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Inorganic nitrate supplementation improves muscle oxygenation, O_2 uptake kinetics, and exercise tolerance at high but not low pedal rates

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Bailey SJ, Varnham RL, DiMenna FJ, Breese BC, Wylie LJ, Jones AM. Inorganic nitrate supplementation improves muscle oxygenation, O_2 uptake kinetics, and exercise tolerance at high but not low pedal rates. *J Appl Physiol* 118: 1396–1405, 2015. First published April 9, 2015; doi:10.1152/japplphysiol.01141.2014.—We tested the hypothesis that inorganic nitrate (NO_3^-) supplementation would improve muscle oxygenation, pulmonary oxygen uptake ($\dot{V}O_2$) kinetics, and exercise tolerance (Tlim) to a greater extent when cycling at high compared with low pedal rates. In a randomized, placebo-controlled cross-over study, seven subjects (mean ± SD, age 21 ± 2 yr, body mass 86 ± 10 kg) completed severe-intensity step cycle tests at pedal cadences of 35 rpm and 115 rpm during separate nine-day supplementation periods with NO_3^--rich beetroot juice (BR) (providing 8.4 mmol NO_3^-/day) and placebo (PLA). Compared with PLA, plasma nitrite concentration increased 178% with BR ($P < 0.01$). There were no significant differences in muscle oxyhemoglobin concentration ([O_2Hb]), phase II $\dot{V}O_2$ kinetics, or Tlim between BR and PLA when cycling at 35 rpm ($P > 0.05$). However, when cycling at 115 rpm, muscle [O_2Hb] was higher at baseline and throughout exercise, phase II $\dot{V}O_2$ kinetics was faster (47 ± 16 s vs. 61 ± 25 s; $P < 0.05$), and Tlim was greater (362 ± 137 s vs. 297 ± 79 s; $P < 0.05$) with BR compared with PLA. These results suggest that short-term BR supplementation can increase muscle oxygenation, expedite the adjustment of oxidative metabolism, and enhance exercise tolerance when cycling at a high, but not a low, pedal cadence in healthy recreationally active subjects. These findings support recent observations that NO_3^- supplementation may be particularly effective at improving physiological and functional responses in type II muscle fibers.

nitric oxide; vascular function; oxidative metabolism; exercise performance; fatigue

NITRIC OXIDE (NO) IS A DIFFUSIBLE GAS that impacts a plethora of physiological responses including skeletal muscle perfusion, metabolism, force production, and fatigue resistance (56). It is well documented that NO is produced by the nitric oxide synthase enzymes, which catalyze the complex five-electron oxidation of the semiessential amino acid, L-arginine (10). More recently, there has been a growing appreciation of the potential for NO synthesis from the simple one-electron reduction of nitrite (NO_2^-), in a reaction catalyzed by numerous NO_2^- reductases (44, 57). Importantly, increasing the intake of dietary inorganic nitrate (NO_3^-), which passes into the enterosalivary circulation for subsequent reduction to NO_2^- by oral anaerobes (19), has been shown to positively impact NO biomarkers, exercise efficiency, and exercise tolerance in recreationally active subjects (3, 4, 12, 42, 43, 58, 61). Therefore, supplementation with NO_3^- appears to represent an effective dietary intervention to improve NO bioavailability, contractile efficiency, and fatigue resistance.

Results from in vitro studies suggest that the influence of NO on mammalian skeletal muscle contractility is impacted by contraction frequency (5, 20, 46, 49). Hernandez et al. (34) harvested single flexor digitorum brevis (FDB) muscle fibers from NO_3^--supplemented mice and reported increased in vitro contractile force up to 50 Hz stimulation and a more rapid rate of force development in the single FDB muscle fibers at 100 Hz stimulation. Similarly, increased evoked contractile force has been observed in human skeletal muscle in vivo during low-frequency submaximal stimulation and over the initial stages of high-frequency maximal stimulation following NO_3^- supplementation (31). However, NO_3^- ingestion has been shown to increase human peak knee extensor torque at 360°/s, but not 90°/s, 180°/s, and 270°/s, in vivo (15). Collectively, these findings suggest that dietary NO_3^- supplementation enhances skeletal muscle contractile function, and that it might be particularly effective at augmenting contractility at higher contraction velocities. However, heretofore, human in vivo studies reporting improvements in exercise tolerance following NO_3^- supplementation have utilized cycle ergometer exercise with pedal cadences of 70–90 rpm (i.e., at "mid-range" contraction frequency/velocity; e.g., see Refs. 4, 12, 58, 61). Consequently, it is unclear whether NO_3^- supplementation may be more effective at improving skeletal muscle fatigue resistance at a higher contraction frequency/velocity.

Relative to a lower contraction frequency (60 rpm), a higher contraction frequency (100 rpm) has been shown to increase oxygen uptake ($\dot{V}O_2$) and to reduce contractile efficiency in human skeletal muscle in vivo (23). Similarly, when contraction duration is shortened, $\dot{V}O_2$ (32, 35) and the ATP cost of force production (35) are increased in mammalian skeletal muscle in situ relative to contractions of longer duration when the same work-to-rest ratio is applied. There is also evidence to suggest that pulmonary $\dot{V}O_2$ kinetics is slower (11, 18) and the $\dot{V}O_2$ slow component is increased (11, 52) when cycling at very high compared with very low pedal cadences. Dietary NO_3^- supplementation has been shown to increase muscle blood flow (24), lower the O_2 (3, 4, 42, 43, 58, 61) and ATP (3) requirements of muscle contraction, and improve the matching between muscle O_2 supply and muscle O_2 utilization (4, 25, 26). Therefore, this potential for enhanced contractile efficiency and perfusion distribution with NO_3^- supplementation might improve muscle oxygenation, $\dot{V}O_2$ kinetics, and exercise toler-

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ance, particularly when more rapid muscle contractions are completed.

It has been suggested that the proportional contribution of fast-twitch muscle fibers to force production is greater at higher pedal cadences (6, 7; but see 1). Importantly, in murine models, dietary NO_3^- supplementation has been shown to *1*) increase bulk muscle blood flow and preferentially distribute this toward fast-twitch muscle fibers (24), and *2*) increase calcium-handling proteins and twitch and tetanic force production in fast-twitch extensor digitorum longus muscle, but not in slow-twitch soleus muscle (34). In support of a targeted effect of NO_3^- supplementation on fast-twitch muscle, Breese et al. (12) have recently reported that NO_3^- supplementation speeds $\dot{V}O_2$ and muscle deoxyhemoglobin [HHb; reflective of the balance between muscle O_2 utilization and muscle O_2 delivery (27, 37)] kinetics during a severe-intensity step exercise test initiated from a moderate-intensity baseline. Conversely, there was no effect when a moderate-intensity step test was initiated from a low-intensity baseline. This is important because, compared with the latter, the former would be expected to involve a greater recruitment of fast-twitch fibers (38, 39). Taken together, these findings suggest that dietary NO_3^- supplementation may preferentially augment physiological responses within, and in the microvasculature surrounding, fast-twitch muscle fibers. Based on these observations, the effects of NO_3^- might be more pronounced when humans cycle at a very high compared with a very low pedal cadence; however, this has yet to be investigated.

The purpose of this study was to evaluate the effects of short-term dietary NO_3^- supplementation on muscle oxygenation, pulmonary $\dot{V}O_2$ kinetics, and exercise tolerance when cycling at a very high (115 rpm) and a very low (35 rpm) pedal cadence at the same relative exercise intensity. Given the effects of cadence on the physiological responses evoked during cycling and the physiological benefits afforded by NO_3^- supplementation (see above), we hypothesized that muscle oxygenation would be increased, pulmonary $\dot{V}O_2$ kinetics speeded, and exercise tolerance extended by a greater magnitude with NO_3^- when cycling at 115 rpm than at 35 rpm.

METHODS

Subjects. Seven healthy male subjects (mean ± SD age, 21 ± 2 yr; body mass, 86 ± 10 kg; height, 1.82 ± 0.08 m) volunteered to participate in this study. None of the subjects were tobacco smokers or users of dietary supplements. The subjects participated in exercise at a recreational level, but were not highly trained. All subjects were familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. The procedures employed in this study were approved by the Institutional Research Ethics Committee, and all subjects were required to give their written informed consent prior to the commencement of the study after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. All tests were performed at the same time of day (±2 h). Each subject was asked to refrain from caffeine and alcohol intake for 6 and 24 h before each test, respectively. Subjects were also provided with a list of foods rich in NO_3^- and instructed to avoid the consumption of these foods and to abstain from the use of antibacterial mouthwash, which eliminates the oral bacteria that reduce NO_3^- to NO_2^- (28) for the duration of the study.

Experimental design. Subjects were required to report to the laboratory on 10 occasions over a 5–7 wk timeframe. Prior to the experimental testing, subjects completed cadence-specific ramp incremental tests at 35 and 115 rpm in a randomized order so that the same relative exercise intensity could be prescribed at both pedal cadences during the experimental tests. Over the remaining eight laboratory visits, subjects completed severe-intensity step exercise tests at 35 and 115 rpm during separate nine-day supplementation periods with beetroot juice (BR) (35-BR and 115-BR, respectively) and placebo (PLA) (35-PLA and 115-PLA, respectively) for determination of muscle oxygenation, $\dot{V}O_2$ kinetics and exercise tolerance. The two supplementation periods were administered as part of a randomized (3 subjects started on BR), cross-over, double-blinded experimental design.

Incremental tests. Before the intervention period, subjects completed a ramp incremental test at 35 and 115 rpm on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) in a randomized order. At least 48 h separated the two ramp incremental tests. Initially, subjects performed 3 min of baseline cycling at 20 W, after which the work rate was increased by 30 W/min until the limit of tolerance. The saddle and handle bar height and configuration during the first incremental test was recorded and reproduced in subsequent tests. During the baseline period and the incremental test, subjects cycled at a predetermined pedal rate (either 35 or 115 rpm) until volitional exhaustion or the cadence fell by >10 rpm for 3 consecutive seconds. Breath-by-breath pulmonary gas exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. The $\dot{V}O_{2\,peak}$ was taken as the highest 30-s mean value attained prior to exhaustion in the test. The gas exchange threshold (GET) was determined from a cluster of measurements including *1*) the first disproportionate increase in CO_2 production ($\dot{V}CO_2$) from visual inspection of individual plots of $\dot{V}CO_2$ vs. $\dot{V}O_2$, *2*) an increase in expired ventilation ($\dot{V}E$)/$\dot{V}O_2$ with no increase in $\dot{V}E/\dot{V}CO_2$, and *3*) an increase in end-tidal O_2 tension with no fall in end-tidal CO_2 tension. The data collected during the incremental tests were used to calculate cadence-specific work rates which were employed during the subsequent severe-intensity step tests. Specifically, the work rates that would require 80% of the difference between the $\dot{V}O_2$ at the GET and $\dot{V}O_{2\,peak}$ (80%Δ) were estimated with account taken of the mean response time of the $\dot{V}O_2$ response to ramp exercise (60).

Step exercise tests. Subjects completed one severe-intensity step test on *days 4*, *5*, *8*, and *9* of each dietary condition (PLA and BR). The same pedal cadence (either 35 or 115 rpm) was applied on *days 4* and *5* of the supplementation period with the other cadence applied on *days 8* and *9* of the supplementation period. The order in which the 35 and 115 rpm tests were administered over the first supplementation period was randomized (3 subjects started on 35 rpm), and this order was replicated over the second supplementation period. The step tests comprised 4 min of baseline cycling at 20 W, followed by a step increment to a severe-intensity (80%Δ) constant work rate that was continued until exhaustion (same criteria as described above for the incremental exercise test). For each condition, the mean time-to-exhaustion was used for subsequent analysis. Two step tests at 35-PLA, 115-PLA, 35-BR, and 115-BR were completed to average the pulmonary $\dot{V}O_2$ responses in the same experimental condition to improve the signal-to-noise ratio and the confidence surrounding the estimation of the $\dot{V}O_2$ kinetic parameters obtained from the exponential modelling procedures (41) (see *Data analysis procedures* below).

Supplementation procedures. Following the initial ramp tests, subjects underwent two 9-day supplementation periods with BR and PLA, with each period separated by at least 10 days of washout. Subjects recorded an 11-day food diary, which commenced 2 days prior to the first 9-day supplementation period, and were asked to use the diary to replicate and record their diet in the second supplemen-

tation period. The BR (Beet It Sport, James White Drinks, Ipswich, UK) was administered in 70-ml doses providing 6.2 mmol NO_3^- per serving. Sodium chloride (NaCl) was administered as the PLA in doses of 0.1 mmol/kg body mass (43). Subjects were informed that the PLA was $NaNO_3$, and that the purpose of the study was to compare the effects of '$NaNO_3$' relative to NO_3^--rich BR. All labels and packaging were removed from the BR supplements, and the PLA was provided in transparent plastic capsules which were placed in a small transparent plastic bag labeled sodium nitrate. Subjects were instructed to open the PLA capsules and mix the content with 200–300 ml of water for consumption. On *days 1–3* and *6–7* of the supplementation periods (when no exercise tests were conducted), one dose of supplement was consumed in the morning and evening. On the days of the experimental testing (*days 4–5* and *8–9*), subjects were instructed to consume both supplement doses 2.5 h before arriving at the laboratory (61). An additional dose of supplement was consumed 2 h following each experimental exercise test to counteract the marked depletion of plasma [NO_2^-] that occurs during intense exhaustive exercise (62).

Measurements. After reporting to the laboratory on *days 4* and *8* of each dietary intervention (the first day that each cadence-specific step test was performed), a venous blood sample was drawn into a lithium-heparin tube and centrifuged at 4,000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was subsequently extracted and immediately frozen at −80°C for later analysis of [NO_2^-] in duplicate via ozone-based chemiluminescence (61).

During all tests, pulmonary gas exchange and ventilation were measured breath-by-breath with subjects wearing a nose clip and breathing through a low-dead-space, low-resistance mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter with paramagnetic (O_2) and infrared (CO_2) analyzers (Jaeger Oxycon Pro, Hoechberg, Germany) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated with a 3-l syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in the capillary gas transit and the analyzer rise time relative to the volume signal. Pulmonary gas exchange and ventilation were calculated and displayed breath-by-breath.

The oxygenation status of the m. vastus lateralis of the right leg was monitored with a commercially available near-infrared spectroscopy system (model NIRO 300, Hamamatsu Photonics KK, Hiugashi-ku, Japan) on *days 4* and *8* of each dietary intervention. The system consisted of an emission probe that irradiates laser beams and a detection probe. Four different wavelength laser diodes provided the light source (776, 826, 845, and 905 nm), and the light returning from the tissue was detected by a photomultiplier tube in the spectrometer. The intensity of incident and transmitted light was recorded continuously at 2 Hz and used to estimate concentration changes from the resting baseline for oxygenated, deoxygenated, and total tissue hemoglobin/myoglobin. Therefore, the NIRS data represent a relative change based on the optical density measured in the first datum collected. The deoxygenated hemoglobin/myoglobin concentration ([HHb]) signal was assumed to provide an estimate of changes in fractional O_2 extraction in the field of interrogation (27, 29, 37). It should be noted here that the contribution of deoxygenated myoglobin to the NIRS signal is presently unclear, and, as such, the terms [O_2Hb] and [HHb] used in this paper should be considered to refer to the combined concentrations of oxygenated and deoxygenated hemoglobin and myoglobin, respectively.

The leg was initially cleaned and shaved around the belly of the muscle, and the optodes were placed in the holder, which was secured to the skin with adhesive at 20 cm above the fibular head. To secure the holder and wires in place, an elastic bandage was wrapped around the subject's leg. The wrap helped to minimize the possibility that extraneous light could influence the signal and also ensured that the optodes did not move during exercise. Indelible pen marks were made around the holder to enable precise reproduction of the placement in subsequent tests. The probe gain was set with the subject at rest in a seated position with the leg extended at down stroke on the cycle ergometer before the first exercise bout, and NIRS data were collected continuously throughout the exercise protocols. The data were subsequently downloaded onto a personal computer, and the resulting text files were stored for later analysis.

Fingertip capillary blood samples (~20 μl) were collected during the final 30 s of baseline cycling and at exhaustion. Blood [lactate] was determined using an automated analyzer (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The mean of the two baseline and exhaustion blood [lactate] values were calculated for each experimental condition prior to analysis. Blood [lactate] accumulation during exercise was taken as the change in the values from baseline to exhaustion.

Data analysis procedures. The breath-by-breath $\dot{V}O_2$ data from each test were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than four standard deviations from the local mean were removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values and, for each individual, the two identical repetitions for each experimental condition were time aligned to the start of exercise and ensemble averaged. The first 20 s of data after the onset of exercise (i.e., the phase I response) were deleted, and a nonlinear least-square algorithm was used to fit the data thereafter until the point of exhaustion. A biexponential model was used to characterize the $\dot{V}O_2$ responses to severe exercise, as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2baseline} + A_p\left[1 - e^{-(t\text{-}TDp)/\tau p}\right] - A_s\left[1 - e^{-(t\text{-}TDs)/\tau s}\right],$$

where $\dot{V}O_2$ (t) represents the absolute $\dot{V}O_2$ at a given time t; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ in the baseline period; A_p, TD_p, and τ_p represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in $\dot{V}O_2$ above baseline; and A_s, TD_s, and τ_s represent the amplitude of, time delay before the onset of, and time constant describing the development of the $\dot{V}O_2$ slow component, respectively.

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. $\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the final 90 s of the resting baseline period. The $\dot{V}O_2$ at the limit of tolerance (T_{lim}) was defined as the mean $\dot{V}O_2$ measured over the final 30 s of the exhaustive exercise bout. Because the asymptotic value (A_s) of the exponential term describing the $\dot{V}O_2$ slow component may represent a higher value than is actually reached at the end of the exercise, the actual amplitude of the $\dot{V}O_2$ slow component at exhaustion was defined as A_s'.

To provide information on muscle oxygenation, we also modelled the [HHb] response to exercise. A monoexponential model was applied to the data with the fitting window commencing at the time at which the [HHb] signal increased 1 SD above the baseline mean. The [HHb] kinetics were determined by constraining the fitting window to the point at which monoexponentiality became distorted, consequent to a [HHb] slow component, as determined by visual inspection of the residual plots. The [HHb] TD and τ values were summed to provide information on the overall [HHb] response dynamics in the fundamental phase of the response.

The [O_2Hb] response does not approximate an exponential and was, therefore, not modelled. Rather, we determined the [O_2Hb] at baseline (90 s preceding step transition), 120 s (30 s mean surrounding 120 s), and exhaustion (mean response over the final 30 s of exercise). The muscle [HHb] and [O_2Hb] responses were summed at these time points ([Hb_{tot}]) to provide information on the total [Hb] in the NIRS area of interrogation.

Statistics. A two-way, treatment (PLA and BR) × cadence (35 and 115 rpm), repeated-measures ANOVA was employed to assess dif-

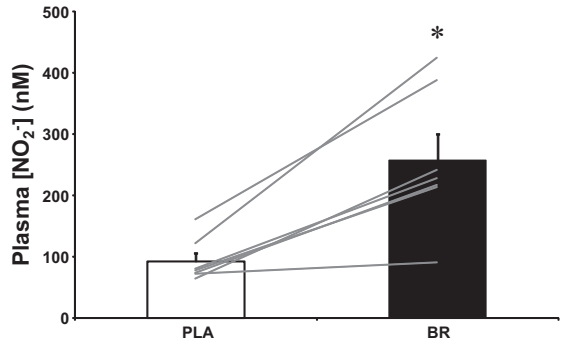

Fig. 1. Resting plasma nitrite concentration ([NO_2^-]) following placebo (PLA) and nitrate-rich beetroot juice (BR) supplementation. The open bar represents the group mean ± SE plasma [NO_2^-] from the PLA trials, while the filled bar represents the group mean ± SE plasma [NO_2^-] from the BR trials. The solid grey lines represent the individual changes in plasma [NO_2^-] following BR supplementation. *Significantly different from PLA ($P < 0.01$). Note the increase in plasma [NO_2^-] with BR supplementation.

ferences in plasma [NO_2^-], $\dot{V}O_2$ kinetics, muscle [HHb], muscle [O_2Hb], muscle [Hb_{tot}], and exercise tolerance across the experimental conditions. Significant effects were further explored using post hoc *t*-tests with the alpha level adjusted via a Fisher's LSD correction. Relationships between the outcome variables were assessed with Pearson's correlation coefficient (*r*). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted when $P < 0.05$.

RESULTS

The PLA and BR supplements administered in this study were well tolerated by all subjects with no negative side effects reported. Subjects consumed all doses of the supplement for each experimental condition, and their diet was consistent across all the dietary interventions. After all experimental testing for the study was completed, all subjects confirmed that they were unaware that the PLA condition was not $NaNO_3$.

The $\dot{V}O_{2\,peak}$ and peak work rate attained in the ramp incremental tests at 35 rpm and 115 rpm were, respectively, 3.84 ± 0.57 liter/min and 4.11 ± 0.56 liter/min, and 304 ± 43 W and 319 ± 52 W. The $\dot{V}O_2$ and work rate at the GET during the incremental tests were 1.62 ± 0.31 liter/min and 122 ± 18 W at 35 rpm, and 2.53 ± 0.33 liter/min and 122 ± 8 W at 115 rpm, respectively. There were no significant differences in $\dot{V}O_{2\,peak}$ or peak work rate attained during the ramp tests at 35 rpm and 115 rpm. However, the $\dot{V}O_2$ at the GET was significantly greater during the 115 rpm incremental test than the 35 rpm incremental test ($P < 0.001$). The work rates which corresponded to 80%Δ, which were imposed during the experimental tests conducted at 35 rpm and 115 rpm, were 244 ± 35 W and 258 ± 47 W, respectively. These work rates were not significantly different from each other ($P > 0.05$).

Plasma [NO_2^-] and blood pressure. There was a significant main effect for supplement on plasma [NO_2^-] ($P < 0.01$), systolic blood pressure (SBP) ($P < 0.05$), and mean arterial pressure (MAP) ($P < 0.05$), but not diastolic blood pressure (DBP) ($P > 0.05$). Plasma [NO_2^-] was significantly higher in both 35-BR and 115-BR compared with 35-PLA and 115-PLA ($P < 0.01$), with plasma [NO_2^-] increased by 179% above PLA conditions with BR when all data were pooled ($P < 0.01$; Fig. 1). There were no differences in plasma [NO_2^-] between 35-PLA and 115-PLA, and 35-BR and 115-BR ($P > 0.05$). Systolic blood pressure and mean arterial pressure were lowered by 8 mmHg and 4 mmHg across the BR conditions when all data were pooled ($P < 0.05$).

$\dot{V}O_2$ kinetics. Pulmonary $\dot{V}O_2$ at designated time points and the key parameters derived from the biexponential modelling are presented in Table 1 and illustrated for a representative individual in Fig. 2. There was a significant main effect for cadence on $\dot{V}O_2$ baseline and the $\dot{V}O_2$ at 120 s and exhaustion ($P < 0.01$) with $\dot{V}O_2$ at these time points being higher in both 115-PLA and 115-BR compared with both 35-PLA and 35-BR ($P < 0.05$; Table 1). The $\dot{V}O_2$ at exhaustion was not significantly different between 35-PLA and 35-BR, and these values were not different to the $\dot{V}O_{2\,peak}$ attained in the incremental test at 35 rpm ($P > 0.05$). Likewise, the $\dot{V}O_2$ at exhaustion in 115-PLA and 115-BR and the $\dot{V}O_{2\,peak}$ attained in the incremental test at 115 rpm were not significantly different from each other ($P > 0.05$). However, the $\dot{V}O_2$ at exhaustion was higher in 115-PLA relative to 35-PLA and 115-BR relative to 35-BR (both $P < 0.01$). There was a significant main effect for cadence on the $\dot{V}O_2$ phase II τ with a higher $\dot{V}O_2$ phase II τ (slower $\dot{V}O_2$ kinetics) in 115-PLA (61 ± 25 s) and 115-BR (47 ± 16 s) compared with both 35-PLA (32 ± 10 s) and 35-BR (32 ± 6 s; $P < 0.05$; Table 1). In addition, there was a significant treatment × cadence interaction effect on the $\dot{V}O_2$ phase II τ ($P < 0.05$). Post hoc analyses demonstrated that there was no significant difference in the $\dot{V}O_2$ phase II τ between 35-PLA and 35-BR ($P > 0.05$), but the $\dot{V}O_2$ phase II τ was lower in 115-BR compared with 115-PLA ($P < 0.05$; Table 1; Fig. 2). There were no significant between-supplement differences in the $\dot{V}O_2$ fundamental amplitude ($P > 0.05$), but there were significant between-cadence differences in the $\dot{V}O_2$ fundamental amplitude ($P < 0.05$; Table 1). The $\dot{V}O_2$ slow component amplitude was not significantly different between any of the experimental conditions ($P < 0.05$; Table 1). There were no significant between-supplement differences in blood [lactate]

Table 1. *Pulmonary oxygen uptake measures during severe-intensity cycle exercise at 35 rpm and 115 rpm after placebo and nitrate-rich beetroot juice supplementation*

Oxygen uptake	35-PLA	35-BR	115-PLA	115-BR
Baseline, liter/min	0.92 ± 0.14	0.89 ± 0.07	1.93 ± 0.34*†	1.89 ± 0.25*†
120 s, liter/min	3.41 ± 0.55	3.45 ± 0.51	3.65 ± 0.49* †	3.68 ± 0.47*†
Exhaustion, liter/min	3.83 ± 0.58	3.86 ± 0.52	4.24 ± 0.61*†	4.32 ± 0.75*†
Phase II τ, s	32 ± 10	32 ± 6	61 ± 25*†	47 ± 16*†‡
Fundamental amplitude, liter/min	2.58 ± 0.40	2.61 ± 0.43	2.01 ± 0.24*†	1.92 ± 0.30*†
Slow component amplitude, liter/min	0.44 ± 0.15	0.35 ± 0.18	0.38 ± 0.32	0.53 ± 0.42

Values are means ± SD. *Significantly different from 35-PLA ($P < 0.05$); †significantly different from 35-BR ($P < 0.05$); ‡significantly different from 115-PLA ($P < 0.05$). Placebo, PLA; beetroot juice, BR.

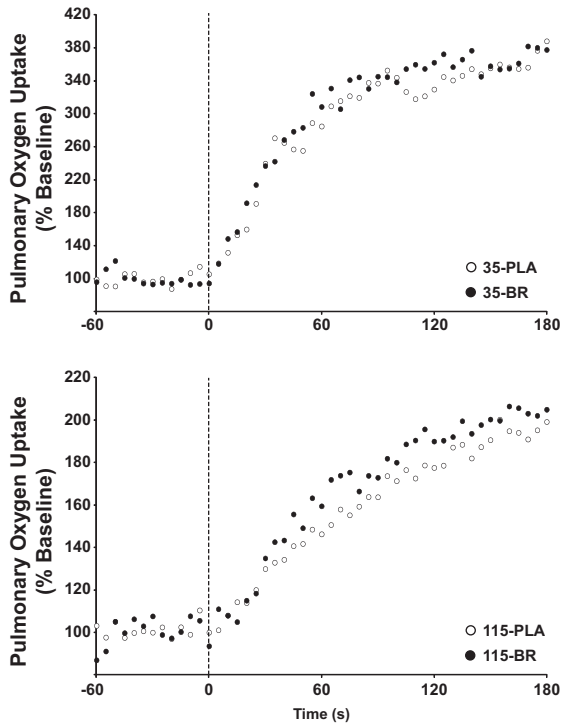

Fig. 2. Pulmonary oxygen uptake ($\dot{V}O_2$) responses to a step increment from an unloaded baseline to a severe-intensity work rate in a representative subject. Upper panel illustrates the $\dot{V}O_2$ responses while cycling at a cadence of 35 rpm following PLA and nitrate-rich BR supplementation. Lower panel shows the $\dot{V}O_2$ responses while cycling at a cadence of 115 rpm following PLA and nitrate-rich BR supplementation. Data are expressed as a percentage of the $\dot{V}O_2$ during baseline cycling and displayed as 5-s averages. The dashed vertical line indicates the point of the abrupt increase in work rate. Note the more rapid increase in $\dot{V}O_2$ with BR following the step increment in work rate at 115 rpm, but not 35 rpm. Data are truncated at 180 s.

accumulation at either cadence (35-PLA: 4.4 ± 1.6, 35-BR: 4.3 ± 1.8, 115-PLA: 4.7 ± 1.1, 115-BR: 4.4 ± 1.3 mM; $P > 0.05$ for all comparisons).

Muscle oxygenation parameters. NIRS-derived [HHb], [O_2Hb], and [Hb_{tot}] responses are presented in Table 2 with group mean [HHb] and [O_2Hb] responses illustrated in Figs. 3 and 4, respectively. There were no significant main effects or interaction effects on the [HHb] at baseline or throughout the step exercise test ($P > 0.05$; Table 2). The ANOVA revealed a significant main effect of supplement on the [HHb] τ + TD ($P < 0.05$) and a significant main effect of cadence on the [HHb] amplitude ($P < 0.05$; Table 2; Fig. 3). There were no significant differences in these variables across the individual experimental conditions ($P > 0.05$). There were significant main effects of cadence and a significant treatment × cadence interaction effect on the [O_2Hb] at baseline, 120 s, and at exhaustion ($P < 0.05$). Further analyses revealed that the [O_2Hb] was greater in 115-BR at baseline compared with 115-PLA, at 120 s compared with all other conditions, and at exhaustion compared with 35-PLA and 35-BR ($P < 0.05$). There were no significant main or interaction effects on [Hb_{tot}].

Exercise tolerance. The effects of BR on Tlim at 35 rpm and 115 rpm are illustrated in Fig. 5. Supplementation with BR significantly increased Tlim when cycling at 115 rpm (115-PLA: 297 ± 79 s vs. 115-BR: 362 ± 137 s; $P < 0.05$), but did not significantly alter Tlim at 35 rpm (35-PLA: 341 ± 99 s vs. 35-BR: 344 ± 74 s; $P > 0.05$; Fig. 5). There was a significant correlation between the change in Tlim and muscle [O_2Hb] at 120 s in 115-BR compared with 115-PLA ($r = 0.76$, $P < 0.05$) and a trend for a correlation between the change in muscle [O_2Hb] at Tlim and the change in Tlim in 115-BR compared with 115-PLA ($r = 0.76$, $P = 0.07$). There were no other correlations between the physiological and performance variables assessed in this study ($P > 0.05$).

DISCUSSION

The principal novel findings from this study are that short-term dietary NO_3^- supplementation, which likely enhanced the potential for O_2-independent NO synthesis by elevating plasma [NO_2^-], increased muscle oxygenation, speeded phase II $\dot{V}O_2$ kinetics, and improved severe-intensity exercise tolerance (Tlim) when cycling at 115 rpm. In contrast, there were no changes in muscle oxygenation, $\dot{V}O_2$ kinetics, or exercise tolerance with BR compared with PL at 35 rpm. Muscle [O_2Hb] was higher in 115-BR compared with 115-PLA, suggesting that BR may have increased muscle O_2 delivery at 115 rpm facilitating faster phase II $\dot{V}O_2$ kinetics and improved severe-intensity exercise tolerance. These findings are consistent with our experimental hypotheses and suggest that BR supplementation is more effective at improving muscle O_2 delivery, phase II $\dot{V}O_2$ kinetics, and fatigue resistance at higher pedal cadences.

In line with other studies administering NO_3^--rich BR (3, 4, 12, 42, 43, 58, 61), plasma [NO_2^-] was increased in this study. Importantly, plasma [NO_2^-] was not significantly different between the two BR trials and between the two PLA trials. This observation is consistent with our previous finding that plasma [NO_2^-] remains consistently elevated above baseline with BR, at least up to 15 days of supplementation (58). An

Table 2. *Near-infrared spectroscopy determined muscle oxyhemoglobin concentration, deoxyhemoglobin concentration, and total hemoglobin concentration measures during severe-intensity cycle exercise at 35 rpm and 115 rpm after PLA and nitrate-rich BR supplementation*

	35-PLA	35-BR	115-PLA	115-BR
Muscle [HHb]				
Baseline, AU	−4.0 ± 3.8	−3.9 ± 2.5	−2.0 ± 4.2	−3.0 ± 3.9
120 s, AU	5.8 ± 6.1	5.4 ± 5.8	4.3 ± 4.2	2.9 ± 5.9
Exhaustion, AU	6.4 ± 6.7	7.0 ± 7.0	4.9 ± 4.3	4.1 ± 6.5
[HHb] τ + TD, s	19 ± 6	23 ± 13	24 ± 14	36 ± 24
[HHb] amplitude, AU	10 ± 5	9 ± 6	6 ± 4	6 ± 3
Muscle [O_2Hb]				
Baseline, AU	1.8 ± 3.1	0.1 ± 4.5	−1.1 ± 3.5*	3.4 ± 4.9‡
120 s, AU	−6.9 ± 2.9	−7.7 ± 7.1	−6.1 ± 3.7	−1.0 ± 6.3*†‡
Exhaustion, AU	−6.9 ± 3.1	−7.0 ± 6.7	−6.0 ± 5.4	−0.6 ± 7.8*†
Muscle [Hb_{tot}]				
Baseline, AU	−2.2 ± 3.2	−3.8 ± 6.4	−3.1 ± 3.1	0.3 ± 5.5
120 s, AU	−1.1 ± 5.9	−2.3 ± 8.1	−1.8 ± 3.2	1.9 ± 5.5
Exhaustion, AU	−0.5 ± 5.7	−0.1 ± 8.9	−1.2 ± 4.3	3.5 ± 7.0

Values are means ± SD. *Significantly different from 35-PLA ($P < 0.05$); †significantly different from 35-BR ($P < 0.05$); ‡significantly different from 115-PLA ($P < 0.05$). AU, arbitrary units. [O_2Hb], oxyhemoglobin concentration; [HHb], deoxyhemoglobin concentration; [Hb_{tot}], total hemoglobin concentration.

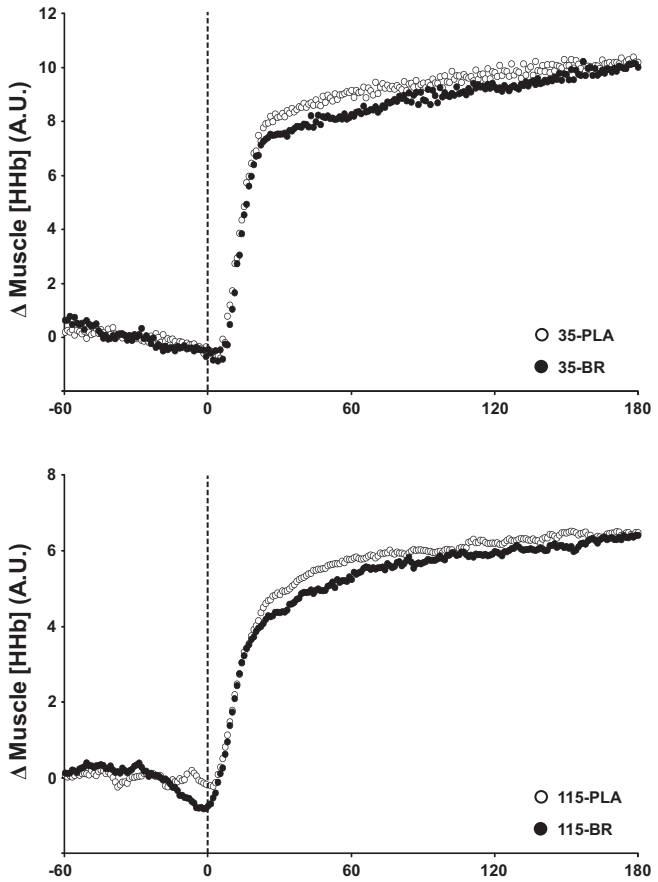

Fig. 3. Muscle deoxyhemoglobin concentration ([HHb]) group mean responses to a step increment from an unloaded baseline to a severe-intensity work rate. Upper panel illustrates the [HHb] responses while cycling at a cadence of 35 rpm following PLA and nitrate-rich BR supplementation. Lower panel shows the [HHb] responses while cycling at a cadence of 115 rpm following PLA and nitrate-rich BR supplementation. Data are expressed as the change in muscle [HHb] above baseline values. The dashed vertical line indicates the point of the abrupt increase in work rate. Note the slower adjustment of muscle [HHb] following the step increment in work rate with BR for both conditions. Data are truncated at 180 s.

increase in the circulating plasma [NO_2^-] reflects an increase in the reserve for NOS-independent NO production given that NO_2^- undergoes a simple one-electron reduction to NO through numerous NO_2^- reductatses (44, 57). The reduction of NO_2^- to NO is augmented as O_2 tension (14) and pH (48) decline and, since muscle PO_2 (53) and pH (3) are lowered during intense muscle contractions, NO_2^- reduction is likely to be an important source of NO during exercise. Aside from its reduction to NO, it should be acknowledged that NO_2^- itself can participate in posttranslational protein modifications that positively impact physiological processes (2). Moreover, a few days of NO_3^- supplementation can increase mitochondrial (42) and calcium-handling (34) proteins promoting enhanced metabolic and contractile function, respectively. Therefore, the short-term exposure to inorganic NO_3^- and the associated increase in plasma [NO_2^-] in this study may have been sufficient to increase NO signaling and elicit favorable physiological and functional responses. Indeed, resting arterial blood pressure was lowered by BR supplementation in this study, in accord with established effects of elevated NO on vascular tone (30).

Pulmonary $\dot{V}O_2$ was significantly higher at baseline and throughout exercise when cycling at 115 rpm compared with 35 rpm, as has been reported previously (18, 27, 63). While subjects were cycling at the same external work rate at baseline (20 W), and the same relative exercise intensity (80%Δ) during the step tests, internal work (59) and muscle ATP turnover rate and $\dot{V}O_2$ (23) are increased at higher contraction frequencies, which accounts for the higher pulmonary $\dot{V}O_2$ observed in the 115 rpm trials. The phase II $\dot{V}O_2$ τ was higher ($\dot{V}O_2$ kinetics was slower) when cycling at 115 rpm compared with 35 rpm, which corroborates findings of a slower adjustment of $\dot{V}O_2$ when cycling at a higher pedal cadence (11, 18). However, despite slower phase II $\dot{V}O_2$ kinetics at the higher pedal cadence, there were no differences in muscle microvascular [HHb] kinetics (τ + TD) between 115 rpm and 35 rpm. Since muscle [HHb] is considered a noninvasive proxy for muscle O_2 extraction (27, 37), this suggests that the slower phase II $\dot{V}O_2$ kinetics at 115 rpm might be a function of slower microvascular blood flow kinetics and, therefore, to a relative shortfall in muscle O_2 delivery compared with muscle O_2 demand over

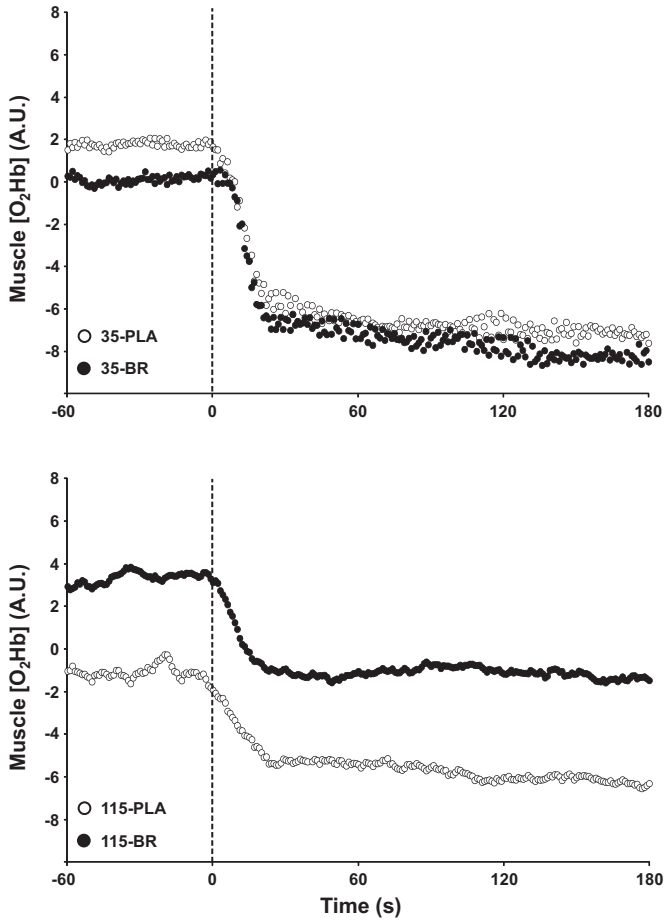

Fig. 4. Muscle oxyhemoglobin concentration ([O_2Hb]) group mean responses to a step increment from an unloaded baseline to a severe-intensity work rate. Upper panel illustrates the [O_2Hb] responses while cycling at a cadence of 35 rpm following PLA and nitrate-rich BR supplementation. Lower panel shows the [O_2Hb] responses while cycling at a cadence of 115 rpm following PLA and nitrate-rich BR supplementation. The dashed vertical line indicates the point of the abrupt increase in work rate. Note the significantly higher [O_2Hb] during baseline cycling and during the severe-intensity cycling bout with BR at 115 rpm, but not 35 rpm.

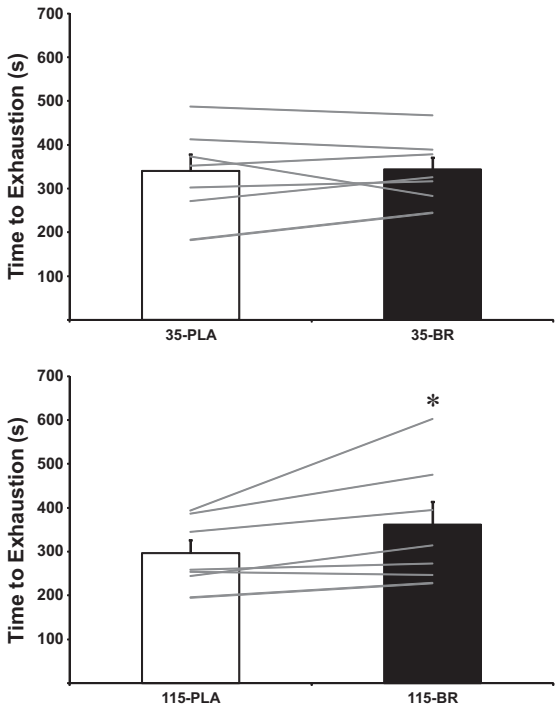

Fig. 5. Time-to-exhaustion responses during a step increment from an unloaded baseline to a severe-intensity constant work rate. Upper panel compares time-to-exhaustion following PLA and nitrate-rich BR supplementation while cycling at 35 rpm. Lower panel compares time-to-exhaustion following PLA and nitrate-rich BR supplementation while cycling at 115 rpm. The open bars represent the group mean ± SE responses after PLA supplementation, while the filled bars represent the group mean ± SE responses after BR supplementation. The solid grey lines represent the individual changes in time-to-exhaustion following BR supplementation. *Significantly different from PLA ($P < 0.05$). Note the significant increase in exercise tolerance after BR at 115 rpm, but not 35 rpm.

the initial stages of the severe-intensity step test. The slower phase II $\dot{V}O_2$ kinetics at the higher pedal cadence might also reflect increased fast-twitch fiber recruitment (7), because fast-twitch fibers are believed to exhibit slower $\dot{V}O_2$ kinetics (17, 40). Alternatively, the higher baseline metabolic rate that is elicited by cycling at a higher pedal cadence might have slowed phase II $\dot{V}O_2$ kinetics because of the altered energetic state (9, 12).

Although our recent study indicated that supplementation with BR speeded $\dot{V}O_2$ kinetics in the upper step of a double step test, where a greater proportional recruitment of type II muscle fibers would be expected (38, 39), muscle fiber recruitment patterns were not directly addressed in that study (11). Therefore, further research was required to address the potential for augmented physiological and functional effects in human type II muscle fibers following NO_3^- supplementation. Given the differences in the power-velocity relationship between disparate skeletal muscle fiber populations (54), contraction velocity is another intervention that has been employed to manipulate the proportional contribution of different muscle fiber types to power output during exercise, with the contribution of type II muscle fibers to power output expected to be increased at higher contraction velocities (6, 7; but see 1). In this study, supplementation with BR did not significantly impact muscle oxygenation, $\dot{V}O_2$ kinetics, or exercise tolerance when cycling at 35 rpm. However, muscle [O_2Hb] was increased at baseline and throughout exercise, phase II $\dot{V}O_2$ kinetics was 23% faster, and exercise tolerance was extended by 22% in 115-BR compared with 115-PLA. Therefore, taken together with our previous findings (12) and consistent with recent results using murine models (24, 25, 34), the results of the current study demonstrate a preferential effect of NO_3^- on physiological responses in type II muscle in humans.

The findings from this study suggest that the faster phase II $\dot{V}O_2$ kinetics in 115-BR compared with 115-PLA might have been linked to increased delivery of O_2 to the muscle microvasculature. Since the slower $\dot{V}O_2$ kinetics in the 115-rpm trials compared with the 35-rpm trials appears to be linked to slower muscle O_2 delivery (as described above), the increase in muscle O_2 delivery at the higher cadence with BR might have partly corrected for the compromised muscle O_2 delivery permitting faster $\dot{V}O_2$ kinetics in 115-BR compared with 115-PLA. Compared with type I muscle, type II muscle exhibits a shortfall in O_2 delivery relative to O_2 demand mandating increased muscle O_2 extraction, particularly at higher contraction intensities (47). In turn, this lowers the microvascular PO_2 and thus the driving pressure for blood-to-myocyte O_2 flux (47). Providing the higher cadence condition in the current study increased the contribution of type II muscle fibers to force production (6, 7; but see 1), the increased muscle [O_2Hb] with BR supplementation at the higher cadence might have attenuated the mismatch between muscle O_2 supply and muscle O_2 demand, facilitating a higher driving pressure for blood-to-myocyte O_2 flux and a more rapid adjustment of $\dot{V}O_2$ during the step exercise test. This interpretation is in keeping with the O_2 'tipping point' theory proposed by Poole and Jones (51), which stipulates that phase II $\dot{V}O_2$ kinetics is only speeded by interventions which enhance muscle O_2 delivery when muscle O_2 delivery is initially limited, at least in healthy adults. Alternatively, or in conjunction with enhanced fast-twitch muscle perfusion, inorganic NO_3^- supplementation has been shown to enhance calcium-handling proteins in fast-twitch muscle and to increase cytoplasmic [calcium] (34). This might provide a greater stimulus for oxidative phosphorylation in fast-twitch muscle fibers, potentially promoting faster $\dot{V}O_2$ kinetics in 115-BR (33). It is also possible that the higher baseline metabolic rate evoked by the higher pedal cadence was responsible for the effects of BR at 115 rpm, but not 35 rpm, in this study (12). Bowen et al. (9) suggested that elevating metabolic rate during cycling exercise negatively impacted the intramuscular energy state leading to slower phase II $\dot{V}O_2$ kinetics. Since NO_3^- supplementation has been shown to blunt intramuscular adenosine diphosphate and inorganic phosphate accumulation, and phosphocreatine utilization during exercise (3), the faster $\dot{V}O_2$ kinetics and improved exercise tolerance in 115-BR might be linked to an improved intramuscular energy state, regardless of muscle fiber recruitment patterns, in the higher cadence condition.

A more rapid adjustment in oxidative metabolism following the onset of exercise would be expected to spare the utilization of the finite anaerobic energy reserves and attenuate the accumulation of fatigue-related metabolites, thereby promoting enhanced exercise tolerance (13, 36). This is supported by our observation that exercise tolerance was enhanced by ~22% when phase II $\dot{V}O_2$ kinetics was speeded by BR (i.e., at 115 rpm where the phase II τ was ~23% shorter after supplementation) and unchanged when no speeding was present (i.e., at

35 rpm; see Fig. 5). Furthermore, the improvements in exercise tolerance and muscle [O_2Hb] at 120 s with BR at 115 rpm were significantly correlated. This suggests that the increase in muscle O_2 delivery with BR was an important contributor to the ergogenic effects of BR at 115 rpm. Plasma [NO_2^-] was increased with BR and since NO_2^- can positively impact on vascular function directly (2), or indirectly through its reduction to NO (44, 57), this is likely to account for any improved muscle O_2 delivery with BR. Indeed, previous studies have shown that NO_3^- supplementation can increase muscle blood flow in the running rat (24) and that NO_2^- infusion can increase blood flow in humans performing forearm exercise (16). The potential for enhanced perfusion with BR is likely to be more pronounced in the microvasculature of fast-twitch muscle, where PO_2 during contractions is lower (8, 47), since the reduction of NO_2^- to NO is augmented as PO_2 declines (14). This might explain why NO_3^- supplementation has been shown to preferentially distribute blood flow toward (24), and increase microvascular PO_2 within (26), the more fatigue-susceptible fast-twitch muscle fibers in rats and to speed phase II $\dot{V}O_2$ kinetics when a greater proportion of fast-twitch muscle fibers are likely to be recruited (12). Therefore, assuming the recruitment of fast-twitch muscle was greater at the higher cadence (6, 7; but see 1), this might account for the increased muscle [O_2Hb] in 115-BR, but not 35-BR, in this study. This increased muscle O_2 delivery in 115-BR likely reduced the mismatch between muscle O_2 delivery and muscle O_2 utilization at the higher pedal cadence which, in turn, promoted a more rapid adjustment of phase II $\dot{V}O_2$ kinetics and improved exercise tolerance in 115-BR relative to 115-PLA.

There is evidence that NO_3^- supplementation can increase force during the initial stages of high-frequency maximal stimulation (31) and at high but not low contraction velocities (15) in human skeletal muscle in vivo. Moreover, NO_3^- supplementation can increase the rate of force development during high-frequency stimulation in mouse skeletal muscle in vitro (34). Importantly, our data extend these observations by suggesting that NO_3^- supplementation also improves muscle O_2 delivery, $\dot{V}O_2$ kinetics, and fatigue resistance to a greater extent as contraction frequency is increased. Taken together, these findings suggest that the effects of NO_3^- supplementation on skeletal muscle vascular function, metabolism, and force production are enhanced at higher contraction velocities and frequencies. It is likely that these positive physiological responses account for the improved fatigue resistance observed with BR at 115 rpm, but not 35 rpm, in the current study. The results of this study might have important implications for improving skeletal muscle fatigue resistance at a high muscle contractile frequency or velocity, when greater recruitment of type II muscle fibers and a lower contractile efficiency might be expected (54). While these findings might have performance implications for recreationally active subjects, this might not necessarily be the case for well-trained endurance athletes with a lower percentage of type II muscle fibers, despite the fact that trained cyclists typically self-select a higher pedal cadence during competition (21, 22). However, since patients with type II diabetes (50), chronic heart failure (55), and chronic obstructive pulmonary disease (45) have a greater percentage of type II muscle fibers, our findings might suggest that muscle oxygenation, $\dot{V}O_2$ kinetics, and exercise tolerance could be increased in these populations following short-term inorganic NO_3^- supplementation. Further research is required to elucidate the efficacy of inorganic NO_3^- supplementation on vascular, metabolic, and functional responses in populations with different muscle fiber compositions and/or recruitment patterns.

In conclusion, short-term dietary supplementation with inorganic NO_3^- increased muscle [O_2Hb] during cycling at 115 rpm, without changing muscle [Hb_{tot}], suggesting improved muscle oxygenation. This increase in muscle oxygenation was accompanied by faster phase II $\dot{V}O_2$ kinetics and was correlated with improved exercise tolerance in 115-BR. There were no changes in muscle oxygenation, phase II $\dot{V}O_2$ kinetics, and exercise tolerance with BR when cycling at very slow pedal cadence (35 rpm). Considered together, our findings indicate that NO_3^- supplementation is more effective at improving muscle microvascular [O_2Hb], pulmonary $\dot{V}O_2$ kinetics, and exercise tolerance during cycle ergometry exercise when a higher pedal cadence is employed. These findings might have important implications for speeding the adjustment of pulmonary $\dot{V}O_2$ and improving fatigue resistance in exercise settings where more type II muscle fibers are recruited and/or muscle O_2 delivery is compromised.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

S.J.B., R.L.V., F.J.D., B.C.B., L.J.W., and A.M.J. conception and design of research; S.J.B., R.L.V., and L.J.W. analyzed data; S.J.B., R.L.V., F.J.D., B.C.B., L.J.W., and A.M.J. interpreted results of experiments; S.J.B. prepared figures; S.J.B., R.L.V., F.J.D., B.C.B., and A.M.J. drafted manuscript; S.J.B., F.J.D., B.C.B., L.J.W., and A.M.J. edited and revised manuscript; S.J.B., R.L.V., F.J.D., B.C.B., L.J.W., and A.M.J. approved final version of manuscript; R.L.V. performed experiments.

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