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Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study

Katherine E. Lansley,1 Paul G. Winyard,2 Jonathan Fulford,2 Anni Vanhatalo,1 Stephen J. Bailey,1 Jamie R. Blackwell,1 Fred J. DiMenna,1 Mark Gilchrist,2 Nigel Benjamin,2 and Andrew M. Jones1

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Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. J Appl Physiol 110: 591–600, 2011. First published November 11, 2010; doi:10.1152/japplphysiol.01070.2010.—Dietary supplementation with beetroot juice (BR) has been shown to reduce resting blood pressure and the O2 cost of submaximal exercise and to increase tolerance to high-intensity cycling. We tested the hypothesis that the physiological effects of BR were consequent to its high NO3 content per se, and not the presence of other potentially bioactive compounds. We investigated changes in blood pressure, mitochondrial oxidative capacity (Qmax), and physiological responses to walking and moderate- and severe-intensity running following dietary supplementation with BR and NO3-depleted BR [placebo (PL)]. After control (nonsupplemented) tests, nine healthy, physically active male subjects were assigned in a randomized, double-blind, crossover design to receive BR (0.5 l/day, containing ~6.2 mmol of NO3) and PL (0.5 l/day, containing ~0.003 mmol of NO3) for 6 days. Subjects completed treadmill exercise tests on days 4 and 5 and knee-extension exercise tests for estimation of Qmax (using 31P-magnetic resonance spectroscopy) on day 6 of the supplementation periods. Relative to PL, BR elevated plasma NO3 concentration (183 ± 119 vs. 373 ± 211 nM, P < 0.05) and reduced systolic blood pressure (129 ± 9 vs. 124 ± 10 mmHg, P < 0.01). Qmax was not different between PL and BR (0.93 ± 0.05 and 1.05 ± 0.22 mM/s, respectively). The O2 cost of walking (0.87 ± 0.12 and 0.70 ± 0.10 l/min in PL and BR, respectively, P < 0.01), moderate-intensity running (2.26 ± 0.27 and 2.10 ± 0.28 l/min in PL and BR, respectively, P < 0.01), and severe-intensity running (end-exercise O2 uptake = 3.77 ± 0.57 and 3.50 ± 0.62 l/min in PL and BR, respectively, P < 0.01) was reduced by BR, and time to exhaustion during severe-intensity running was increased by 15% (7.6 ± 1.5 and 8.7 ± 1.8 min in PL and BR, respectively, P < 0.01). In contrast, relative to control, PL supplementation did not alter plasma NO3 concentration, blood pressure, or the physiological responses to exercise. These results indicate that the positive effects of 6 days of BR supplementation on the physiological responses to exercise can be ascribed to the high NO3 content per se.

Exercise; nitric oxide; magnetic resonance spectroscopy; oxygen uptake kinetics; efficiency; exercise tolerance

A DIET RICH IN VEGETABLES is well documented to have cardiovascular benefits and to be associated with a longer lifespan (1). It has been suggested that these effects might be attributable, at least in part, to the high NO3 content of vegetables, particularly leafy greens and beetroot (25). That dietary NO3 can reduce blood pressure and, thus, potentially be cardioprotective is well established (20, 62). However, evidence is emerging that dietary NO3 supplementation may also positively impact the physiological responses to exercise. It was originally reported that dietary supplementation with pharmacological sodium nitrate (0.1 mmol·kg−1·day−1) resulted in a significant reduction in the O2 cost of submaximal cycling (40). A similar improvement in cycling economy and an improved tolerance to high-intensity cycling were reported when NO3 was administered in the form of beetroot juice (BR; 0.5 l/day, ~5.5 mmol NO3/day) (3). We subsequently demonstrated that the reduced O2 cost of submaximal exercise following BR consumption is consequent to a reduced ATP cost of muscle force production (2) and that the enhanced efficiency is evident acutely (2.5 h following a single 0.5-liter BR dose) and for at least 15 days when supplementation is continued (60).

The improvement in exercise efficiency with BR supplementation (2, 3, 60) has led to the assumption that BR exerts its effects via the metabolic conversion of inorganic NO3 to bioactive NO2 and nitric oxide (NO) (6, 44). However, given that beetroot is rich in several potentially metabolically active compounds, it is unclear whether the cardiovascular and physiological changes observed following BR supplementation can be ascribed exclusively to its high NO3 content. For example, the amino acid betaine, which is present in beetroot, has been used in the treatment of cardiovascular disease (9, 61), and betaine supplementation has been reported to elicit improvements in muscular endurance, strength, and power (23, 45). In addition, beetroot is rich in several polyphenols, of which quercetin and resveratrol have been linked with mitochondrial biogenesis and an associated increase in aerobic capacity (17, 38; cf. 16, 19). The high antioxidant content of beetroot may also provide protection against exercise-induced oxidative stress (31). Consequently, BR supplementation has the potential to affect exercise efficiency and performance via numerous pathways.

The purpose of the present study was threefold. 1) We wished to determine whether the physiological effects of BR supplementation (i.e., reduced blood pressure, lower O2 cost of submaximal exercise, and enhanced tolerance to high-intensity exercise) were consequent to the high NO3 content of BR. We recently developed a process that selectively removes the NO3 from BR using a commercially available resin (M. Gilchrist, N. Benjamin, and P. G. Winyard), while leaving it identical in color, taste, smell, and texture. This important advance enables us to isolate the effects of dietary NO3 from the other potential “active ingredients” found in BR, using a genuinely double-blind experimental design. 2) We wished to investigate the extent to which elevation of NO bioavailability through BR consumption might increase mitochondrial biogenesis (14, 48) and, thus, contribute to the reported improvements in aerobic

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exercise performance (2, 3, 60). Therefore, using 31P-magnetic resonance (MR) spectroscopy (MRS), we estimated muscle oxidative (mitochondrial) capacity (Qmax) following BR supplementation from the maximum rate of phosphocreatine (PCr) resynthesis following high-intensity exercise (Qmax). 3) We wished to extend our previous findings in knee-extension (2) and cycling (3, 60) exercise to walking and running, thereby broadening the potential application of this intervention.

We investigated the influence of dietary NO3-rich BR and NO3-depleted BR [placebo (PL)] supplementation on plasma NO2 concentration ([NO2−]), a biomarker of NO production) (37, 42), blood pressure, and O2 uptake (VO2) dynamics during step transitions from walking to moderate- and severe-intensity running. We hypothesized that, relative to PL, dietary BR supplementation would 1) increase plasma [NO2−] and reduce blood pressure, 2) reduce the O2 cost of walking and running and increase exercise tolerance, measured as the time to task failure, and 3) increase muscle oxidative capacity, as assessed by Qmax. We also hypothesized that the NO3-depleted PL would not alter these physiological indexes relative to the preintervention control measurements, indicating that the physiological effects of short-term (4–6 days) BR supplementation are related to the NO3− content.

METHODS

Subjects. Nine healthy, physically active men (mean ± SD: 22 ± 4 yr old, 69.3 ± 7.2 kg body mass, 1.77 ± 0.06 m stature, 55 ± 7 ml·kg−1·min−1 maximal O2 uptake [VO2max]) volunteered to participate in the study. Subjects gave their written informed consent to participate after the experimental procedures, associated risks, and potential benefits of participation had been explained. All procedures were approved by the Institutional Research Ethics Committee. In the 24 h preceding the first exercise test, subjects recorded their food intake, and this diet was replicated in the 24 h preceding subsequent tests. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, ≥3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Participants were asked to refrain from caffeine for 6 h and from alcohol for 24 h before each test. The subjects also abstained from using antibacterial mouth-wash and chewing gum throughout the study (21).

Experimental overview. The subjects reported to the laboratory on 10 occasions over a 4- to 5-wk period. Subjects initially completed a preliminary incremental treadmill test for determination of VO2max followed by “step” running tests over 2 consecutive days with no dietary supplementation (preintervention control). During these tests, plasma [NO2−], blood pressure, blood lactate concentration ([lactate]), heart rate (HR), pulmonary VO2 dynamics, and time to task failure were measured. Subjects were then assigned in a double-blind, randomized, crossover design to consume 0.5 l/day of NO3-rich BR or NO3-depleted BR (PL) for 6 days. Subjects repeated the step running tests at the same absolute speeds on days 4 and 5 of supplementation.

On the last day of each supplementation period, subjects performed knee-extension exercise in the bore of a 1.5-T superconducting magnet (see below) for the estimation of Qmax (using 31P-MRS).

Exercise tests. All treadmill exercise testing sessions were carried out in a well-ventilated laboratory at 20–22°C on a slat-belt treadmill (PSS-55 Sport, Woodway, Weil am Rhein, Germany) set at a 1% gradient (28). Subjects initially performed a ramp incremental exercise test for the determination of VO2max and the gas exchange threshold (GET). The protocol began with subjects walking at 7 km/h for 6 min; then the belt speed was increased by 1 km/h every minute until volitional exhaustion. The breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged over consecutive 10-s periods. The VO2max was taken as the highest 30-s mean value attained prior to the subject’s volitional exhaustion. The GET was determined as described previously (3, 5). Subsequently, the treadmill speeds that would require 80% of the GET (moderate-intensity exercise) and 75% of the GET (severe-intensity exercise) were calculated, with account taken of the mean response time for VO2 during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the running speeds at GET and VO2max) (63).

Subjects completed step tests during the nonsupplemented control condition and on days 4 and 5 of both supplementation periods for the determination of pulmonary VO2 dynamics (Fig. 1). The protocol on day 4 of supplementation consisted of two 6-min bouts of moderate-intensity running (80% GET) and one exhaustive bout of severe-intensity running (75%Δ) as a measure of exercise tolerance. During the exhaustive bout, the subjects were verbally encouraged to continue for as long as possible. On day 5 of supplementation, the participants returned to the laboratory and completed two 6-min bouts of moder-
ate-intensity running (80% GET) and one 6-min bout of severe-intensity running (75%Δ). All exercise bouts involved an abrupt transition to the target speed initiated from a walking baseline (4 km/h), and exercise bouts were separated by 10 min of walking at 4 km/h. The VO2 responses from like-transitions were averaged before analysis to enhance the signal-to-noise ratio and improve confidence in the parameters derived from the modeling process (39, 64).

On day 6 of each supplementation period, subjects were required to complete an incremental, single-legged, knee-extension exercise test while lying prone inside a 1.5-T superconducting MR scanner (Philips Gyroscan Clinical Intera). Subjects were familiarized with the knee-extension exercise prior to testing. The exercise protocol consisted of unilateral knee extensions with the right leg with use of a custom-built nonferrous ergometer. The foot was fastened securely with Velcro straps to a padded foot brace, which was connected to the ergometer load basket via a simple rope-and-pulley system. Knee extensions over −0.22 m were performed continuously at a constant frequency, set in unison with the magnetic pulse sequence (40 pulses/min) to ensure that the quadriceps muscles were positioned in approximately the same phase of contraction during each MR pulse acquisition. As the MRSsequence during the exercise protocol is pulse-acquired, rather than spatially localized, the signal originates from the muscle lying within the sensitive region of the coil and, thus, is relatively insensitive to subject motion. However, to prevent displacement of the quadriceps relative to the MRS coil during the exercise, leading to sampling of a variable muscle volume, Velcro straps were fastened over the subject’s legs, hips, and lower back. After a 2-min rest period, subjects performed a 42-s exercise bout at 81.2 ± 4.3% of individual maximum (21.7 ± 1.8 W), designed to reduce muscle PCR concentration ([PCr]) without substantially altering muscle pH, for the estimation of Q max. Then, after a further 5-min rest period, subjects completed an incremental test to volitional exhaustion. The basket load was increased in 0.5-kg steps every 30 s until the subject was unable to continue. Knee-extensor displacement was measured using a calibrated optical shaft encoder (model BDK.06.05A 100-5-4, Baumer Electric, Swindon, UK) connected to the weight basket pulley, and load was measured using an aluminum load cell (model F250EBR09HN, Novatech Measurements, St. Leonards-on-Sea, East Sussex, UK). The product of force and distance was used to calculate work done continuously during the knee-extensor exercise.

Supplementation protocol. After completion of the nonsupplemented control condition, subjects were assigned in a double-blind, randomized, crossover design to receive 6 days of dietary supplementation with NO3-rich BR (0.5 l/day organic BR containing −6.2 mmol of NO3; Beet it, James White Drinks, Ipswich, UK) or PL (0.5 l/day organic NO3-depleted BR containing −0.0034 mmol of NO3; Beet it). The PL treatment was designed to match the amount of NO3 ions. Five subjects began with the BR condition, and the other four subjects began with the PL condition. The subjects were instructed to consume the beverages slowly 3 h prior to each exercise test. A 10-day washout period separated each supplementation period. Throughout each study, subjects were instructed to maintain their normal daily activities and food intake. This is in contrast to previous studies (2, 3, 40, 41), in which subjects were instructed to minimize the consumption of NO3-rich foods throughout the study period.

Measurements. Prior to each testing session, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, FL), with subjects in a seated, seated position. Four measurements were recorded, and the mean of the final three measurements was used in subsequent analysis. Plasma [NO3] was used as a biomarker for NO availability (37, 42). Venous blood samples (~4 ml) were drawn into lithium-heparin tubes (7.5 ml Monovette Lithium Heparin, Sarstedt, Leicester, UK), which have very low levels of NO3 (0.89 ± 0.35 μM) and NO2 (0.05 ± 0.01 μM). Samples were centrifuged at 4,000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO2]. All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO2 prior to analysis. After they were thawed at room temperature, plasma samples were initially deproteinized using cold ethanol precipitation. The ethanol was chilled to 0°C, and 1 ml of cold ethanol was added to 0.5 ml of plasma sample; then the sample was vortexed and left to stand at 0°C for 30 min. Thereafter, samples were centrifuged at 14,000 rpm for 5 min, and the supernatant was removed. The [NO3] of the deproteinized plasma samples was determined using a modification (3) of the chemiluminescence technique (4).

During all exercise tests, pulmonary gas exchange and ventilation were measured continuously using a metabolic measurement system (MetaMax 3B, Cortex Biophysics, Leipzig, Germany). A digital vol-
distance from the coil as the P solution, allowing the calculation of muscle P concentration, following corrections for relative coil loading. Absolute values of [PCr] and ATP concentration were subsequently calculated via the ratio of P to PCr and P to ATP. Intracellular pH was calculated using the chemical shift of the P spectral peak relative to the PCr peak (57). ADP concentration ([ADP]) was calculated as described by Kemp et al. (34). Baseline and end-exercise [PCr], [P], and [ADP] were calculated over the last 30 s of the rest or exercise period.

Data analysis procedures. Qmax was calculated as described by Layec et al. (43). Briefly, a time constant (k) was determined to describe the rate of PCr recovery by fitting a single-exponential function to the [PCr] recorded following the 42-s exercise bout, where the intensity was sufficiently low to ensure no substantial reduction in pH relative to baseline. From this, the Qmax was given by

$$Q_{\text{max}} = k[PCr]_0(1 + K_m/\text{[ADP]}_{\text{end}})$$

where [PCr]0 is the depletion in [PCr], [ADP]end is the [ADP] at the end of exercise, and Km is [ADP] at half-maximal oxidation rate, which is assumed to be 30 μM in skeletal muscle (33).

The breath-by-breath V̇O2 data from each test were initially examined to exclude errant breaths, and those values lying >4 SDs 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, identical repetitions were time-aligned to the start of exercise and ensemble-averaged. The two severe-intensity running bouts were of different durations (until volitional exhaustion in the first bout and 6 min in the second bout); so, at this intensity, only the first 6 min of data were averaged and modeled. The first ~20 s of data after the onset of exercise (i.e., the phase I response) were deleted, and a nonlinear least-squares algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the V̇O2 responses to moderate exercise, and a biexponential model was used for severe exercise, as described in the following equations

$$V\dot{O}_2(t) = V\dot{O}_2_{\text{baseline}} + A_p[1 - e^{-(t-TDp)/\tau_p}]$$

and

$$V\dot{O}_2(t) = V\dot{O}_2_{\text{baseline}} + A_p[1 - e^{-(t-TDp)/\tau_p}] + A_s[1 - e^{-(t-TDs)/\tau_s}]$$

where $V\dot{O}_2(t)$ represents the absolute $V\dot{O}_2$ at a given time t; $V\dot{O}_2_{\text{baseline}}$ represents the mean $V\dot{O}_2$ in the baseline period; $A_p$, TDp, and $\tau_p$ represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in $V\dot{O}_2$ above baseline; and $A_s$, TDs, and $\tau_s$ represent the amplitude of, time delay before the onset of, and time constant describing the development of the $V\dot{O}_2$ slow component, respectively.

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. $V\dot{O}_2_{\text{baseline}}$ was defined as the mean $V\dot{O}_2$ measured over the final 90 s of baseline walking. The end-exercise $V\dot{O}_2$ was defined as the mean $V\dot{O}_2$ measured over the final 30 s of exercise. Because the asymptotic value $A'_s$ of the exponential term describing the $V\dot{O}_2$ slow component may represent a higher value than is actually reached at the end of exercise, the actual amplitude of the $V\dot{O}_2$ slow component was defined as $A_s$. The $A'_s$ parameter was compared with the same isotope (360 s) under the three experimental conditions. The amplitude of the $V\dot{O}_2$ slow component was also described relative to the entire $V\dot{O}_2$ response. To determine the overall kinetics of the $V\dot{O}_2$ response for moderate- and severe-intensity exercise, data were fit with a monoeponential model from 0 s to end exercise, without time delay.

The baseline $V\dot{O}_2$ output ($V\dot{CO}_2$), respiratory exchange ratio (RER), and minute ventilation ($V_e$) were calculated as mean values over the final 60 s preceding the start of exercise, and end-exercise values were calculated as mean values over the final 30 s. We also modeled the HR response to exercise in each condition. For this analysis, HR data were linearly interpolated to provide second-by-second values, and, for each individual, identical repetitions from like-transitions were time-aligned to the start of exercise and ensemble-averaged. Nonlinear least-squares mono- and biexponential models without TD were used to fit the data to moderate- and severe-intensity exercise respectively, with the fitting window commencing at 0 s. The HR $\tau$ so derived provides information on the overall HR response dynamics.

Statistical analyses. Effects on the relevant physiological variables were assessed using one-way repeated-measures ANOVAs across the control, BR, and PL supplementation periods. Two-way (treatment-by-time) ANOVAs were used to identify differences in plasma [NO2] and blood pressure across days 4–6 of the control and supplementation conditions. Where significant main effects were detected, Bonferroni-adjusted paired t-tests were used to identify specific differences. All data are presented as means ± SD unless stated otherwise. Statistical significance was accepted at $P < 0.05$.

RESULTS

Plasma [NO2] and blood pressure. The group mean plasma [NO2] in the control condition was 197 ± 184 nM. No significant change was observed following PL supplementation (183 ± 119 nM, $P > 0.05$). However, relative to PL, BR ingestion increased plasma [NO2] by 105% (373 ± 211 nM, $P < 0.05$). A nonsignificant main effect for time indicated that the BR-induced elevations in plasma [NO2] were not different between days 4, 5, and 6 ($F = 1.08, P = 0.315$).

Compared with control, PL supplementation had no effect on systolic, diastolic, or mean arterial blood pressure. The ingestion of BR significantly reduced systolic blood pressure by 4% relative to placebo (129 ± 9 vs. 124 ± 10 mmHg, $P < 0.01$). The BR-reduced reductions in systolic blood pressure were not significantly different between days 4, 5, and 6 ($F = 1.77, P = 0.221$). Diastolic blood pressure (~66 ± 5 mmHg) and mean arterial pressure (~89 ± 5 mmHg) were not significantly affected by BR ingestion ($P > 0.05$).

Moderate-intensity exercise. Pulmonary $V\dot{O}_2$ responses across the control, PL, and BR conditions are presented in Fig. 2, and the parameters derived from the model fit are summarized in Table 1. One-way repeated-measures ANOVAs revealed significant main effects for treatment across the $V\dot{O}_2$ response, and Bonferroni-adjusted paired t-tests revealed no significant differences between control and PL supplementation for any $V\dot{O}_2$ variables (Fig. 2A). Relative to PL, BR supplementation reduced $V\dot{O}_2$ during the baseline walking period by −12% (0.87 ± 0.12 vs. 0.77 ± 0.10 l/min, $P < 0.01$). The absolute $V\dot{O}_2$ values over the final 30 s of moderate-intensity running were also significantly lower than PL (~7%) following BR supplementation (2.26 ± 0.27 vs. 2.10 ± 0.28 l/min, $P < 0.01$). In addition to these changes in the baseline and end-exercise $V\dot{O}_2$ values, there was a reduction in the amplitude of the pulmonary $V\dot{O}_2$ response (~4%, 1.37 ± 0.19 and 1.32 ± 0.23 ml/min for PL and BR, respectively, $P < 0.05$) and in the $O_2$ cost of running 1 km (~6%, 244 ± 16 and 229 ± 17 ml·kg⁻¹·km⁻¹ for PL and BR, respectively, $P < 0.01$). The phase II $V\dot{O}_2$ time constant was not significantly altered by BR supplementation (26 ± 5 and 25 ± 5 s for PL and BR, respectively, $P > 0.05$; Table 1). Baseline and end-exercise $V\dot{CO}_2$, $V_e$, RER, HR, and blood [lactate] were not significantly different between conditions (Tables 1 and 2), although there was a trend for a reduced end-exercise $V\dot{CO}_2$ (~4%, $P = 0.16$), end-exercise $V_e$ (~4%, $P = 0.08$), and...
significant change in the amplitude of the subsequent $\dot{V}O_2$ slow component ($0.38 \pm 0.16$ and $0.37 \pm 0.19$ l/min for PL and BR, respectively, $P = 0.98$). BR supplementation resulted in an enhanced exercise tolerance, as demonstrated by an increased time to task failure of $\sim 15\%$ ($7.5 \pm 1.7$, $7.6 \pm 1.5$, and $8.7 \pm 1.8$ min for control, PL, and BR, respectively, $P < 0.01$). All nine subjects were able to exercise for a longer duration following BR than PL supplementation. The baseline and end-exercise $\dot{V}CO_2$, $V_{\text{E}}$, RER, HR, and blood [lactate] were not significantly different between conditions (Tables 1 and 2).

Muscle metabolic measurements. The 42-s bout of intense exercise resulted in a $\sim 9$ mM reduction in muscle [PCR], with a negligible fall in pH. There was no significant difference in the time constant for the [PCR] recovery kinetics following this bout of exercise ($26 \pm 6, 24 \pm 3$, and $24 \pm 4$ s for control, PL, and BR, respectively, $P > 0.05$; Fig. 4). Moreover, there was no difference in the estimated $Q_{\text{max}}$ between the conditions ($1.08 \pm 0.22, 0.93 \pm 0.05$, and $1.05 \pm 0.22$ m/M/s for control, PL, and BR, respectively, $P > 0.05$). Muscle metabolite concentrations at rest, following 42 s of intense exercise and

end-exercise RER ($0.83 \pm 0.05$ and $0.86 \pm 0.03$ for PL and BR, respectively, $P = 0.08$) following BR supplementation.

Severe-intensity exercise. The pulmonary $\dot{V}O_2$ responses during severe-intensity exercise in the nonsupplemented control condition and following PL and BR supplementation are shown in Fig. 3, and the $\dot{V}O_2$ parameters derived from the biexponential model fit are presented in Table 1. Similar to the effects observed for moderate-intensity exercise, Bonferroni-adjusted paired $t$-tests revealed no differences in $\dot{V}O_2$ variables between control and PL supplementation (Fig. 3A). BR supplementation resulted in a $\sim 14\%$ reduction in $\dot{V}O_2$ during walking relative to placebo ($0.91 \pm 0.13$ vs. $0.78 \pm 0.09$ ml/min, $P < 0.05$) and a $\sim 7\%$ reduction in the end-exercise $\dot{V}O_2$ during the 6-min severe-intensity running bouts ($3.77 \pm 0.57$ vs. $3.50 \pm 0.62$ l/min, $P < 0.01$). The $\dot{V}O_2$ obtained at task failure was $\sim 6\%$ lower following BR supplementation ($3.89 \pm 0.57$ and $3.67 \pm 0.65$ l/min for PL and BR, respectively, $P < 0.01$). The phase II $\dot{V}O_2$ time constant was not significantly altered following BR supplementation ($21 \pm 3$ and $22 \pm 3$ s for PL and BR, respectively, $P > 0.05$). The reduction in the amplitude of the primary $\dot{V}O_2$ response with BR supplementation ($\sim 3\%$) was not significant ($2.58 \pm 0.36$ and $2.50 \pm 0.40$ l/min for PL and BR, respectively, $P = 0.38$), and there was no
reduced the O2 cost of walking and moderate- and severe-intensity running. An important advance in the present study was exercise economy and performance during treadmill walking reports (2, 3, 60) and extend them by demonstrating improved knee-extension exercise. These results confirm our earlier during constant-speed severe-intensity running and incremen-

Table 2. Heart rate and blood lactate responses to moderate- and severe-intensity exercise following the control condition and BR and PL supplementation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PL</th>
<th>BR</th>
</tr>
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<tbody>
<tr>
<td><strong>Heart rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walking baseline, bpm</td>
<td>89 ± 13</td>
<td>93 ± 10</td>
<td>88 ± 12</td>
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<tr>
<td>End, bpm</td>
<td>136 ± 16</td>
<td>139 ± 13</td>
<td>133 ± 15</td>
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<tr>
<td>Time constant, s</td>
<td>56 ± 55</td>
<td>33 ± 18</td>
<td>40 ± 23</td>
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<td>Amplitude, bpm</td>
<td>47 ± 8</td>
<td>44 ± 8</td>
<td>44 ± 6</td>
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<tr>
<td><strong>Blood [lactate]</strong></td>
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</tr>
<tr>
<td>Blood lactate, mM</td>
<td>1.1 ± 0.4</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.4</td>
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<tr>
<td>End, mM</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.6</td>
<td>1.7 ± 0.9</td>
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<tr>
<td>∆, mM</td>
<td>0.5 ± 0.5</td>
<td>0.3 ± 0.5</td>
<td>0.5 ± 0.7</td>
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**Severe-intensity exercise**

<table>
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<th></th>
<th>Control</th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walking baseline, bpm</td>
<td>104 ± 15</td>
<td>111 ± 11</td>
<td>105 ± 14</td>
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<td>End, bpm</td>
<td>192 ± 6</td>
<td>191 ± 4</td>
<td>188 ± 4</td>
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<tr>
<td>Exhaustion, bpm</td>
<td>197 ± 8</td>
<td>195 ± 4</td>
<td>196 ± 4</td>
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<tr>
<td>Time constant, s</td>
<td>26 ± 19</td>
<td>27 ± 18</td>
<td>28 ± 13</td>
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<tr>
<td>Blood lactate, mM</td>
<td>1.0 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>End, mM</td>
<td>8.1 ± 1.7</td>
<td>7.1 ± 0.9</td>
<td>8.0 ± 2.2</td>
</tr>
<tr>
<td>∆, mM</td>
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<td>5.8 ± 0.9</td>
<td>6.1 ± 3.1</td>
</tr>
<tr>
<td>Exhaustion, mM</td>
<td>8.6 ± 1.4</td>
<td>8.6 ± 0.8</td>
<td>9.0 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. [Lactate], lactate concentration.

subsequent recovery, and at the completion of the incremental exercise test are reported in Table 3 for the control condition and following PL and BR supplementation. The time to task failure during incremental knee-extension exercise was significantly longer following 6 days of BR than in control and PL conditions (8.2 ± 0.9, 8.2 ± 0.9, and 8.5 ± 0.8 min for control, PL, and BR, respectively, F = 3.71, P < 0.05). There was no significant difference in the metabolite concentrations measured at end exercise (Table 3).

**DISCUSSION**

The principal findings of this study are that short-term (4–6 days) dietary supplementation with NO3-rich beetroot juice 1) increased plasma [NO2-] and reduced systolic blood pressure, 2) reduced the O2 cost of walking and moderate- and severe-intensity running, and 3) increased the time to task failure during constant-speed severe-intensity running and incremental knee-extension exercise. These results confirm our earlier reports (2, 3, 60) and extend them by demonstrating improved exercise economy and performance during treadmill walking and running. An important advance in the present study was the use of a NO3-depleted PL beverage, which ensured that the study was conducted in a genuinely double-blind fashion. We found that dietary supplementation with NO3-depleted BR did not alter plasma [NO2-], systolic blood pressure, exercise VO2, or the time to task failure, relative to the nonsupplemented control condition. These data therefore indicate that the physiological effects of BR supplementation reported here and previously (2, 3, 60) are consequent to the high NO3 content of the beverage. In the present study, we also measured Qmax using 31P-MRS to estimate muscle oxidative capacity. Although there are several assumptions and limitations to this approach (see Effects of dietary NO3 supplementation on mitochondrial capacity), Qmax was not significantly different between the control, PL, and BR conditions. These data therefore suggest that the enhanced aerobic exercise performance (i.e., increased time to task failure) following short-term BR supplementation is unlikely to be related to possible effects of increased NO bioavailability on mitochondrial biogenesis (14, 48).

Effects of dietary NO3 on plasma [NO2-] and blood pressure. During the NO3-rich BR supplementation period, plasma [NO2-] was increased by 105% and systolic blood pressure was reduced by 5 mmHg (4%) in the normotensive young adults who participated in the study. This change occurred without alterations in diastolic blood pressure or mean arterial pressure, consistent with another recent study that used a similar intervention (3). Other studies reported a reduction in systolic and diastolic blood pressure after 2.5 h (62), 3 days (40), and 15 days (60) of dietary NO3 supplementation. Collectively, these data suggest that consumption of NO3-rich vegetables might confer benefits to cardiovascular health (1, 20, 53). Systolic and diastolic blood pressure were unchanged during PL supplementation, suggesting that the reduction in systolic blood pressure with NO3-rich BR was mediated through the systemic reduction of NO3-derived NO2 to NO (6, 11, 18).

Fig. 3. Pulmonary VO2 responses during step increments to a severe-intensity running speed. A: group mean VO2 responses following nonsupplemented control and placebo supplementation, with error bars shown every 30 s for clarity. B: group mean VO2 response following NO3-rich beetroot (nitrate) and placebo supplementation. Dashed vertical line represents abrupt transition from baseline walking to severe work rate. Insets: group mean ± SE VO2 at task failure (*dietary NO3 supplementation resulted in an increased time to task failure). O2 cost of walking and running is reduced following NO3-rich beetroot juice, but not following NO3-depleted beetroot juice (placebo), supplementation.
Effects of dietary NO₃ supplementation on the physiological responses to moderate-intensity exercise. Consistent with previous studies employing other exercise modalities (2, 3, 40, 41, 60), dietary NO₃ supplementation reduced the O₂ cost of submaximal running. However, a novel finding in the present study was that dietary NO₃ intake resulted in a 12% reduction in the O₂ cost of walking. This is in contrast to previous studies that have reported no effect of NO₃ supplementation on VO₂ during very low-intensity exercise, for example, cycling at 20 W (3, 60). The explanation for these different results is unclear. However, a reduced O₂ cost of walking potentially has important implications for enhancing the ability to complete tasks of daily living within elderly and patient populations. These groups can have a significantly reduced peak VO₂ (VO₂ peak) (46, 52); consequently, the activities of daily living often require them to work toward the higher end of their exercise capacity, resulting in severe metabolic stress. If dietary NO₃ supplementation were to reduce the O₂ cost of these activities, it would have the potential to enhance functional capacity and the quality of life. Further research is required to investigate the effects of dietary NO₃ supplementation on the O₂ cost of walking and functional performance within clinical populations.

The steady-state VO₂ during moderate-intensity running was reduced by ~7% following dietary NO₃ supplementation, while the amplitude of the VO₂ response from baseline to steady state was reduced by ~4%. These changes in the O₂ cost of moderate exercise are less pronounced than in our previous study, where dietary NO₃ supplementation reduced the steady-state VO₂ by 10% and the amplitude of the VO₂ response by ~20% during cycle ergometer exercise (3). The reduced effect in the present study is likely to be due, at least in part, to the fact that habitual dietary NO₃ intake was not restricted during the period of experimentation (60). In previous studies (2, 3, 40, 41), subjects were instructed to exclude NO₃-rich foods (such as certain vegetables and cured meats) from their diet. The present results therefore indicate that the positive effects of NO₃ supplementation on blood pressure and exercise economy are still present when habitual NO₃ intake is not restricted.

The reduced VO₂ during moderate exercise in the present study was reflected in a significant reduction (~6%) in the energy cost required to run 1 km. This result is remarkable, as just 4 days of dietary NO₃ supplementation elicited improvements in running economy comparable to those observed following 6–9 wk of physical training (27, 49). Superior running economy is associated with enhanced endurance running performance (15, 26, 27). Although this was not directly tested in the present study, the results suggest that increased dietary NO₃ intake has the potential to enhance exercise tolerance during longer-term endurance exercise.

In the present investigation, we employed a double-blind experimental design using a PL beverage with negligible NO₃ content. The PL beverage, which was created by passage of the juice through an ion-exchange resin that is selective for NO₃ content, was otherwise similar to the experimental beverage in appearance, odor, and taste. While we cannot exclude the possibility that this treatment had other effects in addition to the removal of NO₃, there were no appreciable differences in sodium, potassium, calcium, or magnesium concentrations, and the proton NMR spectra of the BR and PL beverages were similar (Glichrist et al., unpublished observations). In previous studies, it could only be speculated that the physiological effects of BR consumption were mediated through the reduction of NO₃ to bioactive NO₂ and NO. BR is also rich in several other compounds, including betaine, antioxidants, and polyphenols, that might influence human physiology at rest or during exercise. It has been reported that betaine supplementation may enhance muscular endurance, strength, and power (23, 45). Moreover, two polyphenols found in beets, quercetin and resveratrol, have the potential to increase aerobic capacity through stimulation of mitochondrial biogenesis (17, 38), although this is by no means a consistent finding (16, 19). While we cannot rule out the possibility that NO₃ operates synergis-

| Table 3. Muscle metabolite concentrations and pH at resting baseline and during the exercise protocol following the control condition and BR and PL supplementation |
|-------------------------------------------------|-----------------|-----------------|
|                                                  | Control         | PL              |
|                                                  |                 |                 |
| Baseline  |                  |                 |                 |
| PCr, mM   | 33.0 ± 2.7       | 32.4 ± 2.8      | 32.4 ± 3.0      |
| P₄, mM    | 3.7 ± 0.5        | 3.8 ± 0.4       | 3.9 ± 0.5       |
| ADP, μM   | 5.8 ± 0.9        | 6.6 ± 1.2       | 6.2 ± 1.6       |
| pH        | 7.0 ± 0.0        | 7.1 ± 0.0       | 7.1 ± 0.0       |
| End-exercise (42-s bout) |                  |                 |                 |
| PCr, mM   | 23.5 ± 1.7       | 23.5 ± 1.2      | 23.1 ± 1.4      |
| P₄, mM    | 10.2 ± 1.6       | 10.5 ± 1.7      | 10.8 ± 1.8      |
| ADP, μM   | 32.5 ± 7.8       | 36.8 ± 5.6      | 38.6 ± 9.1      |
| pH        | 7.0 ± 0.0        | 7.0 ± 0.0       | 7.0 ± 0.0       |
| End of recovery |                |                 |                 |
| PCr, mM   | 33.0 ± 2.7       | 32.3 ± 2.8      | 32.4 ± 3.0      |
| P₄, mM    | 3.7 ± 0.6        | 3.9 ± 0.4       | 4.0 ± 0.5       |
| ADP, μM   | 6.0 ± 0.8        | 6.5 ± 0.7       | 6.2 ± 1.3       |
| pH        | 7.0 ± 0.0        | 7.1 ± 0.0       | 7.0 ± 0.0       |
| End-exercise (ramp test) |             |                 |                 |
| PCr, mM   | 14.5 ± 4.6       | 14.0 ± 2.7      | 12.7 ± 4.9      |
| P₄, mM    | 15.8 ± 7.1       | 15.4 ± 4.8      | 15.4 ± 4.3      |
| ADP, μM   | 57.6 ± 15.6      | 91.3 ± 57.5     | 82.1 ± 50.0     |
| pH        | 6.7 ± 0.2        | 6.8 ± 0.2       | 6.8 ± 0.1       |

Values are means ± SD. PCr, phosphocreatine.
tically with one or more of these potentially active compounds, the unchanged plasma [NO$_3^-$], blood pressure, and $\dot{V}_{O_2}$ response with NO$_3^-$-depleted BR supplementation suggests that the physiological effects of BR consumption can be attributed, in large part, to its high NO$_3^-$ content of BR. The significant increase in plasma [NO$_3^-$] following dietary NO$_3^-$ intake combined with a reduced blood pressure suggests that inorganic NO$_3^-$ was metabolized in vivo to form NO$_2^-$ and the physiological signaling molecule and potent vasodilator NO (6, 44).

In a recent study, we reported that the reduced O$_2$ cost of moderate-intensity knee-extensor exercise following NO$_3^-$ supplementation was linked to a reduction in the ATP cost of muscle force production (2). However, the precise manner by which this occurs, i.e., through an NO-mediated reduction in the activity of actomyosin-ATPase, Ca$^{2+}$-ATPase, and/or Na$^+$-$K^+$, is unclear.

**Effects of dietary NO$_3^-$ supplementation on the physiological responses to severe-intensity exercise.** Constant-work-rate exercise performed above the GET results in a progressive loss of muscle efficiency, as reflected in the development of a “slow component” of $\dot{V}_{O_2}$ (30), which has been suggested to be related to the process of muscle fatigue (12). An interesting observation in the present study was that there was no change in the amplitude of the $\dot{V}_{O_2}$ slow component following NO$_3^-$ supplementation. This is in contrast with previous studies from our laboratory showing a significant reduction of the $\dot{V}_{O_2}$ slow component following dietary NO$_3^-$ intake in other exercise modalities (2, 3). Because the $\dot{V}_{O_2}$ slow component amplitude is smaller during running than cycling (7, 29), it might be more difficult to observe changes in this parameter of the $\dot{V}_{O_2}$ kinetics following an intervention.

In the present study we observed a 6% reduction in $\dot{V}_{O_2}$ at the point of task failure following NO$_3^-$ supplementation. This reduction in $\dot{V}_{O_2}$ peak is consistent with the observations of Larsen et al. (41) for combined incremental cycle and arm-crank exercise but contrasts with our previous findings for cycle (3, 60) and knee-extension exercise (2). The explanation for this small reduction in $\dot{V}_{O_2}$ peak in the present study is unclear. However, it is evident that this effect is only evident during “whole body” exercise, such as running (present study) or combined cycle/arm-cranking (41), and not during exercise involving a smaller muscle mass, which results in lower, i.e., non-“maximal,” $\dot{V}_{O_2}$ values (56). During whole body exercise, it is widely accepted that the highest attainable $\dot{V}_{O_2}$ is linked to the maximal cardiac output and, thus, muscle blood flow and $O_2$ delivery (54, 55, 66). Exactly how increased NO availability following dietary NO$_3^-$ supplementation might impact the determinants of $\dot{V}_{O_2}_{max}$ is unclear. However, it has been reported that addition of NO$_3^-$ to the perfusate in a rat heart model reduced left ventricular pressure, contractile function, and $\dot{V}_{O_2}$ (51). The reduced $\dot{V}_{O_2}_{max}$ values reported here and also by Larsen et al. (41) were not related to a significant reduction of maximal HR, suggesting that stroke volume or arteriovenous $O_2$ content difference might have been reduced following NO$_3^-$ supplementation. Further studies are required to investigate this possibility.

Despite the reduction in $\dot{V}_{O_2}$ at task failure, NO$_3^-$ supplementation resulted in a ~15% improvement in the time to task failure during severe-intensity running. This indicates that the enhanced exercise economy following dietary NO$_3^-$ supplementation was more than sufficient to offset the reduced $\dot{V}_{O_2}_{max}$. A ~15% improvement in time to task failure would be expected to be equivalent to a ~1% reduction in the time taken to cover a set distance (24), an effect that is certainly meaningful in elite sport. Importantly, an improvement in time to task failure was evident in all nine subjects. An enhanced exercise tolerance was also observed during the incremental knee-extension exercise test. Although the effect was smaller (5% vs. 15%), this is the expected consequence of the different exercise protocols (i.e., incremental vs. constant-work-rate exercise) (60, 65).

**Effects of dietary NO$_3^-$ supplementation on mitochondrial capacity.** An elevated NO bioavailability has the potential to increase mitochondrial biogenesis through the activation of the cGMP-mediated pathway (14, 48). In the present study, 6 days of dietary NO$_3^-$ supplementation resulted in no significant change in muscle PCr recovery kinetics or Q$_{max}$. This suggests that the estimated maximal rate of mitochondrial ATP synthesis was unchanged (35) and, therefore, no substantial mitochondrial biogenesis occurred during the supplementation period.

We cannot rule out the possibility that a more prolonged NO$_3^-$ supplementation period might induce mitochondrial biogenesis and, thereby, enhance aerobic function and exercise performance independently of the more acute effects on muscle efficiency (60). It should also be acknowledged that NO has the potential to inhibit mitochondrial respiration (10, 13), which may consequently affect certain assumptions inherent in the calculation of Q$_{max}$, such as the $K_{in}$ for ADP and the form of the hyperbolic relationship between the oxidative ATP resynthesis rate and [ADP] (36). Moreover, changes in NO availability might modulate blood flow, especially during recovery from exercise (50), and, thus, impact the muscle PCr recovery profile (22). If present, these effects would complicate the comparison and interpretation of the estimated Q$_{max}$ in the BR and PL conditions. However, it is important to note that the initial PCr recovery kinetics, which are considered to be due entirely to oxidative ATP synthesis (8), and the estimated Q$_{max}$ values were not significantly different from the nonsupplemented control condition following BR or PL, despite the expected differences in the potential for NO production between PL and BR conditions. Additional longer-term studies are needed to explore the possible effects of NO$_3^-$ supplementation on mitochondrial biogenesis and muscle oxidative capacity. However, the present data suggest that the reduced O$_2$ cost of exercise and increased time to task failure during exercise observed after short-term NO$_3^-$ supplementation are related to NO$_3^-$- or NO-mediated effects on muscle contractile function (2), rather than changes in mitochondrial volume.

In conclusion, short-term (4–6 days) dietary NO$_3^-$ supplementation (~0.09 mmol/kg) significantly increased plasma [NO$_3^-$] and reduced systolic blood pressure in normotensive young men consuming a normal, balanced diet. The $\dot{V}_{O_2}$ required for constant-work-rate moderate- and severe-intensity running was reduced (by ~7%), and the time to task failure was increased during severe-intensity running (by ~15%) and incremental knee-extension exercise (by ~5%). These results might have important implications for athletic performance enhancement. A striking finding of the present study was the appreciable (12–14%) reduction in the O$_2$ cost of walking following NO$_3^-$ supplementation. For senescent populations or individuals with pulmonary, cardiovascular, or metabolic disorders, a reduction in the O$_2$ cost of daily activities might
significantly improve functional capacity. Relative to the control (nonsupplemented) condition, NO3−-depleted BR did not alter the physiological variables of interest at rest or during exercise. These data indicate that the positive physiological effects of BR ingestion on blood pressure and exercise performance are consequent to the high NO3− content.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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650 DIETARY NITRATE REDUCES O2 COST OF WALKING AND RUNNING


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