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Jesse C. Craig[1], Ryan M. Broxterman[1,2], Joshua R. Smith[1], Jason D. Allen[3], and Thomas J. Barstow.[1]

[1]Department of Kinesiology, Kansas State University, Manhattan, KS, USA

[2]Department of Anatomy and Physiology, Kansas State University, Manhattan, KS, USA

[3]Department of Medicine, Duke University Medical Center, Durham, NC, USA and Department of Kinesiology, University of Virginia, Charlottesville, VA, USA

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Corresponding author:

Jesse C. Craig

Department of Kinesiology

Kansas State University

Manhattan, KS, 66506

Tel: 785-532-4476

Fax: 785-532-6486

E-mail: jccraig@k-state.edu

Abstract

Dietary nitrate supplementation has positive effects on mitochondrial and muscle contractile efficiency during large muscle mass exercise in humans, and on skeletal muscle blood flow ($\dot{Q}$) in rats. However, concurrent measurement of these effects has not been performed in humans. Therefore, we assessed the influence of nitrate supplementation on $\dot{Q}$ and muscle oxygenation characteristics during moderate (40%peak) and severe (85%peak) intensity handgrip exercise in a randomized, double-blind, crossover-design. Nine healthy men (age: 25±2 yrs) completed four constant-power exercise tests (two per intensity) randomly assigned to condition (nitrate-rich (Nitrate) or nitrate-poor (Placebo) beetroot supplementation) and intensity (40%peak or 85%peak). Resting mean arterial pressure was lower after Nitrate compared to Placebo (84±4 vs 89±4 mmHg; $p<0.01$). All subjects were able to sustain 10 min of exercise at 40%peak in both conditions. Nitrate had no effect on exercise tolerance during 85%peak (Nitrate: 358±29, Placebo: 341±34 s; $p=0.3$). Brachial artery $\dot{Q}$ was not different after Nitrate at rest or any time during exercise. Deoxygenated-[hemoglobin+myoglobin] was not different for 40%peak ($p>0.05$), but was elevated throughout 85%peak ($p<0.05$) after Nitrate. The metabolic cost ($\dot{V}O_2$) was not different at end exercise, however, the $\dot{V}O_2$ primary amplitude at the onset of exercise was elevated after Nitrate for the 85%peak work rate (96±20 vs 72±12 ml/min; $p<0.05$) and had a faster response. These findings suggest that an acute dose of Nitrate reduces resting blood pressure and speeds $\dot{V}O_2$ kinetics in young adults, but does not augment $\dot{Q}$ or reduce steady-state $\dot{V}O_2$ during small muscle mass handgrip exercise.

Keywords: Beetroot juice, NIRS, Kinetics, Dynamic exercise

New and Noteworthy

We show that acute dietary nitrate supplementation via beetroot juice increases the amplitude and speed of local muscle $\dot{V}O_2$ on-kinetics parameters during severe- but not moderate-intensity handgrip exercise. These changes were found in the absence of an increased blood flow response, suggesting the increased $\dot{V}O_2$ was attained via improvements in fractional O_2 extraction and/or spatial distribution of blood flow within the exercising muscle.

Introduction

Dietary nitrate supplementation is well documented to have positive effects during large muscle mass exercise in humans (4, 33, 38, 48, 50). These effects include lowering oxygen consumption ($\dot{V}O_2$) (4, 32, 38, 42), speeding $\dot{V}O_2$ kinetics (3, 6, 31, 32) and reducing the ATP cost of work (2, 26) during submaximal exercise, which may translate to the enhanced exercise tolerance found during severe intensity exercise (6, 31, 37, 50). The precise mechanism(s) for these effects still remains uncertain, but they are facilitated through the reduction of the dietary nitrate to nitrite by commensal bacteria in the mouth (40). Once absorbed into the circulatory system, nitrite is readily converted to nitric oxide (NO) in hypoxic (16, 47) and acidic (41) environments, which are expected to be present at the exercising muscle.

Nitric oxide is a potent vasodilator (20, 22, 45); as such it had been proposed that nitrate supplementation may augment blood flow ($\dot{Q}$) to active muscle. This was first experimentally investigated in rats during submaximal treadmill running (23, 24). These authors found that nitrate supplementation resulted in an increased $\dot{Q}$ to the hindlimb, particularly to muscles composed of greater percentages of type II fibers. These findings demonstrate that nitrate may change the regulation of $\dot{Q}$ relative to $\dot{V}O_2$, as these two variables generally increase in

66 proportion to one another across a range of exercise intensities (1, 43). Recently the effect of
67 nitrate on $\dot{Q}$ was investigated in human subjects (5, 12, 34), but no change in brachial artery
68 blood flow ($\dot{Q}_{BA}$) was found in healthy, young men and women during light-to-moderate
69 intensity handgrip exercise. These previous studies might not have recruited type II fibers in the
70 younger subjects due to lower intensity exercise, potentially missing the preferential effects of
71 dietary nitrate on higher order fiber types (for review see (30)). It should be noted, two of the
72 aforementioned studies did find improvements in compromised populations (i.e., older adults in
73 hypoxia (12) and 'noncompensators' (5)).
74 Importantly, these previous studies in humans using nitrate (12, 34), provided no measure
75 of $\dot{V}O_2$or fractional O_2 extraction (which can be estimated noninvasively via deoxygenated-
76 [hemoglobin + myoglobin] (deoxy-[Hb + Mb]) and used to estimate $\dot{V}O_2$) (7, 18, 19, 35).
77 Moreover, the measurements were made after fixed durations of moderate intensity submaximal
78 exercise and during the steady state, leaving the effects of nitrate on local muscle $\dot{V}O_2$ during the
79 exercise onset transient unknown. Since faster $\dot{V}O_2$ kinetics are associated with a reduction in the
80 O_2 deficit (and thus accumulation of fatigue inducing metabolites), these findings carry important
81 implications for patient populations, such as chronic heart failure (CHF), as accumulating
82 evidence suggests nitrate supplementation may be effective for enhancing quality of life through
83 improvements in exercise and/or daily activity tolerance (21, 25, 51).
84 Therefore, the purpose of this investigation was to resolve whether acute supplementation
85 of nitrate preferentially provided positive effects in small muscle mass exercise during severe
86 intensity exercise, where type II fibers would be recruited and greater hypoxic and acidic muscle
87 environment exists. Specifically, we tested the hypotheses that with nitrate supplementation
88 compared to placebo: 1) $\dot{Q}_{BA}$ would be significantly elevated during severe, but not moderate

intensity exercise; 2) $\dot{V}O_2$ would be elevated during severe intensity exercise and display faster kinetics; and 3) tolerance of exercise (T_{lim}) would be increased during severe intensity exercise.

Materials and Methods

Ten healthy, recreationally active men volunteered for the investigation (mean ± SD: age: 25 ± 2 yrs; height: 178 ± 4 cm; body mass: 80 ± 10 kg; BMI: 25 ± 3 kg/m^2). All experimental procedures in the present study were approved by the Institutional Review Board at Kansas State University and conformed to the standards set forth by the *Declaration of Helsinki*. Prior to participation in the study, all subjects were informed of the protocol, any possible health risks, as well as the probable benefits of the study. All subjects provided written informed consent to participate and completed a medical health history questionnaire to ensure absence of any known cardiovascular or metabolic diseases which would preclude them from the study.

Experimental Protocol

All testing sessions were performed on a custom-built, two-handed handgrip ergometer previously described by our laboratory (7, 8). Briefly, the subjects were seated in an upright position at arm's length from the ergometer with the hands pronated at heart level and directly in front of their torso. All sessions were performed utilizing a 50% duty-cycle (1.5 s contraction, 1.5 s relaxation) and fixed 4 cm linear displacement that was maintained via audio cues. All subjects were familiarized with the exercise, audio cues, and duty-cycle prior to the first testing session. During the first visit, subjects performed an incremental test for the determination of peak power (P_{peak}) starting at 1 Watt (W) and increasing at a rate of 0.5 W·min^{-1}. The test was performed until volitional exhaustion or after three consecutive contraction cycles in which the

subject was unable to maintain the correct tempo or complete full contractions. P_{peak} was recorded as the highest power obtained in which the subjects completed at least 30 s of the stage.

The four subsequent visits were randomly assigned to 40 or 85 %P_{peak} (two tests per intensity) and supplemental condition (see Supplementation below; Figure 1). The four constant power tests were performed for 10 min or until exhaustion for 40 and 85 %P_{peak}, respectively. The coefficient of variation for tolerance of exercise (T_{lim}) at 85 %P_{peak} intensity in our laboratory is ~7% (8). All testing sessions were separated by 48 - 72 h and subjects were asked to abstain from vigorous activity, food, and caffeine prior to testing for 12, 3, and 2 h, respectively. Upon arrival to the laboratory, the subjects sat quietly for 15 min, after which resting blood pressure measurements and subsequent plasma samples were obtained (See Figure 1). All exercise tests were performed at approximately the same time of day (± 1.5 h for each subject) between 1000 and 1500 hours.

Supplementation

The exercise testing sessions were randomly assigned to nitrate or placebo beetroot supplementation conditions (one of each per intensity; i.e., nitrate + 40 %P_{peak} and placebo + 40 %P_{peak}), creating a randomized, double-blind, crossover study design. In each condition, the subjects consumed beetroot concentrate (2 x 70 ml providing ~13 mmol nitrate) or nitrate-depleted beetroot concentrate placebo (2 x 70 ml providing ~0.006 mmol nitrate; both Beet It Sport, James White Drinks, Ipswich, UK). Subjects consumed the shots on their own outside of the laboratory ~2.5 h before testing began to allow for maximal expression of plasma nitrite concentrations ([nitrite]) (49, 50) (See Figure 1). This dose of nitrate was chosen because it was shown to increase T_{lim} with no greater effects seen at higher doses (50). During the study,

subjects were asked to abstain from using mouthwash (29) and toothpaste or chewing gum that contained triclosan, as these products serve to reduce the oral bacteria needed to facilitate the conversion of nitrate to nitrite. Each exercise testing session was separated from the others by at least 48 h to allow plasma [nitrite] adequate time to return to pre-supplementation concentrations (50). Subjects were asked to maintain their normal diet with the exception of limiting foods high in nitrate, such as spinach and arugula (39). No subjects reported taking any multivitamins or anti-oxidant supplements. All subjects self-reported compliance with the supplemental protocol. No subjects reported gastrointestinal discomfort; however, when subjects reported typical symptoms (i.e., beeturia or red stools) they were assured this was a typical side effect of the nitrate supplementation.

Measurements

Venous blood samples (5-6 ml) were separated into 1.5 ml Eppendorf tubes containing 5 µl heparin (concentration 1000U/ml) and centrifuged at 3250 rpm at 4 °C for 5 min within 1 min of withdrawal. Plasma samples were then pipetted into separate Eppendorf tubes, flash frozen in liquid nitrogen, and stored at -80 °C until later analysis.

The measurements of plasma nitrate and nitrite were performed within 30 min of thawing via chemiluminescence with a NO analyzer (NOA 280i, Sievers Instruments, Boulder, CO, USA). In order to obtain plasma nitrite levels and to avoid potential reduction of nitrate, potassium iodide in acetic acid was used as a reductant. This reductant has the ability to reduce nitrite to NO but is incapable of reducing higher oxides of nitrogen (i.e., nitrate), thus increasing the specificity for nitrite. Plasma nitrate concentrations were obtained using the same apparatus with the stronger reductant vanadium chloride in hydrochloric acid at a temperature of 95 °C.

158 This stronger reductant reduces the sum of all nitrogen oxides with an oxidation state of +2 or
159 higher, which is predominately nitrate (μM), but also includes both nitrite (nM) and nitrosothiols
160 (nM).
161 Resting blood pressure was measured in the left arm using an automated patient monitor
162 (S/5 Light Monitor type F-LM1-03, Datex-Ohmeda General Electric, Finland) which makes use
163 of the oscillometric technique. To increase accuracy, this machine utilizes a 3-lead ECG to
164 monitor heart rate. This measurement was taken in triplicate and a mean value was obtained.
165 Exercising blood pressure was taken from the left ankle using the same patient monitor while the
166 subject was seated at the handgrip ergometer. During the measurement, subjects were asked to
167 remain still and allow their leg to relax. A correction factor (pressure = measured pressure –
168 (distance between the heart and ankle in meters x 76 mm Hg) was used to adjust for the
169 increased hydrostatic pressure present between the ankle and heart (27). Pilot work performed in
170 our lab validated the correction factor with measurements taken from the ankle and arm at heart
171 level. This pilot work also revealed that the increase in blood pressure during 85 $\%P_{peak}$ handgrip
172 exercise exceeded the capabilities of the equipment to accurately measure ankle pressure so
173 pressure was only obtained for the 40 $\%P_{peak}$ intensity.
174 The raw blood velocity profiles were measured in the right brachial artery using Doppler
175 ultrasound (Vivid 3, GE Medical Systems, Milwaukee, WI, USA) operating in pulse wave mode
176 at a Doppler frequency of 4.0 MHz with a phased linear array transducer probe operating at an
177 imaging frequency of 6.7 MHz, and were stored for *post-hoc* analysis. For all testing sessions the
178 Doppler gate was set to the full width of the brachial artery to ensure complete insonation and all
179 Doppler velocity measurements were corrected for the angle of insonation, which was adjusted
180 to be less than 60°. Measurements were made at least 3 cm above the antecubital fossa to avoid

bifurcation of the brachial artery. Brachial artery diameters were measured in the transverse axis using two-dimensional sonography.

Muscle and microvascular oxygenation status were measured noninvasively using a frequency-domain multi-distance near infrared spectroscopy (NIRS) system (OxiplexTS, ISS, Champaign, IL, USA) positioned over the belly of the left *flexor digitorum superficialis* (FDS). Details of this technique have been described previously (7, 11). Briefly, this device consists of one detector fiber bundle and eight light-emitting diodes (LED) operating at wavelengths of 690 and 830 nm (four LEDs per wavelength). The LED-detector fiber bundle separation distances are 2.0, 2.5, 3.0, and 3.5 cm. This NIRS device measures and incorporates the reduced scattering coefficient (μ_s'), measured dynamically, to provide absolute concentrations (μM) for deoxy-[Hb + Mb] and total-[Hb + Mb]. The NIRS probe was calibrated prior to each test according to the manufacturer's specifications. The belly of the FDS of the left arm was identified using palpation and EMG. The NIRS probe was secured along the belly of the FDS and was wrapped with an elastic bandage to prevent shifting of the probe. The placement of the NIRS probe was marked with permanent ink for reproducible positioning throughout the study. The NIRS data were collected at 50 Hz and stored for *post-hoc* analysis.

The $\dot{V}O_2$ (ml $O_2 \cdot min^{-1}$) of the FDS was calculated for each minute of exercise using the technique described previously (7), which integrates deoxy-[Hb + Mb] and $\dot{Q}_{BA}$. It was assumed that the deoxy-[Hb + Mb] signal reflects exclusively deoxy-[Hb] [we acknowledge that the signal contains deoxy-[Mb] as well (17)] and that the entire signal arises only from the muscle (i.e., not from any interposing adipose or skin tissue). With these assumptions the deoxy-[Hb] may be converted into an estimated $\dot{V}O_2$. The deoxy-[Hb] values are in units of μmole heme/*l* tissue, where the tissue is assumed to be muscle. These deoxy-[Hb] units can be converted into

μmole heme/*l* blood using the conversion 1.36% capillary blood volume/muscle volume [derived from 400 cap/mm^2, 28.3 μm^2 CSA, and a coefficient of 1.2 correcting for tortuosity and branching of the capillaries (44)]. These units can then be converted into mole O_2/*l* blood assuming 1 mole O_2/mole heme and further to *l* O_2/*l* blood using the conversion 22.4 *l* O_2/mole O_2. $\dot{V}O_2$ values in *l* O_2/min may then be obtained by multiplying this value by the measured $\dot{Q}_{BA}$ values. This calculation was performed with the understanding that $\dot{Q}_{BA}$ likely overestimates $\dot{Q}$ through the capillaries under the NIRS probe. However, because the same calculation (and subsequent assumptions) was used across subjects and the primary comparison was within subjects, the error associated with this assumption was minimized. Further, these assumptions were held constant across both supplemental conditions.

Data analysis

Mean blood velocity ($\dot{V}_{mean}$; cm·s^{-1}) was defined as the time-averaged mean velocity over each 3 s contraction cycle. $\dot{Q}_{BA}$ (ml·min^{-1}) was calculated using the product of $\dot{V}_{mean}$ and vessel cross-sectional area (CSA = πr^2). CSA (cm^2) was calculated each minute of exercise using brachial artery diameters measured at the beginning of each minute. The $\dot{Q}_{BA}$ data were analyzed using three consecutive contraction cycles (i.e., 9 s) for rest and at the end of each minute of exercise. The NIRS data were first multiplied by 4 to convert the values from hemoglobin equivalents back to total heme units (15) and were subsequently analyzed using 1 s mean values that were converted to 30 s mean bins for resting values and 9 s time-binned mean values at the end of each minute of exercise and at exhaustion. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured at least three times at rest and once every 2 min during

exercise and were then used to calculate MAP. Vascular conductance (VC) ($ml \cdot min^{-1} \cdot (100\ mmHg)^{-1}$) was calculated using the quotient of $\dot{Q}_{BA}$/MAP, multiplied by 100.

Kinetics analyses were conducted for the $\dot{V}O_2$ data using 6 s time-binned mean values over the initial 120 s of exercise and 9 s time-binned mean values at 180 and 240 s with a mono-exponential model:

$$y(t) = y(b) + A(1 - e^{-(t - TD)/\tau})$$

where $y(t)$ is the $\dot{V}O_2$ at any point in time, $y(b)$ is the baseline before the onset of exercise, A is the primary amplitude of the response, TD is the time delay proceeding the increase in, and τ is the time constant. The rate constant (RC) was calculated as A divided by τ (giving ml/min/s) to give an indication of the acceleration of the response.

Statistical analysis

All curve fitting and statistical analyses were performed using a commercially available software package (SigmaPlot 12.5, Systat Software, San Jose, CA, USA). Differences in resting values and T_{lim} were analyzed using Student's paired t-tests. Differences within condition (i.e., 40% nitrate and 85% nitrate) for resting plasma [NO_x] and MAP were compared and if no differences were found, these values were averaged to represent the mean resting value for that condition. Exercising values (i.e., $\dot{Q}_{BA}$, deoxy-[Hb + Mb], total-[Hb + Mb], and $\dot{V}O_2$) were analyzed using two-way ANOVAs with repeated measures (supplement x time) using Tukey's *post hoc* tests when main effects were detected. $\dot{V}O_2$ kinetics parameters were analyzed using two-way ANOVA with repeated measures (supplement x work rate) using Tukey's *post hoc* tests when main effects were detected. Differences were considered significant when $p < 0.05$. Data are presented as means ± standard error unless otherwise noted.

Results

Ten subjects completed the protocol. One subject was determined to be an outlier based on their 85 $\%P_{peak}$ T_{lim} change score (-285 s) being more than 3 SD outside the group mean change score (15 ± 84 s). This subject was removed from all data analyses.

Plasma [nitrate] & [nitrite] and resting blood pressure

Plasma [nitrate] and [nitrite] was measured in six participants (see *Limitations* section below for explanation). Plasma [nitrate] was elevated 26-fold over placebo after acute nitrate supplementation (784 ± 32 vs 29 ± 2 μM, $p < 0.001$). All subjects demonstrated elevated plasma [nitrite] after acute nitrate supplementation (456 ± 60 vs 68 ± 7 nM, $p < 0.001$, Fig. 2) resulting in a 5.7-fold increase over placebo. Resting blood pressure values are presented in Table 1. Acute nitrate supplementation was associated with a lowering of SBP, DBP, and MAP by 7%, 4%, and 6%, respectively (all $p < 0.05$) compared to placebo.

40 $\%P_{peak}$ exercise

The mean power for 40 $\%P_{peak}$ was 2.2 ± 0.1 W. All subjects were able to sustain 10 min of exercise at 40 $\%P_{peak}$ in both conditions. $\dot{Q}_{BA}$ increased rapidly from exercise onset in both conditions before approaching a steady-state of approximately 260 $ml \cdot min^{-1}$ by 240 s. $\dot{Q}_{BA}$ was not different after nitrate supplementation at rest or at any time during exercise compared to placebo (Fig. 3). MAP was measured during exercise in eight of nine subjects. There was no main effect of nitrate on MAP during exercise compared to placebo ($p = 0.11$, Fig. 4), although MAP was 4 mmHg lower on average throughout exercise before reaching the peak values 90 ± 4

and 98 ± 5 mmHg (p = 0.02) for nitrate and placebo, respectively. There was no effect of nitrate on VC (p = 0.14, Fig. 4); both groups increased to end exercise values of 314 ± 58 and 279 ± 28 ml/min/100 mmHg (p = 0.08) for nitrate and placebo, respectively.

Deoxy-[Hb + Mb] increased following exercise onset in both conditions, with no differences between conditions. End exercise deoxy-[Hb + Mb] was not different between nitrate and placebo (154 ± 15 vs 156 ± 19 μM, p = 0.83, Fig. 5). Total-[Hb + Mb] was not different after nitrate supplementation at any min during exercise or at the end of exercise compared to placebo (408 ± 15 vs 402 ± 25 μM, p = 0.76, Fig. 5). $\dot{V}O_2$ was not different at any min during exercise or at the end of exercise (73.1 ± 16.7 vs 75.8 ± 18.0 ml/min, p = 0.68, Fig. 6). The results of the $\dot{V}O_2$ kinetics analysis are presented in Table 2 (n = 7).

85 %P_{peak} exercise

The mean power for 85 %P_{peak} was 4.7 ± 0.2 W. Nitrate had no effect on T_{lim} compared to placebo (358 ± 29 vs 341 ± 34 s, p = 0.3, Fig. 7). $\dot{Q}_{BA}$ was not different at rest or any time during exercise after nitrate supplementation. $\dot{Q}_{BA}$ increased at exercise onset and attained end exercise values of 368 ± 42 and 353 ± 46 ml/min (p = 0.56, Fig. 3), for nitrate and placebo, respectively.

Deoxy-[Hb + Mb] was not different at rest and increased at exercise onset in both conditions, with nitrate elevated over placebo for time points preceding end exercise (60 – 180 s, p < 0.05), but not 240 s (p = 0.08). At T_{lim}, nitrate and placebo were different (203 ± 26 vs 180 ± 19 μM, p = 0.03, Fig. 5). Total-[Hb + Mb] was not different after nitrate supplementation, both conditions showed a progressive increase toward the end exercise values (447 ± 30 vs 440 ± 31 μM, p = 0.65). $\dot{V}O_2$ increased 897 ± 183% and 838 ± 191% (p = 0.83) from rest to T_{lim} for nitrate

and placebo, respectively. There was no difference for end exercise $\dot{V}O_2$ after nitrate supplementation (112 ± 12 vs 107 ± 14 ml/min, p = 0.62, Fig. 6). The results of the $\dot{V}O_2$ kinetics analysis are presented in Table 2 (n = 7). Both supplemental conditions had significantly higher primary amplitudes during 85% P_{peak} compared to 40% P_{peak} ($p < 0.05$). Nitrate supplementation also increased the primary amplitude within 85% P_{peak} ($p = 0.02$) and reduced the time constant (τ; $p = 0.04$) compared to placebo.

Discussion

The present study investigated the effects of acute nitrate supplementation on conduit artery $\dot{Q}$ concurrently with local muscle microvascular oxygenation characteristics during moderate and severe intensity handgrip exercise. The acute dosage utilized (~13 mmol nitrate), elevated plasma [nitrite] more than 5-fold higher than that seen with placebo and was associated with reductions in blood pressure at rest of 4-8%. Contrary to our first hypothesis, nitrate had no effect on $\dot{Q}_{BA}$ at rest or any time point during moderate or severe intensity handgrip exercise compared to placebo. The primary novel finding of the present study, in agreement with our second hypothesis, was that the $\dot{V}O_2$ primary amplitude was elevated and the kinetics were faster after nitrate during severe intensity handgrip exercise consequent to an increased O_2 extraction (deoxy-[Hb + Mb]) . Additionally, nitrate had no effect on T_{lim} when exercise was performed in the severe intensity domain.

Effect on control of blood flow

Ferguson and colleagues (23, 24) discovered that nitrate supplementation increased bulk hindlimb $\dot{Q}$ in rats with the largest effect in muscles composed of a high percentage of type IIb

and IIx fibers (23, 24). To date, the previous studies (5, 12, 34) and the present investigation that directly measured $\dot{Q}$ in young healthy humans during small muscle mass (handgrip) exercise, have been unable to replicate the findings of Ferguson *et al*. (23, 24) or Cosby *et al*. (14). The work of Kim and colleagues (34) had young healthy subjects perform rhythmic exercise under both nitrate and placebo conditions; however the work done was performed at fairly low work rates. The greatest $\dot{Q}_{BA}$ achieved in the work of Kim et al. (34) was approximately 200 ml/min for both supplementations, which was lower than the $\dot{Q}_{BA}$ measured in the present investigation at 40 $\%P_{peak}$ (~260 ml/min). If dietary nitrate does in fact have preferential effects in high order fiber types, it is likely that Kim *et al*. (34) and the lower work rate in the present study did not recruit said fibers.

The other two studies (5, 12) and the present investigation performed higher intensity exercise that increased the likelihood of recruiting higher order fibers. However, in agreement with the lower intensity data, nitrate supplementation had no effect on the steady state $\dot{Q}_{BA}$ in healthy young subjects. The present investigation advanced these previous studies by measuring the dynamic response during the onset of exercise. While there was no difference in the speed of the $\dot{Q}_{BA}$ adjustment to exercise, there was evidence of improved O_2 delivery within the exercising muscle (see *Effect on tissue oxygenation and $\dot{V}O_2$ below*). Casey and colleagues (12) attempted to maximize the stimulus for nitrite conversion to NO (and thus maximize the potential augmentation of $\dot{Q}_{BA}$) by putting their subjects in hypoxia, but there was still no difference between nitrate and placebo. The study by Bentley and colleagues (5) used a hydrostatic challenge to alter O_2 delivery and found no differences in the absolute $\dot{Q}_{BA}$ following nitrate supplementation. These authors did find there was less attenuation of $\dot{Q}_{BA}$ induced by the hydrostatic challenge following nitrate, which the authors attributed to an increased

compensatory vasodilation (5). The supine exercise model used in these aforementioned studies (5, 12, 34) differed from the present investigation in that our subjects were seated upright with the arms at heart level. It has been shown that the seated posture increases muscle sympathetic activity and reduced central venous pressure compared to the supine posture (9). Since the present findings are largely in agreement with these previous studies (5, 12, 34) and handgrip exercise is not limited by cardiac output, these postural differences were likely inconsequential.

Nevertheless, a recent study found that acute nitrate supplementation increased peak cardiac output and $\dot{V}O_2$ in CHF patients with preserved ejection fraction during a supine peak incremental exercise test (51). This study was not designed to resolve the spatial distribution of the ~10% increase in cardiac output. If nitrate does favorably affect VC and $\dot{Q}$ to type II fibers, as suggested by Ferguson and colleagues (24), the increased reliance on type II fibers with CHF, and other diseases (28, 46) supports the notion that nitrate supplementation may be more effective in these populations with O_2 delivery challenges. The discovery that nitrate can increase $\dot{Q}_{BA}$ in older adults in hypoxia (12) further bolsters this hypothesis.

Effect on tissue oxygenation and $\dot{V}O_2$

Larsen and colleagues (38) were the first to show that a dietary nitrate salt supplement could reduce the $\dot{V}O_2$ associated with a given work rate. Subsequent studies utilizing beetroot supplementation have yielded mixed results across a variety of exercise modalities, with some showing ~3-5% reductions in $\dot{V}O_2$ (2, 4, 37, 42, 48, 50), and others no change (6, 13, 31, 32) after supplementation. NIRS-derived variables measured concurrently with $\dot{V}O_2$ paralleled the change in $\dot{V}O_2$ when it occurred (4, 6).

Given the above, attempting to interpret the present findings in the context of whole body exercise is difficult. In the present investigation, deoxy-[Hb + Mb] was elevated after nitrate supplementation throughout severe-, but not moderate-, intensity exercise. Moreover, total-[Hb + Mb] was not impacted by nitrate supplementation during both exercise intensities. Changes in total-[Hb + Mb] from rest to exercise are thought to reflect the change in microvascular hematocrit (17). To the best of our knowledge, the current study is the first to observe an increased primary amplitude, exercising level, and T_{lim} value for deoxy-[Hb + Mb] after nitrate supplementation. It should be noted that Breese et al. (6) reported a higher value on beet root juice across the transition from moderate to severe exercise due to faster kinetics, but no differences in the amplitude or T_{lim} were observed (6). Increased deoxy-[Hb + Mb] relative to unchanged total-[Hb + Mb] (and $\dot{Q}_{BA}$), suggests an increased fractional O_2 extraction. The 50% duty-cycle used in the present investigation has been shown to mechanically constrain $\dot{Q}_{BA}$ and $\dot{V}O_2$ during severe intensity handgrip exercise (7), such that the present changes should be viewed as positive and suggest improvements in the microvascular distribution of O_2 rather than a decrease in efficiency. Nitrate may facilitate the delivery of O_2 to regions that were otherwise under perfused in this exercise model. This improved O_2 extraction was manifest in the kinetic response (see discussion below); however, there was no difference in $\dot{V}O_2$ at the end of exercise after nitrate supplementation in the present investigation. This implies that the efficiency of the work was neither positively nor negatively impacted and the improvements in fractional O_2 extraction were likely obscured by the mechanical limitations of the exercise. Future work could usefully attempt to elucidate if there is a 'threshold' type effect of the duty cycle used for the exercise (i.e., employing 20, 30, 40% duty cycles in the severe intensity domain).

Effect on $\dot{V}O_2$ kinetics parameters and tolerance to exercise

No differences in the end exercise amplitude of local muscle $\dot{V}O_2$ were found in the present study, but kinetics analyses revealed that the initial amplitude of $\dot{V}O_2$ was increased during exercise at 85% P_{peak} after nitrate supplementation. Nitrate supplementation resulted in a faster τ and a substantially greater rate constant (amplitude/τ; ~73% increase). These findings are in agreement with the speeding of pulmonary $\dot{V}O_2$ kinetics shown during whole body exercise in instances of compromised O_2 delivery and/or recruitment of higher order Type II muscle fibers (3, 6, 31, 32) and the equivalent microvascular PO_2 response in rats (23). The present investigation is the first to show significant differences in kinetics parameters after an acute dosage of nitrate, where other studies used chronic supplementation to see effects (3, 6, 31). However, these improvements in $\dot{V}O_2$ amplitude and speed of adjustment did not lead to improved T_{lim} in the present investigation. Previous work has found that speeding $\dot{V}O_2$ kinetics during whole body exercise does not always result in improvements to T_{lim} (10, 36), indicating that the relationship between these two variables is not a simple relationship. Indeed, interactions between $\dot{V}O_2$ kinetics and other physiological parameters are likely requisite to see improvements in exercise tolerance. Had there been a summation of the improved kinetics across multiple transitions (such as that seen during daily activity), a greater sparing of the O_2 deficit could result in an accumulated improvement.

Limitations

We acknowledge that the method used to estimate $\dot{V}O_2$ herein utilizes several assumptions (7) and likely overestimated the $\dot{V}O_2$. We contend that these assumptions, held constant throughout, should not obscure an impact of nitrate on $\dot{V}O_2$. Additionally, our sample

size was small and as such could have resulted in the present investigation not being sufficiently powered to detect differences in some variables (e.g., exercising MAP and VC). Finally, we did not measure plasma [nitrite] in all nine subjects (access to the NOA was precluded during later data collection); however, the three subjects without this measurement exhibited similar differences in blood pressure to the six with plasma [nitrite] measurements. It should be noted that no pre-dose blood pressure measurements were made in the present investigation. However, each subject served as their own control and thus had two separate days of blood pressure measurements for each condition (i.e., nitrate and placebo), increasing our confidence that nitrate influenced blood pressure herein.

Conclusions

The present study reaffirmed previous findings that an acute dose of nitrate is associated with lower SBP, DBP, and MAP in healthy, young men. The acute dose was also an effective method to increase and speed the local muscle $\dot{V}O_2$ on-kinetics parameters during severe intensity handgrip exercise, primarily through an increased fractional O_2 extraction rather than increased blood flow. However, the ergogenic effects associated with nitrate supplementation (i.e., improved tolerance to exercise) during large muscle mass exercise were not seen when the exercise was performed in small muscle mass handgrip exercise. These findings warrant future studies investigating the effects of nitrate supplementation during the dynamic adjustment at the onset of exercise in populations at risk of O_2 delivery impairment and reduced NO bioavailability.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Table 1. Resting Blood Pressure After Acute Dietary Nitrate Supplementation

	Placebo	Nitrate
SBP (mmHg)	130 ± 4	121 ± 4 †
DBP (mmHg)	69 ± 4	66 ± 5 *****
MAP (mmHg)	89 ± 4	84 ± 4 *****

SBP, DBP, and MAP denote systolic blood pressure, diastolic blood pressure, and mean arterial pressure, respectively. Values are expressed as means ± SE. † significantly different from placebo ($p < 0.01$), ***** significantly different from placebo ($p < 0.05$)

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Table 2. $\dot{V}O_2$ Kinetics Parameters for the Onset of Handgrip Exercise

40% P_{peak}	Placebo	Nitrate
Baseline (ml/min)	17 ± 3	16 ± 4
Amplitude (ml/min)	52 ± 13	64 ± 8
τ (s)	38 ± 5	34 ± 9
TD (s)	1 ± 1	3 ± 1
RC (ml/min/s)	1.6 ± 0.4	1.9 ± 0.6
85% P_{peak}		
Baseline (ml/min)	15 ± 4	17 ± 2
Amplitude (ml/min)	72 ± 16 †	99 ± 22 *†
τ (s)	37 ± 8	25 ± 3 *
TD (s)	4 ± 2	6 ± 2
RC (ml/min/s)	2.2 ± 0.4	3.8 ± 0.6 *

τ, TD, and RC denote time constant, time delay, and rate constant, respectively. Values are expressed as means ± SE. ***** significantly different from placebo within work rate, † significantly different from 40% P_{peak} within supplement (both $p < 0.05$). Analysis completed on 7 of 9 subjects.

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Figure Legends

Figure 1. Schematic representation of experimental protocol
Left: overall protocol showing the timing of the five laboratory visits in relation to one another. **Right:** expansion of an individual testing day (in this case, Testing day #1; each subsequent testing session followed the same timeline). Each testing session was assigned randomly to the supplemental condition (i.e., nitrate or placebo) and exercise intensity (i.e., 40 or 85 $\%P_{peak}$). All exercise tests began approximately 2.5 h after supplement consumption.

Figure 2. Resting nitrite concentrations
Plasma nitrite concentration ([nitrite]) for individual subjects (gray lines) and group means (both, n = 6; see text for discussion of reduced subject number). Plasma nitrate concentrations were similar to [nitrite], these data are presented in text only. Error bars represent SE. ***** significantly different from placebo ($p < 0.001$).

Figure 3. Brachial artery blood flow during exercise
A: Mean brachial artery blood flow ($\dot{Q}_{BA}$) at the end of each minute of 40 $\%P_{peak}$ exercise. **B**: Mean $\dot{Q}_{BA}$ at the end of each minute of 85 $\%P_{peak}$ exercise and the limit of exercise tolerance (T_{lim}). In both graphs, filled circles represent placebo and open circles represent nitrate supplementation (both, n = 9). Error bars represent SE.

Figure 4. Mean blood pressure and vascular conductance responses to 40 $\%P_{peak}$ exercise
A: Mean arterial pressure (MAP) taken every 120 s during exercise. **B**: Vascular conductance (VC) calculated as the product of brachial artery blood flow and MAP every 120 s during exercise. In both graphs, filled circles represent placebo and open circles represent nitrate supplementation (both, n = 8). Error bars represent SE.

Figure 5. NIRS-derived muscle and microvascular oxygenation responses during exercise
Left: 40 $\%P_{peak}$ exercise A: Mean deoxygenated-[hemoglobin + myoglobin] (deoxy-[Hb + Mb]) at the end of each minute of exercise. **B**: Mean total-[hemoglobin + myoglobin] (total-[Hb + Mb]) at the end of each minute of exercise (both, n = 9). **Right: 85 $\%P_{peak}$ exercise C**: Mean deoxy-[Hb + Mb] at the end of each minute of exercise and at the limit of exercise tolerance (T_{lim}). **D**: Mean total-[Hb + Mb] at the end of each minute of exercise and at T_{lim}. In all graphs, filled circles represent placebo and open circles represent nitrate supplementation (both, n = 9). Error bars represent SE. ***** significantly different from placebo ($p < 0.05$).

Figure 6. Estimated $\dot{V}O_2$ during exercise
A: Mean estimated $\dot{V}O_2$ at the end of each minute of 40 $\%P_{peak}$ exercise. **B**: Mean estimated $\dot{V}O_2$ at the end of each minute of 85 $\%P_{peak}$ exercise and at the limit of exercise tolerance (T_{lim}). In

both graphs, filled circles represent placebo and open circles represent nitrate supplementation (both, n = 9). Error bars represent SE.

Figure 7. Effect of supplementation on tolerance to exercise

Individual (solid gray lines) and mean (n = 9) tolerance to exercise (T_{lim}) responses under both supplementations during 85 $\%P_{peak}$ exercise. Error bars represent SE.

Figure 1

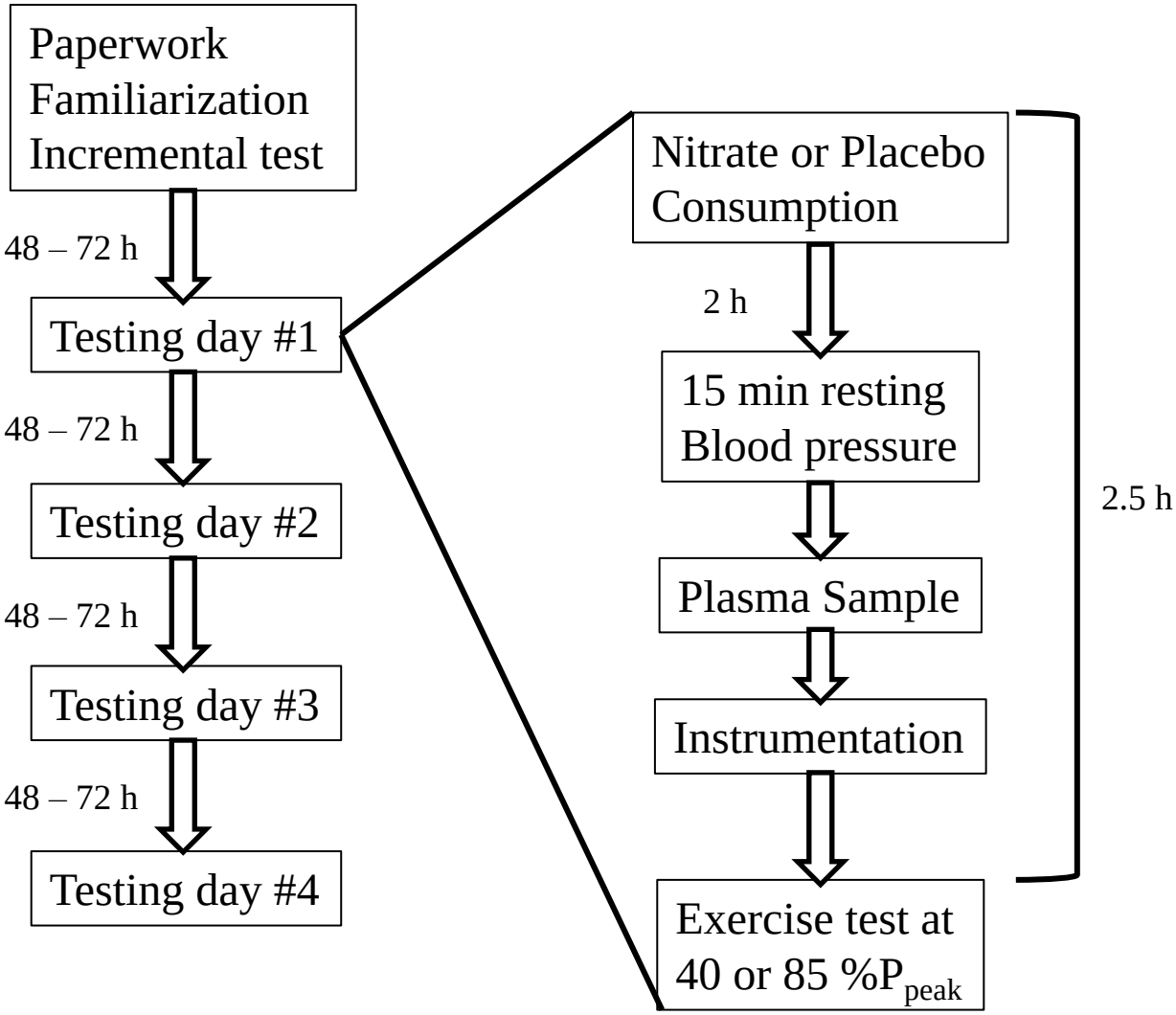

Paperwork
Familiarization
Incremental test
48 – 72 h
Testing day #1
48 – 72 h
Testing day #2
48 – 72 h
Testing day #3
48 – 72 h
Testing day #4
Nitrate or Placebo
Consumption
2 h
15 min resting
Blood pressure
Plasma Sample
Instrumentation
Exercise test at
40 or 85 %P peak
2.5 h

Figure 2

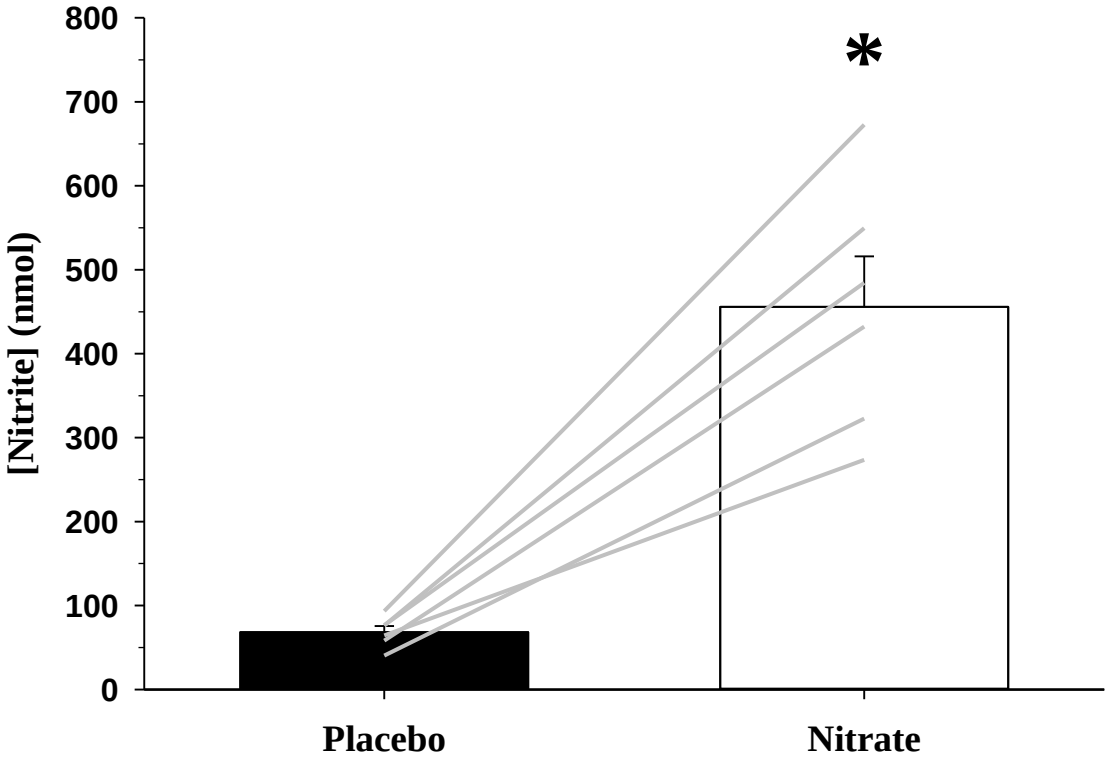

800
700
600
500
400
300
200
100
0
[Nitrite] (nmol)
*
Placebo
Nitrate

Figure 3

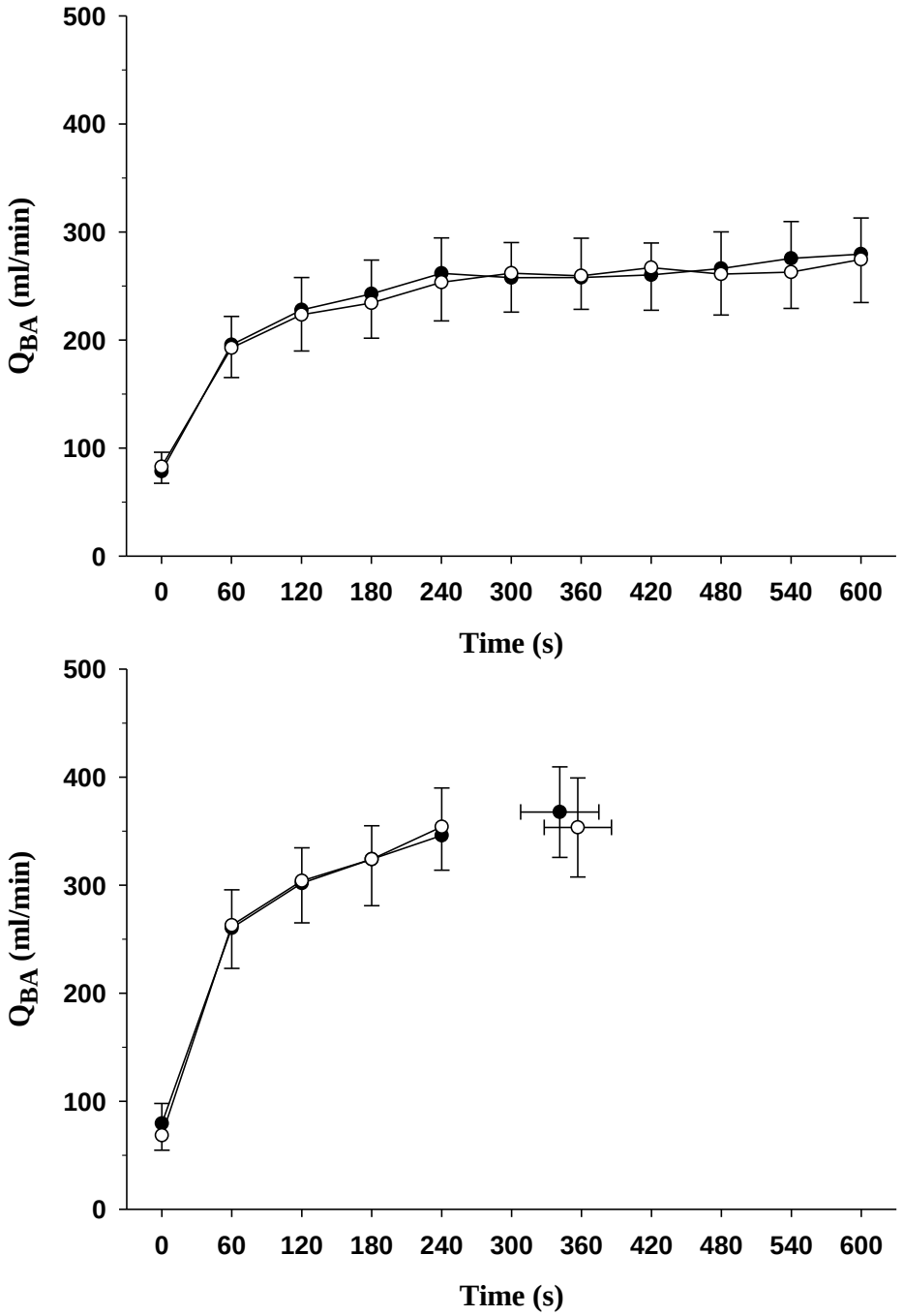
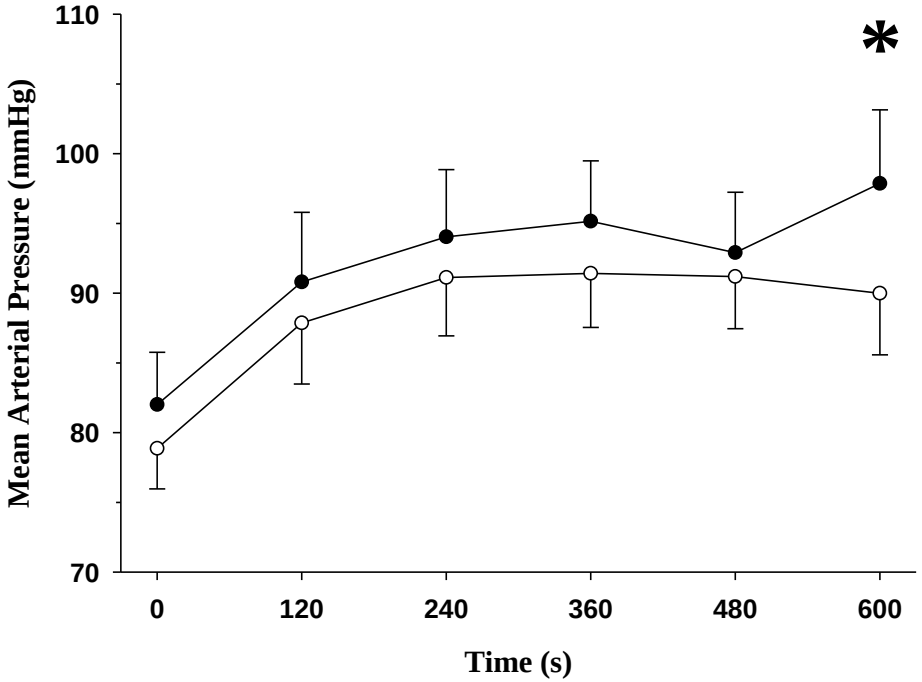

500
400
300
200
100
0
Q_{BA} (ml/min)
0
60
120
180
240
300
360
420
480
540
600
Time (s)
500
400
300
200
100
0
Q_{BA} (ml/min)
0
60
120
180
240
300
360
420
480
540
600
Time (s)

Figure 4


110
100
90
80
70
Mean Arterial Pressure (mmHg)
*
0
120
240
360
480
600
Time (s)

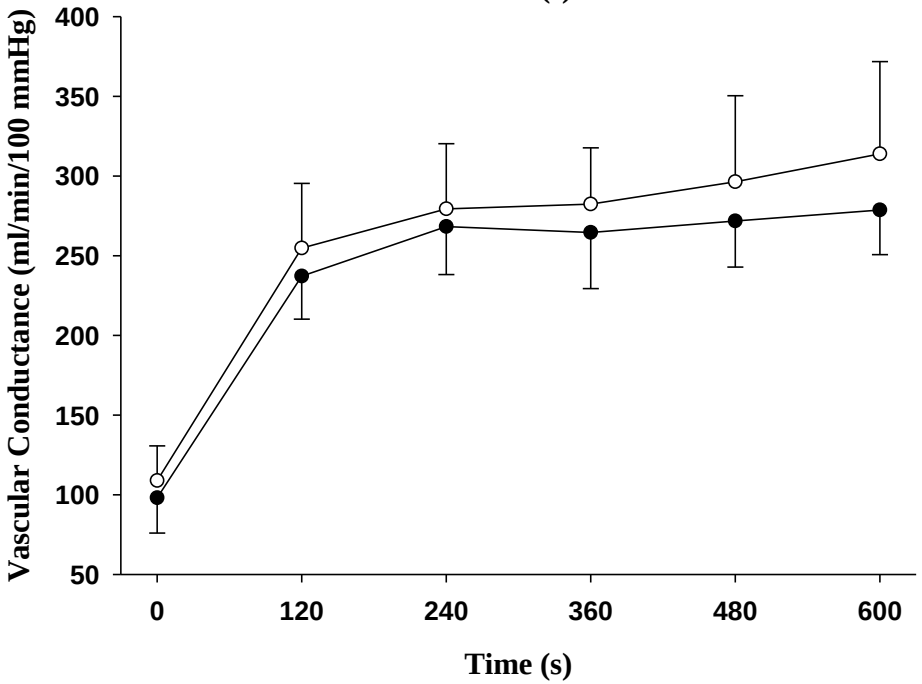

400
350
300
250
200
150
100
50
Vascular Conductance (ml/min/100 mmHg)
0
120
240
360
480
600
Time (s)

Figure 5

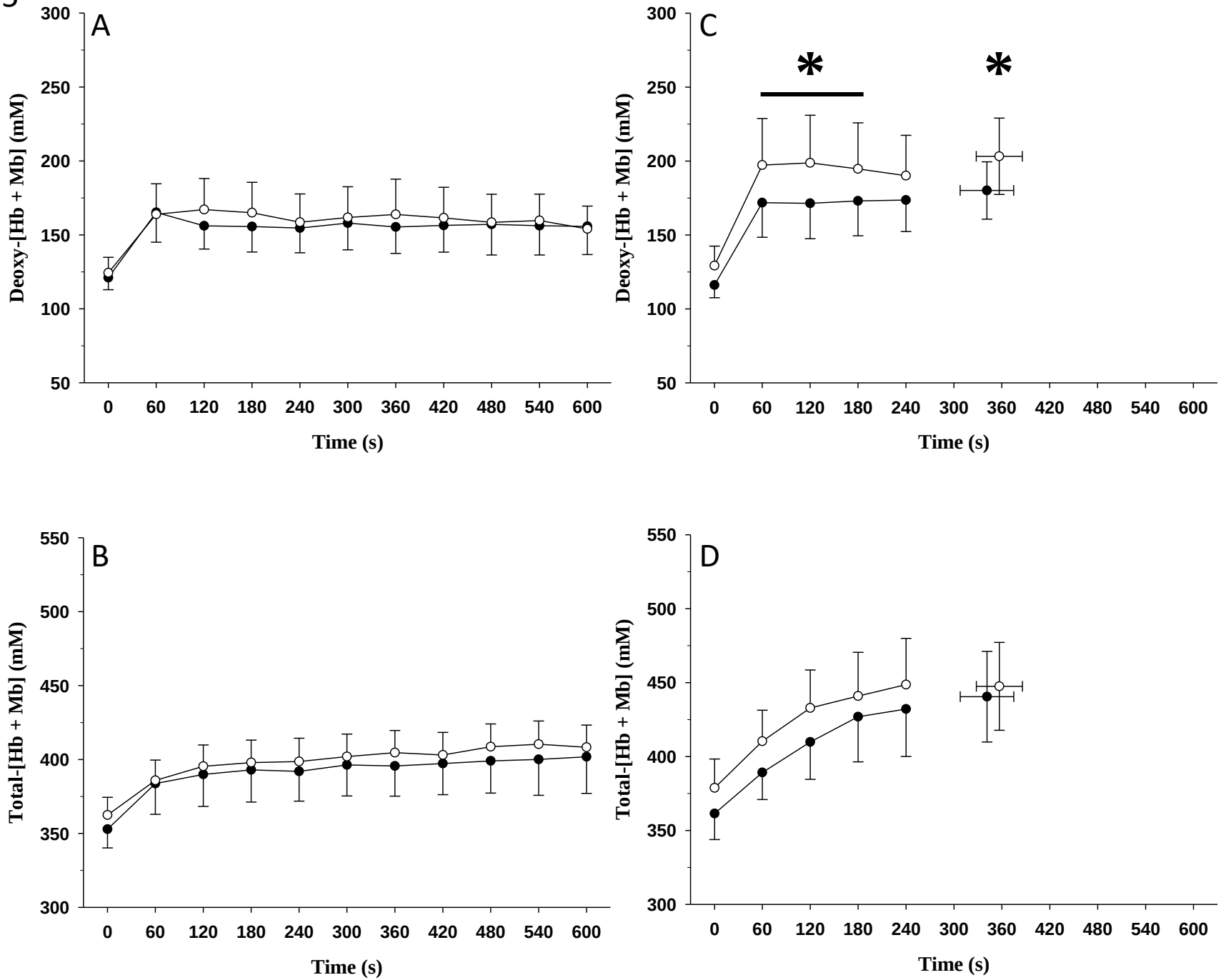

A
B
C
D
Deoxy-[Hb + Mb] (mM)
Total-[Hb + Mb] (mM)
Time (s)
300
250
200
150
100
50
550
500
450
400
350
0
60
120
180
240
300
360
420
480
540
600
*
*

Figure 6

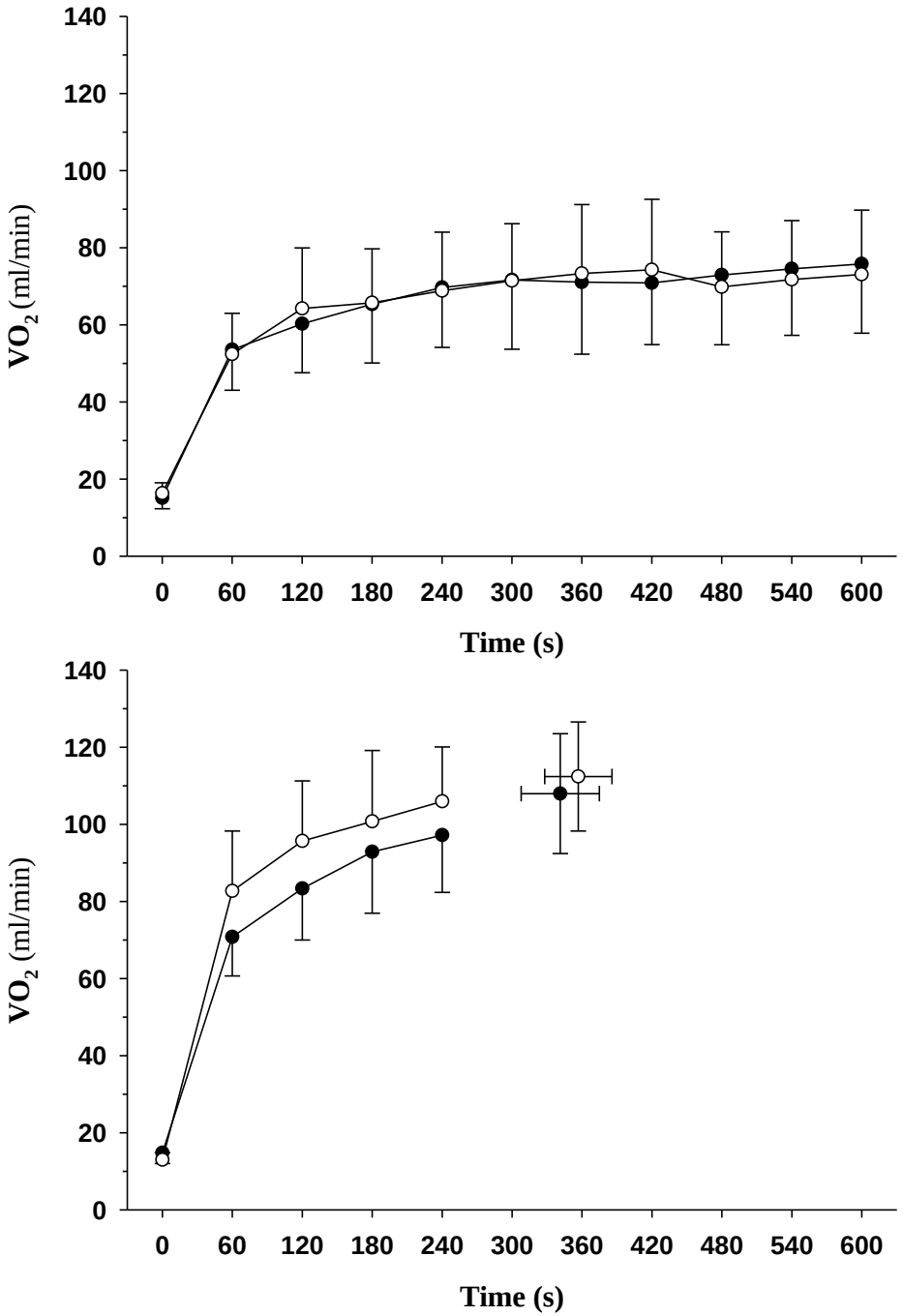
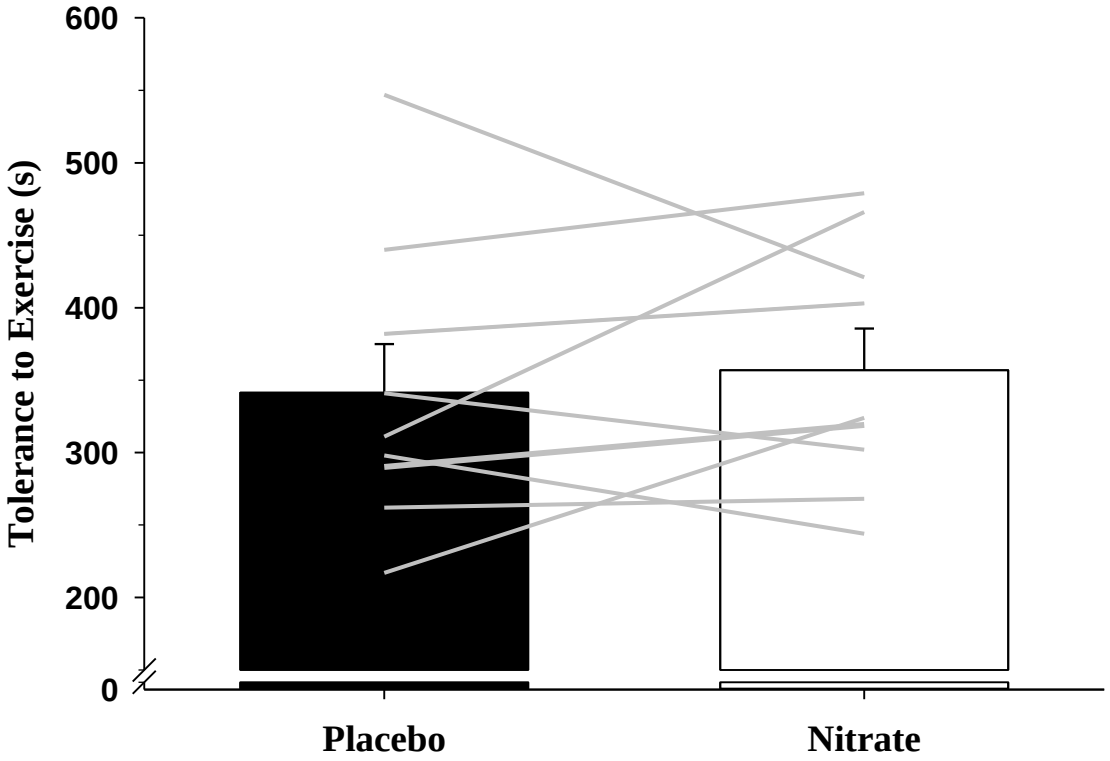

140
120
100
80
60
40
20
0
VO2 (ml/min)
0
60
120
180
240
300
360
420
480
540
600
Time (s)
140
120
100
80
60
40
20
0
VO2 (ml/min)
0
60
120
180
240
300
360
420
480
540
600
Time (s)

Figure 7


600
500
400
300
200
0
Tolerance to Exercise (s)
Placebo
Nitrate