Nitrate supplementation and exercise tolerance in patients with type 2 diabetes

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Nitrate supplementation and exercise tolerance in patients with type 2 diabetes

Principal Investigator: Darren Casey, Ph.D.

Co-investigator(s): Kenichi Ueda, M.D.

Vitor Lira, Ph.D.

Richard Shields, Ph.D.

Dale Abel, M.D., Ph.D.

Abstract

Patients with type 2 diabetes (T2D) demonstrate a reduced exercise capacity, a powerful predictor of cardiovascular mortality, which may be due to reductions in skeletal muscle perfusion and mitochondrial dysfunction. Nitric oxide (NO) is a key molecule involved in in the regulation of blood flow to contracting muscles, as well as a critical mediator in mitochondrial respiration. However, there appears to be a decreased enzymatic synthesis of NO and an overall reduction of bioavailable NO in patients with T2D, which likely contributes to the reduced exercise capacity and tolerance. Accumulating evidence suggests that exogenous nitrate supplementation is an effective option for increasing NO bioavailability in vivo. The purpose of the proposal is to use a randomized, double-blind, placebo controlled study design to comprehensively investigate the impact of 8-weeks of dietary nitrate supplementation on skeletal muscle blood flow, mitochondrial function, and exercise capacity in patients with T2D. The *central hypothesis* is that increasing NO bioavailability via dietary nitrate supplementation in patients with T2D will lead to improved oxygen delivery and utilization during exercise. To address this hypothesis a highly mechanistic and translational experimental strategy will be used to explore whether increased NO bioavailability via dietary nitrate supplementation improves skeletal muscle perfusion during exercise (**Aim 1**), enhances mitochondrial biogenesis and function (**Aim 2**), and improves exercise capacity and efficiency (**Aim 3**). Collectively, these studies will provide important mechanistic insight into the therapeutic potential of dietary nitrate supplementation for improving skeletal muscle blood flow, mitochondrial function and exercise capacity in patients with T2D.

Research Plan

I. Specific Aims:

Aim 1: Determine if increased NO bioavailability via 8 weeks of dietary nitrate supplementation improves skeletal muscle perfusion during exercise in patients with T2D. The increase in muscle blood flow during exercise is substantially attenuated in T2D compared with relatively healthy subjects, which has been attributed to impaired endothelium-dependent (NO-mediated) vasodilation and/or potentially greater sympathetic restraint of the microvasculature.[1-4] Our ***primary hypothesis*** is that increasing NO bioavailability through the nitrate-nitrite-NO pathway will improve blood flow to the contracting muscles of T2D patients. Our ***secondary hypothesis*** is that the improved exercise blood flow will be due in part to an enhanced ability to blunt sympathetic vasoconstriction within the vascular beds of contracting skeletal muscle during exercise.

Aim 2: Determine if dietary nitrate supplementation and subsequent increases in NO bioavailability lead to improved mitochondrial function in patients with T2D. NO stimulates mitochondrial biogenesis and contributes to mitochondrial respiration. Individuals with T2D demonstrate a reduced content and impaired functional capacity of skeletal muscle mitochondria,[5] possibly due to reductions in bioavailable NO. Our ***hypothesis*** is that 8 weeks of dietary nitrate supplementation will improve various measures associated with skeletal muscle mitochondrial content and function (i.e. respiration) in patients with T2D.

Aim 3: Determine if exercise capacity and efficiency are improved following 8 weeks of dietary nitrate supplementation in patients with T2D and examine whether these improvements are associated with changes in oxygen delivery and utilization (as assessed in Aims 1 and 2). Relative to age- and weight-matched subjects, exercise capacity is reduced in T2D patients.[6-9] Recent evidence suggests that nitrate supplementation improves exercise efficiency and tolerance in humans.[10-14] Our ***primary hypothesis*** is that dietary nitrate supplementation will improve maximal exercise capacity, as measured by whole body maximal oxygen uptake (VO_2), as well as improve exercise efficiency (reduce the oxygen cost for a given workload).

II. Background

***Overview*.** It is currently estimated that nearly 30 million Americans have diabetes mellitus, with the majority (up to 90%) of cases being Type 2 diabetes (T2D).[15] Individuals with T2D are at a greater risk of developing CV disease;[16, 17] with mortality rates from CV related disease being up to four times higher in this patient population.[18] Compared to age-matched controls, patients with T2D often demonstrate a reduced exercise capacity, as measured by whole body maximal oxygen uptake (VO_2).[6-9] This is critical from a clinical standpoint, as VO_2peak and measures of cardiorespiratory fitness are inversely related to mortality risk in healthy individuals and those with CV diseases.[19-21] Moreover, among patients with T2D those with the lowest VO_2peak have a greater tendency to develop future CV disease related events.[22] Although the mechanism by which exercise capacity is reduced in persons with T2D is not completely understood, there is evidence to suggest that impairments in skeletal muscle perfusion or oxygen utilization play a significant role.[23] Along these lines, patients with T2D often demonstrate an attenuated blood flow response to exercise compared to age- and weight-matched subjects.[1-4] It has also been demonstrated that the ability to utilize available oxygen within skeletal muscle via mitochondrial respiration is also altered in T2D.[5] Collectively, impairment in each of these parameters can contribute to the decreased exercise capacity associated with T2D.

T2D and Nitric Oxide (NO) bioavailability. Since its initial discovery in the late 1980s,[24] NO has been demonstrated to have multiple biological actions and can regulate physiology acutely or lead to long-term changes in cell function. Accumulating evidence suggests a decreased enzymatic synthesis of NO and an overall reduction of bioavailable NO in T2D.[25, 26] From a vascular standpoint, patients with T2D demonstrate impairments in endothelial dependent vasodilation, which is largely a NO mediated response.[1, 27-29] Important to this proposal, endothelial dysfunction has been proposed as a critical component involved with the reduced exercise tolerance in T2D.[23] Increasing evidence also demonstrates that endogenously produced NO plays an important role in regulating metabolism of energy substrates, various aspects of mitochondrial respiration, and in the activation of mitochondrial biogenesis.[30-35] Taken together, a reduction in bioavailable NO in T2D could 1) limit vasodilation in active skeletal muscle and therefore attenuate the blood flow and oxygen delivery response to exercise and/or 2) alter skeletal muscle mitochondrial respiration and oxygen utilization, which in turn contributes to the exercise deficits observed in patients with T2D

***Source of NO and benefits of NO boosting compounds*.** In addition to the L-arginine-NOS system for endogenous synthesis of NO, evidence suggests that nitrite (NO_2^-) and nitrate (NO_3^-) metabolism occurs in blood and various tissues and contributes to the overall bioavailability of NO.[36-38] Thus, NO_2^- reduction (i.e. nitrate-nitrite-NO pathway) represents an alternative and differentially regulated system for NO generation that operates in parallel to the classical L-arginine-NOS pathway.[39] A relatively easy method to increase plasma NO_2^- is through increasing

dietary NO_3^- intake. Recent evidence suggests that dietary nitrate supplementation can have physiological and therapeutic effects in both animal models and humans.[40] Briefly, several of the reported physiological benefits of acute oral nitrate supplementation in young and older humans include but are not limited to improvements in exercise efficiency and tolerance,[10-14] vascular function,[41-43] and blood pressure.[12, 14, 43-45] ***Considering the important link between NO deficiency and impairments in muscle blood flow and mitochondrial function, stimulation of the nitrate-nitrite-NO pathway may enhance NO bioavailability and thus have beneficial effects on exercise capacity in patients with T2D. Therefore, the findings of the present study may be rapidly translated to changes in therapy for patients with T2D.***

III. Research Design and methods

APPROACH

Figure 1 is an overview of the key concepts underpinning this application. The idea is that a decreased NO bioavailability contributes to I) a reduced blood flow in contracting muscle, II) impaired skeletal muscle mitochondrial function, and III) ultimately a reduced exercise capacity in patients with T2D. We will use a novel therapeutic option (8 weeks of dietary nitrate) in an effort to boost bioavailable NO and subsequently help improve exercise tolerance. The use of state-of-the-art human physiology and pharmacology techniques will allow us to examine the potential beneficial effects of dietary nitrate supplementation *in vivo*, while our studies utilizing muscle tissue from patients with T2D will provide novel information at the molecular and cellular level regarding skeletal muscle mitochondrial function.

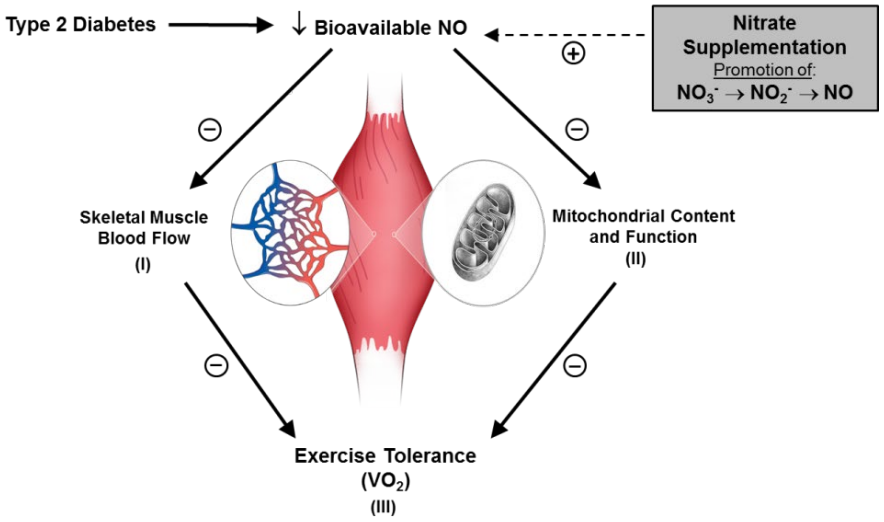

Figure 1. Conceptual overview of application and protocols. *Dashed line represents dietary nitrate supplementation as a potential therapy to improve bioavailable nitric oxide (NO).*

Patients. 50 patients with documented Type 2 diabetes will be studied. We will recruit patients (40-70 years of age) from the University of Iowa Hospitals and Clinics database. ***The Institute for Clinical and Translational Science (ICTS) at the University of Iowa identifies potential research participants within the database based on specific inclusion and exclusion criteria (described below) provided by the investigator.*** Exclusion criteria will include diagnosis of type 2 diabetes < 3 years prior to enrollment, HbA1c <6.0% or >10.0%, body mass index > 40 kg/m^2, incident cardiovascular events in the last year (heart attack, stroke), symptomatic coronary artery disease and/or heart failure, uncontrolled hypertension, hypotension (resting systolic BP < 90 mmHg), renal impairment with creatinine clearance (eGFR) of <50 ml/min, insulin use, smoking or history of smoking within past one year, and the use of medication

which contain nitrates. We will also recruit 15 age- and weight-matched nondiabetic control subjects which will allow us to 1) quantify the degree of impairment in T2D prior to intervention and 2) assess the effectiveness of the nitrate therapy in restoring the primary outcome measures back to 'normal'.

Measurements: All studies required to accomplish the three specific aims of this application outlined below will employ state of-the-art techniques to assess blood flow, exercise capacity, and mitochondrial content and function in humans. Relevant publications from other groups and ours are listed next to the following primary measures/techniques to be used in the proposed studies:

- *Subject monitoring.* Heart rate will be monitored via a three-lead electrocardiogram. Beat-by-beat arterial blood pressure will be measured and monitored via artery catheter (described below).
- *Brachial Artery Catheterization*. An indwelling 20-gauge Teflon catheter will be placed in the brachial artery using aseptic techniques after local anesthesia (1% lidocaine).[46-51] The catheter will be connected to a 3-port connector as well as a pressure transducer, which will permit the simultaneous measurement of beat-by-beat brachial arterial pressure, blood draws and infusion of study drugs. Placement of the arterial catheter will be performed by Dr. Kenichi Ueda (board-certified anesthesiologist) who has significant experience in this technique in research studies and clinical practice.
- *Intra-arterial Drug Administration*.
  - *Vasodilator responsiveness in the forearm* using standard intra-arterial doses of acetylcholine (ACh) and sodium nitroprusside (NTP).[46, 48, 50] Ach (a nonspecific muscarinic agonist) will be infused intra-arterially at 4.0, and 8.0 µg/dl forearm volume/min for 2 min each. Intra-arterial infusion of NTP will be infused at 1.0 and 2.0 µg/dl forearm volume/min for 2 min each.
  - *Standard intra-arterial doses of phenylephrine (α_1-agonist) and dexmedetomidine (α_2-agonist)* will be administered to examine postjunctional alpha-adrenergic vasoconstrictor responses during exercise.[52, 53] In male subjects, phenylephrine will be infused at 0.03125 µg/deciliter of tissue/min for the final 3 min at rest, exercise and during the high flow trial. In male subjects dexmedetomidine will be infused at 6.25 ng/deciliter of tissue/min for the final 3 min at rest, exercise and during the passive vasodilation trial. The doses of phenylephrine and dexmedetomidine will be doubled for young and older female participants because they typically have reduced vasoconstrictor responses to α-receptor stimulation compared with men. In an effort to normalize the concentration of each drug in the blood perfusing the forearm, the infusions will be adjusted on the basis of forearm blood flow (measured via Doppler ultrasonography) and forearm volume (measured via water displacement).
  - *Standard intra-arterial doses of adenosine* will be administered as a control non-exercise vasodilator condition.[47, 49] Adenosine will be infused at 6.25 µg/deciliter of tissue/min for a duration of 6 min.
- *Rhythmic forearm exercise* will be performed using a handgrip device at 20% maximum voluntary contraction (MVC), as previously described.[46-50] Briefly, subjects will be studied while in the supine position under standardized conditions. Maximal voluntary contraction (MVC) of the nondominant hand will be measured by having subjects squeeze a handgrip device at maximal effort three separate times and then averaged. The MVC will be used to calculate a relative work rate of 20% MVC for the experimental protocol. Based on data from our lab, this intensity is considered "moderate" and therefore, all subjects are expected to complete this intensity under each exercise condition. Rhythmic forearm exercise will be conducted with a handgrip dynamometer by squeezing and releasing two handles together by 4-5 cm which will then raise and lower a weight over a pulley connected by rope. Forearm contractions will be

at a rate of 20 times min^{-1} (7 minutes each exercise trial). In addition, this technique has been adapted to facilitate beat-to-beat measurements of brachial artery blood flow via Doppler ultrasound.

- *Forearm blood flow (FBF)* will be determined using Doppler ultrasound. Briefly, brachial artery diameter and blood velocity will be measured with a probe insonation angle at 60 degrees. Velocity measurements will be recorded continuously; diastolic diameter measurements will be made under each condition. FBF will be calculated as the product of mean blood velocity and artery cross sectional area. Forearm vascular conductance (FVC) will be calculated using FBF and mean arterial pressure. For more details see references[46-51].
- *Arterial and deep vein blood samples* (retrograde catheter technique) will be obtained during the forearm exercise trials for determination of blood gases and calculation of oxygen delivery and extraction.
- *Exercise Testing.* A 12-lead ECG, symptom limited cardiopulmonary exercise testing with gas exchange measurements (Parvo Medics, Sandy, UT) will be performed on a cycle ergometer (Lode Corival Bike Ergometer) using a ramp protocol to determine peak aerobic capacity (VO_2peak). In addition to VO_2peak, exercise efficiency will be analyzed at various submaximal workloads (i.e. VO_2 for a given workload).
- *Venous blood samples* will be obtained before and after the intervention for determination of blood glucose, HbA1C, plasma nitrate and nitrite, and cGMP concentration. Plasma nitrate and nitrite concentration will be measured using ozone chemiluminescence and quantified using a NO analyzer (NOA 280i, Sievers, Boulder, CO USA) as previously described.[44] cGMP will be determined as an indicator for generation of bioactive NO using an enzyme immunoassay (cGMP EIA kit; Cayman Chemical, Ann Arbor, MI, USA). All subjects will follow a 3 day low nitrate diet prior to each study day.
- *Muscle biopsies* will be obtained from the vastus lateralis using a modified Bergstrom needle with suction under local anesthesia, which will yield ~200-250mg of tissue.[54, 55]
- *Mitochondrial function*. Fiber bundles will be chemically permeabilized with saponin and mitochondrial respiration will be analyzed by *in situ* high-resolution respirometry at 37°.[56] Briefly, state 3 (with ADP) and state 4 (without ADP) respiration will be measured using succinate as a substrate and the respiratory control index and P/O ratio will be calculated as previously described.[5, 57] Additionally, mitochondrial ATP production will be measured.[58-60]
- *Mitochondrial biogenesis and content.* Enzyme activity (citrate synthase), mitochondrial DNA and respiratory chain protein content (i.e. Complexes I - V), as well as peroxisome-proliferator-activated receptor γ co-activator-1α (PGC-1α) mRNA and protein will be assessed as previously described. [35, 61]
- *Oxidative Stress.* Previous studies have demonstrated a link between increased mitochondrial oxidative stress and the development of insulin resistance.[62] Whether this increased oxidative stress contributes to reduced exercise tolerance in T2D remains unknown. Because nitrate supplementation is expected to improve mitochondrial function, it may also reduce oxidative stress in skeletal muscle. Therefore, 4-Hydroxynonenal (4-HNE) adducts, indicative of lipid peroxidation, and protein carbonylation will be assessed in muscle homogenates via western blots as previously described.[63]
- *Autophagy.* NO has been shown to improve mitochondrial function in different cell models. Because turnover of damaged/dysfunctional mitochondria can contribute to improved respiratory function we will assess autophagy levels in skeletal muscle. Autophagy flux will be assessed via western blot and quantification of LC3-II/LC3-I ratio and p62 levels from muscle homogenates, as previously shown.[64, 65] In addition, we will assess levels of AMP-associated protein kinase (AMPK, and its phosphorylation at T172) and of its downstream target unc-51 like autophagy activating kinase (ULK1, and its phosphorylation at S555). The rationale for these measurements is provided under Aim 2.

Exploratory measures (*Not necessary to address specific aims*)

- *Oral glucose tolerance tests (OGTT).* Recent evidence suggests that nitrite augments glucose uptake in adipocytes.[66] Therefore OGTTs will be performed to assess if nitrate supplementation improves blood glucose clearance in T2D. Plasma glucose and insulin will be measured before and at 1 and 2 hours after consumption of 75 g dextrose drink as previously described.[67]

Inorganic Nitrate Supplementation

Patients will be randomized in a double-blind fashion to either a inorganic nitrate (beetroot juice with nitrate) or placebo (beetroot juice without nitrate) group for 8 weeks. The daily dose of oral nitrate is similar to the doses previously used in human studies,[11, 14, 45] which is effective at boosting plasma levels of nitrate and nitrite (Figure 2).

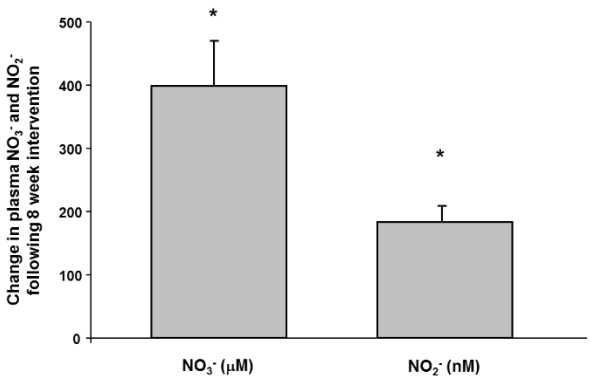

Figure 2. Preliminary data demonstrating the effectiveness of 8 weeks of $NaNO_3$ supplementation in boosting plasma levels of nitrate (NO_3^-) and nitrite (NO_2^-). (n=7). *P < 0.001 vs baseline (pre intervention) values.

Overview of Analysis Plan: For all analyses distributional assumptions will be assessed with data transformations or non-parametric methods used as appropriate. Test statistics with an association probability of ≤ 0.05 will be considered statistically significant. When the stated hypothesis indicates a direction for the anticipated association a one-tailed test will be used; if a direction is not stated a two-tailed test will be used. The primary outcome variables outlined for each aim will be analyzed as the change from baseline and compared between groups (inorganic nitrate vs. placebo) using repeated measures ANOVA. Pearson product-moment correlations will be used to assess whether improvements in exercise efficiency/capacity are associated with changes in skeletal muscle blood flow and/or mitochondrial function following the nitrate intervention. Additionally, unpaired t-tests will be used to compare group means between the patients with T2D at baseline (prior to 8 week intervention) and the age- and weight-matched nondiabetic control subjects. This will allow us to 1) quantify the degree of impairment in T2D prior to intervention and 2) assess the effectiveness of the nitrate therapy in restoring the primary outcome measures back to 'normal'.

Sample-size/ Statistical Power: One of the primary aims of this investigation is to assess the potential efficacy of dietary nitrate supplementation for improving muscle blood flow during exercise in patients with T2D (*Aim 1*). In a previous study of 12 older adults (see **Figure 4**) we found that the mean ± SD within subject change in forearm blood flow at 20% exercise following acute dietary nitrate supplementation was 32±30 ml/min corresponding to an effect size of 1.07 standard deviation units. In general, for a repeated measures analysis (e.g. paired t-test) a sample-size in the treatment group of N=25 will provide statistical power (two-tailed, alpha=0.05) of 94% to detect a difference of 1 SD. For *Aims 2 and 3* we plan to use the same patient groups as proposed in Aim 1. Based on previously published data related to dietary nitrate and exercise efficiency,[58] we assume the standard deviation of the change in whole body VO_2 for a given workload from baseline to end of treatment will be approximately 0.09 l/min. Under the assumption that the standard deviation is 0.09 l/min; if dietary nitrate supplementation improves VO_2 for a

given workload by 0.06 l/min compared to placebo, a total sample size of N=50 (25 per group) will provide statistical power (two-tailed, alpha=0.05) of 85%.

General Overview of Protocol

Overall Strategy: Figure 3 on the following page is a schematic of our overall strategy. In keeping with ADA guidelines we focus on our overall approach and avoid excessive detail on well-established methods we have used previously.

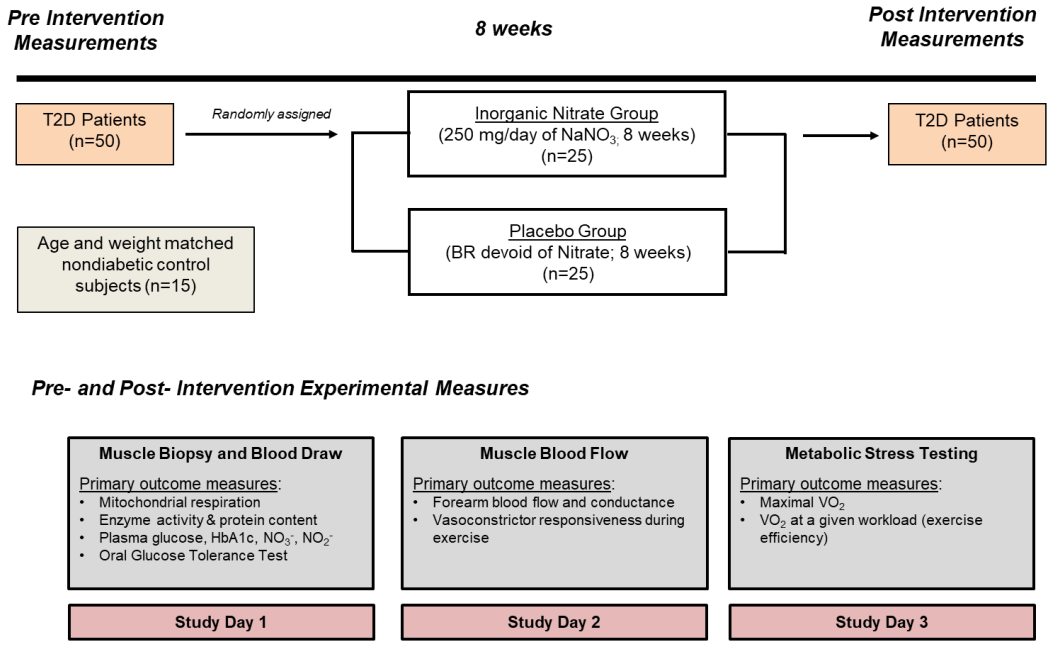

Figure 3. Proposed experimental approach. Patients with T2D will undergo pre intervention experimental measurements (3 separate study days) prior to being randomized to either 8 weeks of 1) dietary nitrate supplementation or 2) placebo. All patients with T2D enrolled in the study will complete both pre and post intervention measurements. It is important to note that the age and weight matched nondiabetic subjects will only undergo pre intervention experimental measurements. The inclusion of the control subjects will allow us to 1) quantify the degree of impairment in T2D prior to intervention and 2) assess the effectiveness of the nitrate therapy in restoring the primary outcome measures back to 'normal'.

Aim 1 – Determine if increased NO bioavailability via 8 weeks of dietary nitrate supplementation improves skeletal muscle perfusion during exercise in patients with T2D

Rationale and hypothesis: Relative to age- and weight-matched subjects, blood flow to exercising muscles is reduced in patients with T2D,[1-4] which has been attributed to impairments in NO-mediated vasodilation.[1] Recent evidence suggests that dietary nitrate supplementation can increase NO bioavailability and subsequently improve various measures of vascular function,[41-43] including muscle blood flow during exercise in animals.[68, 69] **Figure 4** is recent data from our lab demonstrating that acute dietary nitrate supplementation augments muscle blood flow during hypoxic exercise in older adults.[70] It is also important to note that the magnitude of change in

vasodilation during hypoxic exercise was related to the rise in plasma nitrite post supplementation in aging humans (r=0.69; P<0.05). Moreover, our preliminary data in a small sample of patients with peripheral artery disease (PAD), some of which had T2D, suggest that long term (8 weeks) dietary nitrate supplementation improves blood flow to the contracting muscles at multiple exercise intensities (**Figure 5**). Thus, our ***primary hypothesis*** is that 8-weeks of nitrate supplementation will effectively increase NO bioavailability via the nitrate-nitrite-NO pathway and improve blood flow to the contracting muscles of T2D patients.

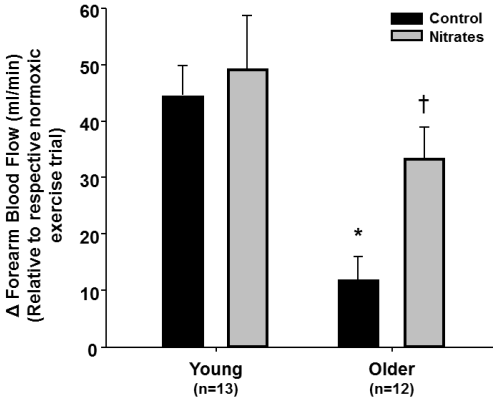

Figure 4. Our recent data showing blunted blood flow during hypoxic exercise with aging, which is improved following acute dietary nitrate intake. *P<0.05 vs. young; †P<0.05 vs. control.[70]

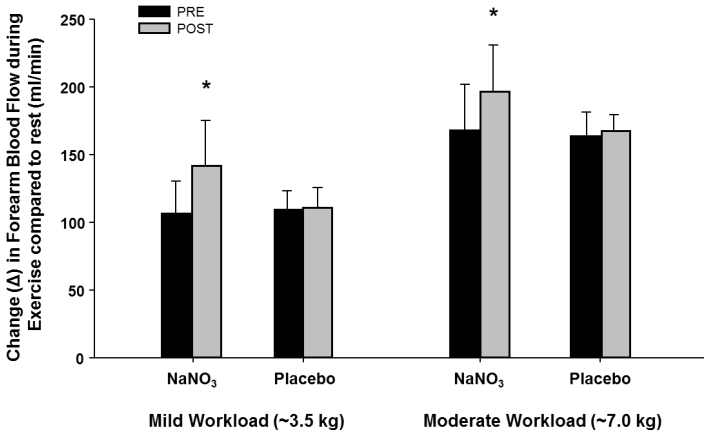

Figure 5. Preliminary data showing an increase in blood flow during mild to moderate intensity forearm exercise following 8 weeks of $NaNO_3$ in patients with PAD. (n=7 for $NaNO_3$ and n=5 for placebo) *P<0.05 vs PRE

In healthy young adults, sympathetic vasoconstrictor responses are blunted in the vascular beds of contracting skeletal muscle,[71-75] a phenomenon referred to as "functional sympatholysis." The ability to blunt sympathetic vasoconstriction (i.e. functional sympatholysis) in vascular beds of contracting muscle is a key local regulatory mechanism to ensure adequate blood flow and oxygen delivery to the contracting muscle. Along these lines, NO has been shown to inhibit sympathetic vasoconstriction in contracting skeletal muscle of experimental animals and humans.[76-79] Therefore a decreased NO bioavailability[1, 29] coupled with sympathetic overactivity[80, 81] commonly observed in patients with T2D might lead to a decreased ability to blunt sympathetic vasoconstriction in contracting skeletal muscle and consequently contribute to the attenuated blood flow responses to exercise.[1-4] Thus, our ***secondary hypothesis*** is that the improved exercise blood flow following nitrate supplementation will be due in part to an enhanced ability to blunt sympathetic vasoconstriction within the vascular beds of contracting skeletal muscle during dynamic exercise. Our approach (described below) will allow us to determine whether these potential changes are selective for α_1- or α_2-adrenergic receptors.

General Approach: All patients will be instrumented with a brachial artery catheter before and after the 8 week intervention. On each study day (before and after intervention) they will perform a total of two bouts of rhythmic handgrip exercise at 20% of maximum. The exercise bouts will be performed to assess 1) the effects of dietary nitrate supplementation on exercise blood flow (**primary hypothesis**), and 2) the vasoconstrictor responsiveness during exercise (i.e. functional sympatholysis) to direct α_1- and α_2-adrenergic receptor stimulation (via phenylephrine and dexmedetomidine, respectively – **secondary hypothesis**). Intra-arterial adenosine will be

administered to elevate blood flow to values similar to those observed during exercise and serve as 'high flow-functional sympatholysis' control trials. The order of adenosine vs. exercise, as well as phenylephrine vs. dexmedetomidine will be varied across subjects. ACh and NTP will be infused to assess endothelial-dependent and-independent vascular responsiveness. Non-invasive measurements of arterial stiffness will also be performed using applanation tonometry.

Potential Results, Interpretation & Follow-Up Studies: We expect that 8-weeks of dietary nitrate supplementation will increase plasma levels of NO_3^- and NO_2^- relative to the placebo group. In the nitrate group we also anticipate increases in forearm blood flow (FBF) and vascular conductance (FVC) post treatment as a result of an enhanced ability to blunt sympathetic vasoconstriction (i.e. improved functional sympatholysis). The use of both intra-arterial phenylephrine (α_1-agonist) and dexmedetomidine (α_2-agonist) will permit us to determine if improvements in functional sympatholysis are selective to a specific α-adrenergic receptor subtype. Additionally, we have the experimental tools to pharmacodissect a number of potential dilator and constrictor pathways that might contribute to improvements in the FBF and FVC responses not explained by changes in sympathetic vasoconstriction or enzymatically produced NO. These studies will also set the stage for future mechanistic studies on how NO may modulate sympathetic vascular transduction in T2D.

Aim 2 – Determine if dietary nitrate supplementation and subsequent increases in NO bioavailability lead to improved mitochondrial function in patients with T2D.

Rationale and hypothesis: Mitochondria are important dynamic organelles for cell survival and function, which often become dysfunctional and/or reduced in various disorders, including diabetes.[5, 82] NO appears to play a critical role in mitochondrial function (i.e. respiration) as well as acts as a key messenger to activate mitochondrial biogenesis.[30, 33-35] Interestingly, a recent study led by Dr. Vitor Lira (co-investigator in this application) demonstrated that in healthy insulin sensitive muscle mitochondrial biogenesis and autophagy, a catabolic process responsible for aggregate protein and organelle degradation, are regulated in parallel. This conclusion is based on a series of experiments that showed: a) oxidative muscles, which have higher mitochondrial content, also present higher autophagy machinery (i.e., expression of autophagy proteins) and basal autophagy flux when compared to glycolytic muscles; b) endurance exercise training leads to increased mitochondrial content in primarily glycolytic muscles, which also adopt basal autophagy levels that more closely resemble those of oxidative muscles; and c) muscle specific overexpression of peroxisome-proliferator-activated receptor γ co-activator-1α (PGC-1α), a master regulator of mitochondrial biogenesis, results in increased autophagy flux primarily associated with mitochondrial autophagy (i.e., autophagy targeting mitochondria).[65] The most logical interpretation of these findings is that a fine balance between mitochondrial biogenesis and removal, via autophagy, enables optimal organelle turnover, which seems instrumental for an ideal mitochondrial function in muscle.

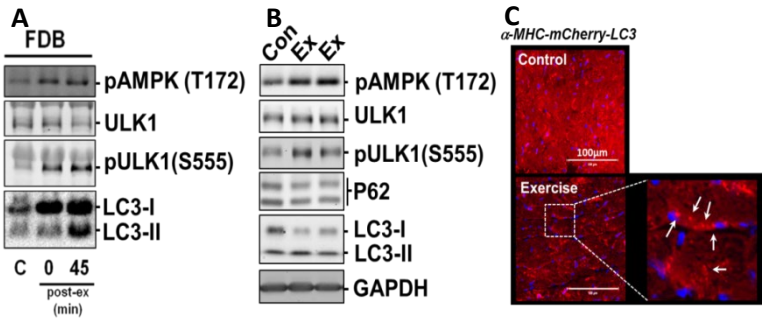

Figure 6. Preliminary data showing the induction of AMPK-associated signals for autophagy activation in mouse skeletal muscle (A) and heart (B and C). In these studies an acute bout of treadmill exercise was used to induce autophagy in skeletal muscle and in the heart (n=3 in each group). Representative images denoting increased phosphorylation of AMPK (T172) and of its downstream target unc-51 like autophagy activating kinase 1 (ULK1, at S555) occurring in both skeletal muscle (A) and heart (B) in response to exercise. (A) Flexor digitorum brevis (FDB) muscle was removed from sedentary control mice (c), as well as from mice that exercised immediately and 45 min post-exercise (0 and 45, respectively); (B) Left ventricle lysates from sedentary control mice (con) and from mice at the end of

Mechanistically, the processes of mitochondrial biogenesis and autophagy can be stimulated by AMP-activated protein kinase (AMPK).[83, 84] This is further supported by our preliminary data shown in **Figure 6**. Dr. Lira has also demonstrated that NO donors induce mitochondrial biogenesis and Glut4 expression in rat skeletal muscle cells via AMPK-mediated activation of PGC-1α.[35, 85] Important to this proposal, short term (3 days) supplementation of $NaNO_3$ improves intrinsic mitochondrial efficiency in young humans.[58] However, whether $NaNO_3$ improves mitochondrial function in T2D, and to what degree that is associated with increased mitochondrial biogenesis and autophagy remains unknown. Thus we ***hypothesize*** that longer term (8 weeks) nitrate supplementation will be effective in improving mitochondrial respiration as well as enhancing mitochondrial content in patients with T2D. We further postulate that the processes of mitochondrial biogenesis and autophagy will both be upregulated by the nitrate supplementation intervention.

General Approach: We will obtain a biopsy of the vastus lateralis. The sample will be obtained under local anesthesia by Dr. Richard Shields (co-investigator) with the assistance of Dr. Kenichi Ueda (licensed physician and co-investigator), using a modified Bergstrom needle with suction, which will yield ~200-250mg of tissue. A portion (~50-100mg) of the biopsy will be dissected and rapidly frozen in liquid nitrogen and stored at -80°C for later analysis of enzymatic activity and protein concentration. The major part of the biopsy (~150mg) will be chemically permeabilized with saponin and mitochondrial function will be assessed through isolation of the electron transport chain complexes *in situ*.[56]

Potential Results, Interpretation & Follow-Up Studies: We expect that an increase in bioavailable NO following nitrate supplementation will lead to an enhanced maximal ADP-initiated mitochondrial respiration (state 3). We also expect that there will be an increase in citrate synthase activity, suggestive of mitochondrial biogenesis following dietary nitrate supplementation. We further expect an increase in basal autophagy flux (i.e. increased LC3-I conversion into LC3-II, assessed via LC3-II/LC3-I ratio, and reduced p62 levels[64] as shown in Figure 6) in response to nitrate supplementation. Increases in both mitochondrial content and autophagy flux will mechanistically point to improved mitochondrial turnover, which is dependent on both mitochondrial biogenesis and degradation, as a central molecular outcome of nitrate supplementation in improving mitochondrial function in T2D. Examination of various protein and mRNA levels within the biopsied muscle tissue will also provide us with insight how NO and

associated metabolites may impact mitochondria function at different levels. Lastly, we have the experimental tools to conduct follow-up microarray RNA studies to identify muscle transcriptional signatures associated with reduced baseline mitochondrial function, as well as improved function as a result of nitrate supplementation in T2D. This non-biased approach will allow us to develop future mechanistic proposals to identify novel therapeutic targets for diabetic patients.

Aim 3 – Determine if exercise capacity and efficiency are improved following 8 weeks of dietary nitrate supplementation in patients with T2D and examine whether these improvements are associated with changes in oxygen delivery and utilization (as assessed in Aims 1 and 2).

Rationale and hypothesis: Several studies suggest that patients with T2D have an impaired exercise tolerance,[6-9] which is not associated with the degree of glycemic control.[9] Of clinical significance, a lower VO_2peak is associated with a higher rate of subsequent cardiovascular events in patients with T2D.[22] Recent evidence suggests that dietary nitrate can improve exercise efficiency and tolerance in young and older adults.[10-14] Therefore, we ***hypothesize*** that increasing bioavailable NO via nitrate supplementation will improve maximal exercise capacity and efficiency (e.g. reduce the oxygen cost for a given workload). In support of this hypothesis, our preliminary data suggests that 8 weeks of nitrate supplementation is effective in improving 6 minute walk test (6MWT) distance, a clinical measure of functional capacity, in patients with peripheral artery disease (**Figure 7**). Ultimately, exercise tolerance is dependent on the delivery and utilization of oxygen to the working muscles, both of which involve NO-mediated mechanisms.[30, 86] Therefore, we also ***hypothesize*** that the improvements in exercise efficiency will be associated with an increased blood flow response in contracting muscles (Aim 1) and/or mitochondrial function (Aim 2). Along these lines, our preliminary data in older adults (**Figure 8**) support the idea that a greater blood flow response to muscle contractions, a NO-mediated response,[51] is associated with enhanced exercise efficiency.

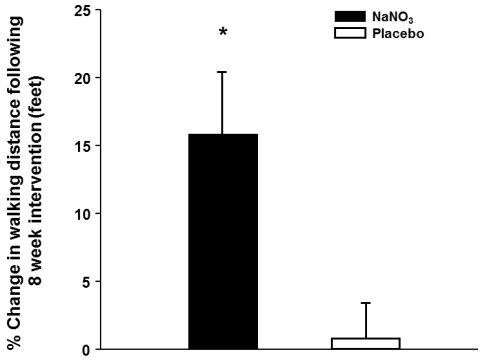

Figure 7. Preliminary data demonstrating a significant increase in walking distance during a 6-mintue walk test following 8 weeks of $NaNO_3$ in a clinical population with severe exercise intolerance. *P<0.05 vs Placebo

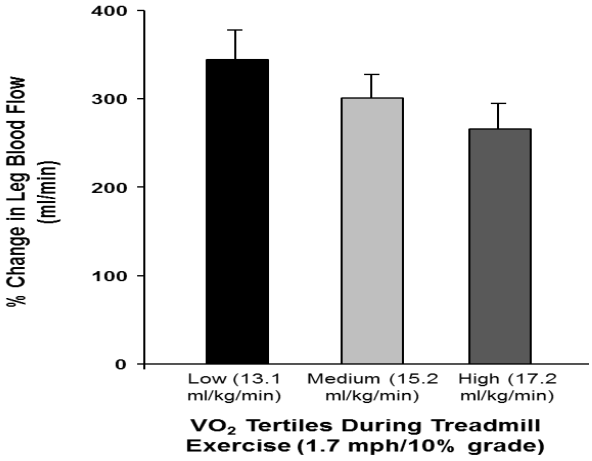

Figure 8. Preliminary data highlighting the relationship between contraction-induced blood flow and exercise efficiency in humans.

General Approach: Exercise efficiency and capacity will be assessed using a 12-lead ECG, symptom limited exercise test on a cycle ergometer with gas exchange measurements before and after 8 weeks of inorganic nitrate or placebo. The main comparisons will be changes in VO_2peak and exercise efficiency after 8 weeks of dietary nitrate supplementation. The associations between improvements in exercise capacity/efficiency and muscle blood flow will also be explored.

Potential Results, Interpretation & Follow-Up Studies: We expect that the dietary nitrate intervention will increase exercise capacity (VO_2peak) as well as exercise efficiency. Coupled with the protocols outlined in *Aims 1 and 2*, we will be able to determine if the potential improvements in exercise capacity and efficiency following nitrate supplementation are associated with changes in blood flow, mitochondrial function, or both. However, it is possible improvements in exercise capacity and efficiency may be observed without changes in local control of skeletal muscle blood flow or mitochondrial function. If this is the case, follow-up studies could be aimed at looking at potential changes in myocardial function following dietary nitrate supplementation.

Summary of Protocols: We have outlined a series of protocols to test our Aims and anticipate conducting ~40 sets of studies (either pre or post) per year to address our Aims. We have preliminary data that supports the questions we raise and demonstrates the technical ability needed to address them. Each protocol has clearly stated hypotheses with clear outcomes linked to each hypothesis and we have outlined key potential results, data interpretation and ideas about follow-up studies.

Technical Expertise of Investigative Team. The table below shows the team's experience with key elements of the proposal.

Technique/Approach	Investigators	# Performed	Years of Experience
Exercise blood flow studies	Darren Casey, PhD	>500	10
Arterial catheterization	Kenichi Ueda, MD	>500	10
Drugs to Study Human Physiology	Darren Casey, PhD	>500	8
Exercise Testing	Darren Casey, PhD Kenichi Ueda, MD	>500 >50	12 2
Muscle Biopsies	Richard Shields, PhD	>200	25
Mitochondrial content, enzyme activity and autophagy measures	Vitor Lira, PhD	~100	8
Mitochondrial function studies	E. Dale Abel, MD PhD	>500	>10

Potential Risks.
Risks from participation in these studies are not considered minimal.

1. Brachial artery catheterization. The brachial artery will be catheterized for ~4.5 hours in the proposed experiments. A 2-inch standard 20-gauge Teflon catheter will be used, and continuously flushed with low dose saline (2 units/ml, 3 cc/hr). Cannulation will be done using

ultrasound guidance under aseptic technique after infiltration of the area with 1-2 ml of lidocaine. Minor risk associated with this procedure would be the pain at the site of insertion, hematoma after withdrawal, and soreness over the site. These should be transient and resolve after 2-10 days. Major complications associated with brachial artery catheterization would include vasospasm, thromboembolism, or infection. Two large reviews (N = 4,000 patients) of brachial artery catheterization for 24 or more hours in older patients with cardiovascular disease have been published. These reviews report only two serious complications (one occlusive clot and one aneurysm) and indicate that brachial catheterization has less risk than radial artery catheterization. Dr. Kenichi Ueda has extensive experience (10+ years) with these procedures as part of his routine clinical duties. Dr. Casey has been involved with >300 arterial catheterization studies in the last 8 years without any major complications.

2. Forearm drug infusions. Because the doses used are very small and infused locally via the arterial catheter, there should be little or no effect on systemic hemodynamics (e.g., blood pressure and heart rate). Intra-arterially administration of adenosine and acetylcholine will cause an increase in blood flow (i.e. vasodilators) and thus carry minimal risk. Phenylephrine and dexmedetomidine cause a reduction in limb blood flow (i.e. vasoconstriction; < 25-50%) similar to that associated with the vasoconstriction seen when moving from the supine to upright posture. These compounds are well tolerated and we are unaware of any allergic or other untoward reactions when administered in this fashion. Dr. Casey and Dr. Ueda have extensive experience over the last several years with administration of vasoactive drugs via arterial catheter or IV.

3. Lidocaine: 2% lidocaine will be used to numb the area on the arm for insertion of the brachial artery needle. Likely and expected effects include mild, temporary burning at the site of injection and a sensation of coolness or numbness at the site of the injection. Unlikely, but possible reactions include lightheadedness, dizziness, ringing in the ears, nervousness, and blurred vision. Individuals known to be allergic to anesthetics like lidocaine (e.g., novocaine- used for tooth fillings) should notify the investigator before participating and will not undergo the forearm blood flow test.

4. **Venipuncture/IV catheters.** Risks of venipuncture or placing IV catheters in the arm include bruise or clot formation and infection.

5. Forearm exercise. There are no long term risks associated with rhythmic forearm handgrip contractions. Rhythmic handgrip exercise may lead to minor fatigue in the hand and/or forearm and soreness for a day or two.

6. Doppler ultrasound. There is no risk associated with Doppler ultrasound measurements.

7. Applanation tonometry. There is no risk associated with arterial tonometry measurements.

8. Maximal exercise testing. This procedure will be used to determine each subjects exercise capacity. There is minor discomfort associated with maximal exercise testing including temporary fatigue, shortness of breath, and muscle soreness. These sensations resolve within minutes after the test or exercise session is completed. In healthy older subjects screened with a questionnaire who are normotensive, non-smokers, not obese, and non-diabetic the risk of a catastrophic event during graded exercise tests for screening purposes are thought to be less than 1 in 10,000 (Squires, personal communication). According to the American College of Sports Medicine the risk of a cardiovascular (CV) event during maximal exercise testing increases in individuals with CV disease and/or risk factors for CV disease. However, safe testing with appropriate monitoring (blood pressure, ECG, and physician supervised) is still recommended in this patient population.

The exercise tests performed in this study will be conducted by well-trained personnel and supervised by medical personnel (Dr. Ueda) who are familiar with the use of graded exercise testing. Moreover heart rate and rhythm will be monitored via 12-lead ECG and blood pressure measured throughout the test as well as during a recovery period following termination of exercise. After maximal exercise, serious risks during submaximal exercise are extremely small, except for temporary fatigue and muscle soreness.

9. Muscle biopsies: Biopsies will be performed under sterile conditions to minimize the risk of infection. However, there is a risk of bleeding, bruising, pain and/or swelling after the muscle sample is taken. To further minimize such risks subjects will be instructed to leave the bandage on the biopsy site for 24 hours and to not allow the area to become wet for 48 hours after the procedure.

Summary of risks: The proposed study will include both invasive and non-invasive techniques. Regarding invasive approaches Drs. Casey and Ueda have extensive experience conducting brachial artery catheterization studies and Dr. Shields has extensive experience in performing muscle biopsies. Along these lines, we are confident that at the very least, the standard care of practice will be met before, during and after artery catheterization, drug infusions, and muscle biopsies. Additionally, with regard to the non-invasive approaches of the proposed study, the team has a long history of conducting these types of studies in conscious humans, and we have standard operating procedures in our laboratory to minimize the risks and to ensure that these studies can be done safely.

Protection.
Each subject will give written informed consent after all questions have been answered by an investigator or study coordinator. Confidentiality of data will be assured by coding of subject identities, and that coding will be known only to the investigators. No identified individual data will be presented or published, rather group mean data will serve as the basis for comparisons. Participation in these studies is safe, with minimal short-term (days) complications and minimal anticipated long-term risks. Procedures used in this study are performed regularly in the laboratory or clinical setting by the investigators.

Inclusion of Women and Minorities.
The proposed studies will include older (40-70 yr) men and women of all races and ethnic backgrounds recruited from the University of Iowa and from persons in Iowa City, Iowa and surrounding communities. We will strive to enroll an equal number of males and females and recruit minority participants at least to the extent representative of the participation of minority populations in Iowa City and Johnson County.

Inclusion of Children
The proposed studies will only include older (40-70 yr) men and women. Therefore, children will be excluded from the proposed studies.

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