e -(-t-td/\tau1)]+ A2 [1-e-(-t-td2/\tau2)]+A3 [1-e-(-t-td3/\tau3)]$ (2, 3, 6, 7, 13)

Leg Blood Flow. Leg Blood flow will be determined by pulsed wave Doppler ultrasound in the common femoral artery from a site 3-5 cm proximal to the inguinal ligament as described (32, 40, 50). Doppler measurements will be carried out using a 5MHz Doppler probe by an experienced technician. Serial estimates of mean blood flow will be calculated over each contraction duty cycle (0.5Hz) using the mean blood velocity and diameter measurements to enable the determination of blood flow dynamics (50).

Low Level Physical Activity Recall Questionnaire (LOPAR): This questionnaire has been validated for use in persons with T2DM and peripheral arterial disease as well as in sedentary controls by the Investigator as previously reported and is being used as an outcome measure by Diabetes Prevention Program. The LOPAR asks about physical activity level over the previous week. Subjects are asked in a series of questions to itemize their time (reporting specific activities) into work, leisure and housework categories. Questionnaire results are calculated in metabolic equivalents (METS) where one MET equals resting VO_2 (3.5 ml/kg/min). This questionnaire will primarily be used in this study as a screen to ensure that all participants are sedentary but will also be used as a measure of change in habitual physical activity levels (Attachment).

Magnetic Resonance Imaging (MRI): For the soleus and tibialis anterior MRS, the subject is positioned in the MRI magnet feet first, prone, with the soleus muscle centered in the extremity coil. Scout images (usually T1-weighted) position the volume-of interest (usually 2cm x 2cm x 2cm), to avoid regions of gross adipose and vascular structures. Spectroscopy acquisition is performed using the PRESS pulse sequence without water suppression (TR/TE = 2000/24 ms, 64 averages, total acquisition time 2 minutes), and analyzed by SAGE spectroscopy analysis (GE). The water peak is fit as a Lorentzian line using Marquardt routine, and subtracted from the spectrum. The remaining peaks (creatinine and choline) are then fit using the Marquardt routine. IMC and EMC triglyceride peaks (1.3 and 1.5 ppm, respectively) are obtained from the fit results, corrected for T1 and T2 relaxation, and expressed as a percentage of the water content(15). Rates of mitochondrial phosphorylation will be assessed by ^{31}P magnetic resonance spectroscopy saturation transfer performed at 36.31 MHz with the use of a flat, concentric probe made of an inner coil 9 cm in diameter (for ^{31}P) and a 13-cm outer coil tuned to proton frequency for scout imaging and shimming as previously described(33). Unidirectional rates of ATP synthesis will be measured with the use of the saturation-transfer method applied to the exchange between inorganic phosphate and ATP. The steady-state magnetization of inorganic phosphate is measured in the presence of a selective irradiation of the γ resonance of ATP and compared with the magnetization of inorganic phosphate

at equilibrium in a control spectrum (without irradiation of the γ resonance of ATP)(33).The ratio of inorganic phosphate to phosphocreatine in the soleus muscle is also measured by ^{31}P magnetic resonance spectroscopy as previously described (39,52). Subjects will perform two brief (90 second) muscle contractions against resistance separated by rest while the ^{31}P MRS is performed. Dr. Mark Brown, of UCHSC radiology will be responsible for the interpretation of the leg MRS and quantitation of IMCL and EMCL as well as mitochondrial measures. Dr. Brown is experienced in there procedures and has performed similar interpretations on previous CTRC exercise study of adolescents, in subjects with and without diabetes.

Measurement of Pulmonary Gas Exchange. Rates of oxygen uptake (VO_2) and carbon dioxide output will be measured breath-by-breath for the determination of peak VO_2 and VCO_2 kinetics using a Medical Graphics Ultima CPX metabolic system (Medical Graphics Corporation, St. Paul, MN). For all exercise tests, breath-by-breath and data will be collected for three minutes at rest, during the entire exercise test, and for a period of seven minutes following exercise. Heart rate (HR) will be recorded simultaneously with ventilatory data via TTL signaling from the ECG recorder.

Near infrared spectroscopy. Skeletal muscle [HHb] will be assessed by a frequency domain multi-distance NIRS monitor (Optiplex, Iss, Inc. Chapaign, IL) during each CWR exercise test. The use and limitations of NIRS have been extensively reviewed. The NIRS monitor uses two wavelengths of NIR light (690 and 830nm) and four light source-detector distances at 2.0, 2.5, 3.0 and 3.5 cm. Local muscle O2 extraction was determined as the change in [HHb] as previously described. The NIR data will be sampled continuously at rest and during exercise and recorded at 50Hz. The device probe will be positioned on the lower third of the rectus femoris muscle (approximately 12 cm above the knee joint) of the dominant limb and secured using a Velcro strap. The position of the probe on the limb will be marked and photographed for identical placement between exercise testing visits. The NIRS monitor is calibrated prior to each visit using a calibration phantom of known scattering and optical properties. Tissue oxygenation data will be collected every 50ms by a dedicated data collection computer and saved as text files to a hard disk for later analysis. This device is non-invasive and will be placed only on intact skin.

Plethysmographic measurements: Calf blood flow in response to hyperemia will be determined in by venous occlusion strain gauge plethysmography (D.E. Hokanson Inc. Issaquah, WA), using calibrated mercury-in-silastic strain gauges and expressed as ml/100ml tissue/min as previously reported in our laboratory (23,20). Measurements will be made at rest and after reactive hyperemia, with the patient supine in bed, in a quiet room at approximately 25^{o}C. The patient will be asked not to eat or drink (NPO) for four hours before the procedure. The leg will be supported at the heart level and a mercury filled silastic strain gauge will be placed on the widest part of the calf. The gauge will be connected to the plethysmograph, calibrated to measure the percent change in volume; the plethysmograph in turn will be connected to a chart recording the flow measurements in the arm. For each measurement, a cuff will be placed above the knee and will be inflated to approx 35 mm Hg with a rapid cuff inflator (model E-10, Hokanson) to occlude venous outflow from the leg. Every 15 seconds, flow measurements will be recorded for about 7 secs; at least 5 separate readings will be taken. For the reactive hyperemic flows, the leg cuff will be inflated to 50 mm Hg above systolic pressure for 5 minutes.

Pulmonary function test: The metabolic cart (Minneapolis, Minnesota) will be used to perform a pulmonary function test. Patient will perform the breathing tests until two FVCs and two FEV1s within 200 ml of each other have been obtained. After a few seconds of normal breathing, following the patient's breathing pattern as displayed, we will instruct the patient to take a big inhale and a big exhale followed a complete inhale and exhale. On the complete exhale, patient should blow out as quickly, forcefully, and completely as possible and continue to blow until instructed to stop (at least 6 seconds). This will be repeated as needed.

Stationary Bicycle Graded Exercise Test. To determine the VO_2max and anaerobic threshold, in all participants, a graded bicycle protocol to exhaustion will be carried out (44,45). Each test will have the subject seated on the cycle ergometer (Medgraphics, Minneapolis, MN) breathing into the mouthpiece of the metabolic cart (Medgraphics, Minneapolis, MN) at rest. Five minutes of resting data will be collected to obtain baseline measurements prior to exercise. The rest period may be prolonged at the discretion of the investigator if additional time is required for adjustment to the mouthpiece and stabilization of physiologic variables. At the start of exercise the work rate will be incremented in 10-25 watt/min increments and the incremental portion of the test will be 10-14 minutes in duration.

Completion of the maximal exercise test on the stationary bicycle in Visit 2 will be utilized to determine the maximal workrate, and will use the VO_2 data for that time interval to also confirm criteria for VO_2max. If criteria for VO_2max are not met in the last 15 seconds, we will determine the latest 15-second interval where VO_2max criteria were met and average the workrate for that 15-second period as the maximal workrate. VO_2max will be defined in the proposed study as VO_2 remaining unchanged or increasing less than 1 ml/kg/min for 30 seconds or more despite an increment in work load (53). If VO_2max is not reached by this criterium, it will be repeated on a separate day. The lactate threshold will be estimated from ventilatory data using the V-slope method. We will confirm 35% maximal workload is less than the lactate threshold as calculated by the V-slope method. One maximal exercise test will be done while breathing 60% O_2.

Submaximal exercise test: To make VO_2 kinetic measurements, constant load bicycling test will performed at 85 percent of work-loads below the lactate threshold (steady state). Each test will begin with resting period. Following this period, the subject will pedal 60 rpm against unloaded resistance (e.g. 0 watts) for four minutes immediately prior to beginning the pre-selected work load (85% of individual lactate threshold as determined by the V-slope technique) for 5-8 minutes. This will allow all subjects to reach steady-state and echocardiogram measures to be recorded. The work load will be performed up to four times on a given day (with at least 8-30 min rest periods between bouts) to enable averaging of VO_2 kinetic data within a work load to reduce variability of results. These bout will be performed on two separate visits. The first visit will be done while breathing regular room air and the second visit will be done while breathing 60% O_2.

Tests of autonomic insufficiency: To evaluate autonomic insufficiency, we will measure variation in RR intervals with cycled breathing as previously reported using accepted methods established in our laboratory. The standard method for obtaining RR variability is as follows: The participant, while resting supine, breathes five times per minute, coordinating breaths with a visual electronic signal. This is repeated for five minutes. To obtain data, maximum inspiratory heart rate (RRi) is subtracted from the minimum expiratory heart rate (RRe). Variations of >30 bpm are considered normal, values <20 bpm are considered abnormal. In addition, autonomic insufficiency will be screened measuring lying and standing heart rates and blood pressures (>20 mm fall in upright systolic blood pressure without a change in heart rate) as well as immediate heart rate response test. In this test, analysis of the R-R intervals is utilized when going from lying to standing expressed as the 30-15 ratio where R-R interval is measured at beats 15 and 30 with a ruler.

Ventricular Strains: In addition to standard chamber volumes measurements, evaluation of RV & LV size and function by strain will be completed by the sonographer. Off-line speckle tracking analysis will be performed by a trained echocardiographer. Speckle tracking of the ventricles will focus on the apical four chamber view (LV & RV longitudinal) and the short axis view (LV circumferential). Using dedicated software (EchoPak, Vingmed Horton, Norway), the ventricular walls will be traced and divided into 6 segments. The tracing will be assessed for adequate tracking. In order to obtain valid results, adequate tracking in at least 4 of the 6 segments will be verified; otherwise, values from that view will be discarded. Peak strain (S) will be defined as the greatest negative deflection on the global strain curve. Peak systolic strain rate (SrS) will be defined as the greatest negative deflection on the global strain rate curve. Peak early (SrE) diastolic strain rate will be the first positive defection on the strain rate curves. Regional values will be an average within each segment. Global values will be obtained from each ventricle as a whole.

Risks

Insulin Clamp: With the insulin clamp there is the risk of hypoglycemia. The symptoms of hypoglycemia are excessive sweating, faintness, headache, pounding heart, trembling, and impaired vision. Under very extreme degrees of hypoglycemia, a person may lose consciousness. Blood glucose levels will be monitored throughout this test to prevent hypoglycemia from occurring. I have performed >50 clamps and have caused no episodes of symptomatic hypoglycemia.

Blood Draws: Pain may be felt when the needle goes into the vein. A bruise may form at the site. There is a risk of infection. Standard precautions will be used to minimize these risks and only CTRC nurses will perform blood draws.

Fasting: Subjects who are taking a secretagogue run a risk of hypoglycemia during fasting. Symptoms listed above will be described in advance and subjects will be instructed not to take their AM secretagogue dose on fasting days.

DEXA: During DEXA testing the amount of radiation received is less than 25mRem total body equivalent dose per measurement. This is estimated to be less than one-half the amount received from a chest x-ray. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risk is not known. Women who are or could be pregnant should receive no unnecessary radiation, and should not participate in this study.

IV Risks: When the needle carrying the plastic tube goes into a vein, it hurts for a short time. The needle is then removed and only the plastic tube remains in the vein. There will also be the minor discomfort of having the plastic tube taped to the arm. In about one in 10 cases a small amount of bleeding under the skin will produce a bruise. The risk of a blood clot forming in the vein is about one in 100, while the risk of infection or significant blood loss is one in 1000. Standard precautions will be used to minimize risk and only trained CTRC personnel will place IVs.

GXT (Graded Exercise Test): The exercise test is a standard procedure, which we routinely perform on hundreds of cardiac subjects every year for diagnosis and evaluation of treatment of their condition. The death rate of this test is approximately one in 10,000 tests and the incidence of serious complications including prolonged arrhythmias (irregular heart beats) or prolonged chest pain is approximately 4 in 10,000 tests. This test will be performed on a bicycle. The subject may experience slight resistance to breathing due to breathing through mouthpiece. Some additional risks of exercising are sore muscles, fatigue, shortness of breath, and lightheadedness. People with diabetes may also experience low blood sugar (hypoglycemia).

Plethysmography and Femoral Artery FMD: There may be a slight discomfort while the arm cuff is inflated.

Muscle biopsy – A small cut in the skin will be made after using numbing medication on the incision site. A small sample will be taken from the calf muscle by pressing a hollow needle into the muscle. There is a small chance that subjects could get an infection where the needles goes in. There is also a small chance that subjects could have an allergic reaction to the numbing medicine.

Magnetic Resonance Imaging: The MRI is a non-invasive scan of your abdominal area and calf muscle, respectively, using a magnet. There is no radiation and no risk involved with the MRI. The MRI may be loud, therefore the subject is provided with audio protection and optional television to help increase comfort. Some subjects might feel claustrophobic while having an MRI and the scan will be stopped if it can not be tolerated. In addition, any subjects with implanted metal can not have an MRI due to the magnet involved.

Echocardiogram: The risks of the echocardiogram (performed for research purposes) are negligible.

Nitroglycerin: Common side effects of nitroglycerin are bitter taste, headache, lightheadedness and palpitations (rapid beating of the heart).

Arterial Stiffness Measurement:
There is a very slight chance that fainting or stroke may occur. No actual fainting or stroke has ever been reported. The risk of these happening has been estimated to be less than 1/1,000,000.

Submaximal exercise test: Submaximal exercise test is a standard procedure. The participant risk of an abnormal heartbeat or death with this exercise would be similar to the risk from walking for 5 minutes at a medium speed, and is far less than the 1 in 10,000 risk for the graded exercise test because this test is done at lower work levels.The subject may experience slight resistance to breathing due to breathing through mouthpiece. Subjects may also experience muscle soreness, fatigue, shortness of breath, chest pain, and lightheadedness during submaximal exercise of the calf muscle. Subjects with diabetes may also experience low blood sugar (hypoglycemia) while performing submaximal exercise.

Unforeseen Risks: This study may include risks that are unknown at this time.

Violation of privacy and loss of confidentiality are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected. Therefore, using the aforementioned methods, we will strive to maintain patient confidentiality. No

sensitive information (domestic violence, child abuse, infectious diseases) will be exposed as part of this research.

Protected Health Information. Protected health information collected for this study includes: portions of medical records relevant to the study, lab and procedure results (i.e. blood results, ultrasound report, exercise test results and the Physical Activity Recall Questionnaire). Copies of all protected health information will be provided to the subject upon request.

D. Potential Scientific Problems:

Pitfalls. The limitations of NIRS have been extensively detailed in the literature (37). One of the major limitations of this method is that, at present, NIRS cannot differentiate between hemoglobin in the vascular space and intramuscular myoglobin. In the proposed experiments, we will utilize a quantitative tissue oxygen spectrometer (Inspectra, Hutchinson Technologies, Hutchinson,MN) and a phase-modulated, spatially-resolved NIRS spectrometer (Optiplex TS, ISS, Chicago, IL). We are currently validating the non-invasive NIRS methods with invasive hemoglobin saturation and blood gas monitoring. Alternatively, we could utilze the invasive methods of rapid areterial-venous blood gas sampling and continuous thermodilution across the exercising leg to addres Aim1.

E. Data Analysis Plan:

Sample Size and Analysis Plan.

Sample size: The sample size for this study is based upon our ability to detect a significant difference in time constants at 80% power, ά=0.05 and similar variance in the measured time constants of leg blood flow and muscle deoxygenation as derived from our previously published VO_2 kinetics in T2DM and control subjects. Previous VO_2 kinetic data are shown in table 1 (6). The time constants for kinetic analysis are determined as ($\tau_{1,2,or\ 3}$) for the relevant parameter analysis (blood flow or muscle deoxygenation) in the equations listed above (see *Kinetic Analysis*). An estimate of the expected blood flow kinetics in T2DM and health was calculated from the VO_2 and muscle deoxygenation responses using the Fick Principle ($Q = VO_2/O_2$ extraction) (Table 2.). The pooled variance of the time constants for muscle deoxygenation and leg blood flow are assumed to be similar to that for VO_2 kinetics (e.g. SD = 5.0s). With 15 subjects per group, we will be able to detect a 5.3sec. difference in time constants between groups (at Power =80%, ά = 0.05). Given the predicted time constants for leg blood flow (Table 2) and the expected difference between T2DM and control from the muscle deoxygenation preliminary data, our planned sample size of 15 subjects per group should be adequate to address the primary hypothesis. Specifically, we will have nearly 100% power to detect the expected difference between T2DM and controls for leg blood flow kinetics and 86% power to detect the expected difference in muscle deoxygenation kinetics in T2DM and control subjects. *Analysis Plan.* The primary endpoints are the respective time constants of leg conduit artery blood flow kinetics and muscle deoxygenation kinetics as measured during the transition from rest to constant work rate exercise. The time constant of each parameter will be used to compare the healthy and T2DM groups using a two sample independent t-test with equal variances. Given the small planned sample sizes, should the assumption of normality or equal variance of the time constants appear unreasonable based on group plots, non-parametric tests will be utilized. The secondary endpoint is the time constant of pulmonary VO_2 kinetics. To examine the planned correlations between measured exercise parameters, correlations (Pearson's r) and scatter plots will be utilized. These exploratory analyses will serve the primary purpose of hypothesis generation for future investigations.

Table 1. VO2 kinetics Study Group	N	Time constant (sec) Mean±SD p<0.05
T2DM	8	47.7±4.7*
Control	10	28.8±5.3

Table 2. BF kinetics Study Group		Time constant (sec) Calc Mean
T2DM		50.0
Control		27.2

F. Summarize Knowledge to be Gained:

Expected results and interpretation: Based upon our previous work, we anticipate VO_2 kinetics will be slowed during KE exercise in T2DM compared with controls. Slowed kinetics of leg blood flow in T2DM compared with healthy controls would be interpreted as evidence that bulk blood flow, and hence oxygen delivery, is impaired following the onset of exercise in T2DM skeletal muscle. As previously discussed in the Background section, if we observe more rapid muscle deoxygenation in T2DM muscle, this result would be consistent with an impairment of local oxygen delivery (and blood flow) relative to oxygen utilization. Conversely, if we observe slowed muscle deoxygenation, this result would be consistent with an inherent abnormality in local muscle oxygen utilization relative to oxygen delivery in muscle. Thus, in the proposed experiment, a slowed leg blood flow response *and* more rapid muscle deoxygenation in T2DM muscle would be interpreted as support for our working hypothesis (i.e. at least part of the slowed VO_2 kinetic response in T2DM is attributable to impaired muscle blood flow and oxygen delivery).

G. References:

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