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Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise

Anni Vanhatalo,¹ Stephen J. Bailey,¹ Jamie R. Blackwell,¹ Fred J. DiMenna,¹ Toby G. Pavey,² Daryl P. Wilkerson,¹ Nigel Benjamin,² Paul G. Winyard,² and Andrew M. Jones¹

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Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, Benjamin N, Winyard PG, Jones AM. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol* 299: R1121–R1131, 2010. First published August 11, 2010; doi:10.1152/ajpregu.00206.2010.—Dietary nitrate (NO₃⁻) supplementation with beetroot juice (BR) over 4–6 days has been shown to reduce the O₂ cost of submaximal exercise and to improve exercise tolerance. However, it is not known whether shorter (or longer) periods of supplementation have similar (or greater) effects. We therefore investigated the effects of acute and chronic NO₃⁻ supplementation on resting blood pressure (BP) and the physiological responses to moderate-intensity exercise and ramp incremental cycle exercise in eight healthy subjects. Following baseline tests, the subjects were assigned in a balanced crossover design to receive BR (0.5 l/day; 5.2 mmol of NO₃⁻/day) and placebo (PL; 0.5 l/day low-calorie juice cordial) treatments. The exercise protocol (two moderate-intensity step tests followed by a ramp test) was repeated 2.5 h following first ingestion (0.5 liter) and after 5 and 15 days of BR and PL. Plasma nitrite concentration (baseline: 454 ± 81 nM) was significantly elevated (+39% at 2.5 h postingestion; +25% at 5 days; +46% at 15 days; *P* < 0.05) and systolic and diastolic BP (baseline: 127 ± 6 and 72 ± 5 mmHg, respectively) were reduced by ~4% throughout the BR supplementation period (*P* < 0.05). Compared with PL, the steady-state $\dot{V}O_2$ during moderate exercise was reduced by ~4% after 2.5 h and remained similarly reduced after 5 and 15 days of BR (*P* < 0.05). The ramp test peak power and the work rate at the gas exchange threshold (baseline: 322 ± 67 W and 89 ± 15 W, respectively) were elevated after 15 days of BR (331 ± 68 W and 105 ± 28 W; *P* < 0.05) but not PL (323 ± 68 W and 84 ± 18 W). These results indicate that dietary NO₃⁻ supplementation acutely reduces BP and the O₂ cost of submaximal exercise and that these effects are maintained for at least 15 days if supplementation is continued.

beetroot juice; O₂ uptake; ramp exercise; performance; nitric oxide

IT IS WIDELY RECOGNIZED THAT a diet rich in vegetables is beneficial for human health and is associated with a long life span (44). Nitrate (NO₃⁻), which is found in all vegetables and is particularly abundant in leafy greens and beetroot, has emerged as a possible mediating component for the cardiovascular health benefits associated with high vegetable consumption, such as is typical for the Mediterranean diet (13, 18, 32). Dietary nitrate is reduced to bioactive nitrite NO₂⁻ by facultative anaerobic bacteria in the saliva and further to nitric oxide (NO) via various pathways (7, 14, 50). NO has numerous

functions in the body, including the regulation of blood flow, muscle contractility, myocyte differentiation, glucose and calcium homeostasis, and mitochondrial respiration and biogenesis (11, 13, 41).

Recent investigations suggest that the therapeutic potential of increased NO bioavailability may extend beyond the well-known hemodynamic effects (1, 2, 27, 28). Specifically, it has been shown that dietary supplementation with sodium nitrate (0.1 mmol·kg⁻¹·d⁻¹) resulted in a significant reduction in pulmonary oxygen uptake ($\dot{V}O_2$) during submaximal exercise (28). The use of pharmacological sodium nitrate is regulated in many countries as a result of some earlier reports of a link between foods containing nitrate and nitrite (via conversion to *N*-nitrosamines) and cancer in laboratory animals (e.g., 34). This restriction has been questioned by more recent findings elucidating the benefits of nitrate-rich fruits and vegetables in the diet (reviewed in Refs. 16 and 32), and it is known that vegetables such as beetroot provide a natural and healthy source of nitrate, which is widely available. We recently demonstrated that 4–6 days of dietary supplementation with nitrate-rich beetroot juice (BR; 0.5 l/day, equivalent to ~6 mmol/day of NO₃⁻), which resulted in a significant increase in plasma nitrite concentration ([NO₂⁻]), reduced the O₂ cost of exercise at the same submaximal work rate (i.e., improved exercise efficiency) and resulted in an extended time to exhaustion during high-intensity exercise (1). The results of these studies are striking, particularly when considering that the $\dot{V}O_2$ -work rate relationship during submaximal exercise is considered to be largely independent of factors such as age, health status, aerobic fitness, and training status (22, 33).

In previous studies, the nitrate supplementation protocol has involved around 3 days of supplementation (~0.07–0.10 mmol·kg⁻¹·day⁻¹ of NO₃⁻) prior to any exercise testing with restrictions placed on habitual dietary nitrate intake in both the placebo and experimental conditions (1, 27, 28). However, although 4–6 days of NO₃⁻ supplementation results in beneficial effects on blood pressure (BP) and the O₂ cost of moderate exercise compared with a reduced NO₃⁻ intake condition, it is presently not known whether these same effects are evident following shorter or longer supplementation periods. If improvements in exercise efficiency were evident following acute (i.e., single bolus) NO₃⁻ supplementation, this would have important implications for performance enhancement. Also, it is possible that continued dietary nitrate supplementation (i.e., beyond 6 days) might result in further improvements in exercise efficiency. It is also important to investigate whether the effects of dietary nitrate supplementation on ex-

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ercise efficiency are still present when habitual dietary nitrate intake is not restricted during the control condition.

In a recent study, Webb et al. (45) assessed the effects of a single dose of BR on plasma $[\text{NO}_2^-]$ and BP over 24 h. Plasma $[\text{NO}_2^-]$ reached a peak concentration at 3 h postingestion, remained close to this peak value until 5 h postingestion, and returned to baseline after 24 h (45). The systolic and diastolic BP and the mean arterial pressure (MAP) were significantly reduced 2.5 to 3 h after BR intake, and the change in systolic BP was temporally linked to plasma $[\text{NO}_2^-]$. If the reduced O_2 cost of submaximal exercise is similarly linked to plasma $[\text{NO}_2^-]$, this effect should also be detectable within 2.5 to 3 h after BR ingestion. There are currently no published reports on the effects of continued, daily nitrate supplementation on plasma $[\text{NO}_2^-]$ and related effects on BP and exercise efficiency beyond 6 days. Interestingly, sustained exposure of mammalian cells to NO over 6 days has been shown to induce mitochondrial biogenesis through cGMP-dependent pathways (10, 35). An increase in mitochondrial content might be manifested at the systemic level as an increased maximal oxygen uptake ($\dot{V}\text{O}_{2\text{max}}$) and/or gas exchange threshold (GET; analogous with the lactate threshold) (5). Such effects would not be expected following a single nitrate dose, but may be detectable after prolonged supplementation.

The purpose of this study was to investigate the acute (2.5 h) and chronic (up to 15 days) effects of dietary nitrate supplementation on the O_2 cost of moderate-intensity constant-work-rate cycle exercise, and on the GET, $\dot{V}\text{O}_{2\text{max}}$, and peak work rate measured during a ramp incremental test to exhaustion. The following hypotheses were tested: 1) that the plasma $[\text{NO}_2^-]$ would be elevated, and the BP would be reduced 2.5 h after one 0.5-liter dose of BR and after 5 and 15 days of supplementation at 0.5 l/day; 2) that the O_2 cost of moderate-intensity exercise would be reduced 2.5 h after the first dose and after 5 and 15 days of supplementation; and 3) that the $\dot{V}\text{O}_{2\text{max}}$ and the GET would not be affected 2.5 h after the first dose but would be increased after 5 and 15 days of supplementation.

METHODS

Subjects. Eight healthy participants (including 3 females) volunteered for the study (means \pm SD: age 29 ± 6 yr, body mass 71.8 ± 11.5 kg, height 1.75 ± 0.05 m). Prior to testing, subjects were informed of the protocol and the possible risks and benefits of participation before written informed consent was obtained. All procedures were approved by the institutional research ethics committee and were conducted in accordance with the Declaration of Helsinki. Subjects were physically active but not highly trained in any particular sport. Subjects were instructed to adhere to their normal exercise routine and diet throughout the experimentation. The subjects kept a physical activity and dietary diary and were asked to perform similar activities and consume similar meals in the first and second supplementation periods. Prior to data collection, subjects were fully familiarized with exercise testing and performed a preliminary ramp incremental test (which was not included in the data analyses) to minimize any possible learning effect. Subjects were instructed to avoid strenuous exercise for 24 h prior to each testing session. Exercise tests were conducted at the same time of the day \pm 2 h for each subject. Subjects were asked to arrive into the laboratory adequately hydrated and having refrained from consuming alcohol for 24 h and food or caffeine for 3 h before each test.

Presupplementation tests. Subjects made two visits to the laboratory prior to the commencement of dietary supplementation. All exercise tests were performed on an electrically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During the first visit, a ramp incremental cycling test was performed for the assessment of the GET and $\dot{V}\text{O}_{2\text{max}}$. Subjects pedaled at a self-selected constant cadence (80 rpm, $n = 3$; 70 rpm, $n = 5$). The cadence and saddle and handlebar settings were replicated for all subsequent tests. Following 3 min of unloaded pedaling, the work rate was increased by 1 W every 2 s (i.e., 30 W/min) until the subject reached volitional exhaustion. The test was terminated when the cadence fell by > 10 rpm below the chosen cadence, despite strong verbal encouragement. The power output reached at the point of exhaustion was recorded as the peak power output. All timing devices were covered from the test subject and no feedback was given on the elapsed time or power output during the test. Feedback on performance was only given once all experimentation for the entire study had been completed. Pulmonary gas exchange was measured breath by breath and recorded throughout the test.

During the second visit, subjects performed two-step transitions from a 20-W baseline to moderate-intensity cycling at 90% GET. Each bout lasted for 5 min, and bouts were separated by 10 min of rest. Pulmonary $\dot{V}\text{O}_2$ was measured breath by breath, and the data from the two bouts were time aligned and averaged to improve the signal-to-noise ratio (26). This test provided a baseline measurement of the $\dot{V}\text{O}_2$ dynamics and the O_2 cost ($\Delta\dot{V}\text{O}_2/\Delta\text{work-rate}$) of moderate exercise. During both presupplementation visits, the resting BP was measured, and a resting venous blood sample was taken for the measurement of plasma $[\text{NO}_2^-]$. Plasma $[\text{NO}_2^-]$ was used as a biomarker for NO availability (13, 19, 25, 29). The mean values of all presupplementation measurements were used as presupplementation baseline for BP variables and plasma $[\text{NO}_2^-]$.

Supplementation period. Following completion of the presupplementation tests, subjects were assigned using a balanced, randomized crossover design to receive organic BR (0.5 l/day containing ~ 5.2 mmol of NO_3^- , Beet It; James White Drinks, Ipswich, UK) and placebo (PL; low-calorie blackcurrant juice cordial with negligible NO_3^- content). Thus, all eight subjects underwent both BR and PL supplementations with the 15-day supplementation periods separated by a 10-day washout period (Fig. 1A). Four subjects started with BR, and the other four started with PL. Throughout the study period, subjects were instructed to maintain their normal daily activities and food intake, i.e., unlike in previous studies (1, 2, 27, 28) where subjects were not instructed to minimize consumption of nitrate-rich foods. The subjects were not aware of the experimental hypotheses to be tested but were informed that the purpose of the study was to compare the physiological responses to exercise following the consumption of two commercially available sports beverages. This study was completed before the publication of our earlier studies (1, 2), such that the subjects were not aware that BR might be ergogenic.

On *day 1* of the supplementation period, subjects provided a venous blood sample for the measurement of plasma $[\text{NO}_2^-]$, and the resting BP was measured. The subjects then consumed a 0.5-liter dose of the supplement over a 15-min period and rested for 2.5 h. During this time subjects were allowed to drink water, but no other beverages or food were ingested. Two and a half hours after the ingestion of the supplement a second blood sample was obtained, BP was measured, and the exercise test was commenced. The test protocol consisted of two 5-min bouts of moderate-intensity cycling and a ramp incremental test to exhaustion (as described for the presupplementation tests), with all bouts separated by 10-min recovery (Fig. 1). Subjects were then prescribed a dose of 0.5 l/day of supplement (BR or PL) and instructed to consume it in two equal doses in the morning and in the evening. Subjects returned to the laboratory on *days 2, 5, 8, 12, and 15* for the measurement of plasma $[\text{NO}_2^-]$ and BP, ensuring that the latest supplement intake had occurred 2.5–5 h prior to the start of testing. The exercise protocol (Fig. 1) was also repeated on *days 5 and 15*.

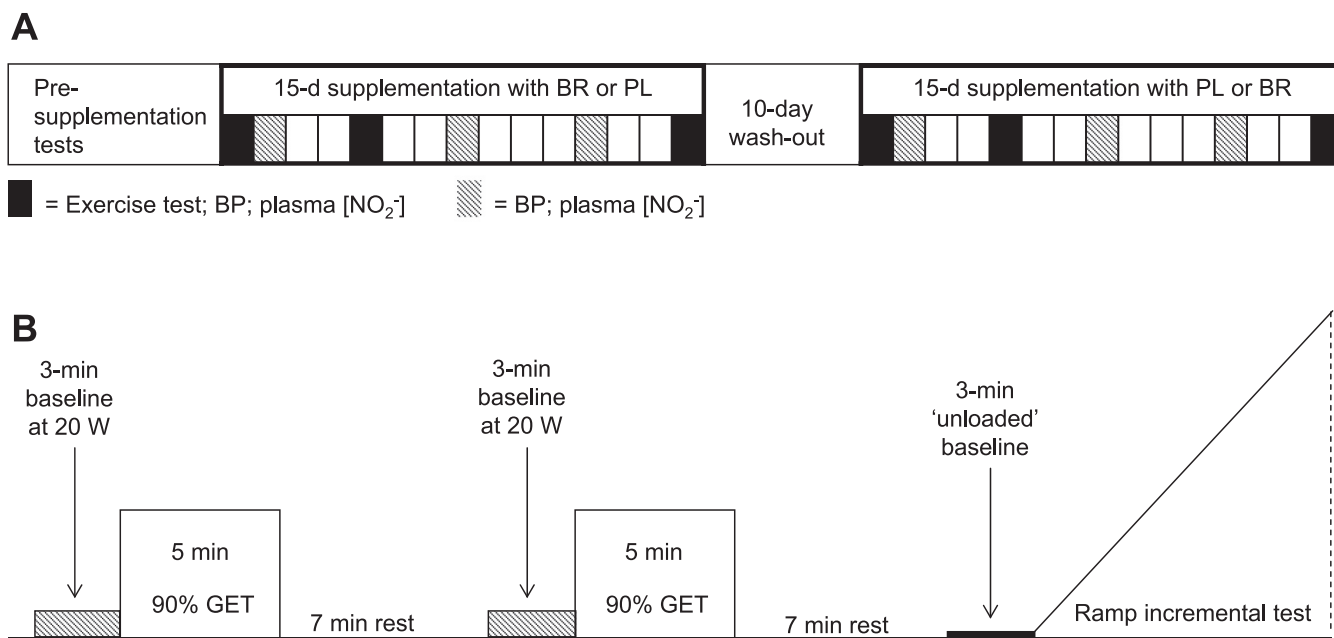


Fig. 1. Schematic illustration of the crossover design (A) and the exercise protocol performed 2.5 h postingestion (*day 1*), and after 5 and 15 days of beetroot juice (BR) and placebo (PL) supplementation (B). In this design, all 8 subjects underwent both BR and PL supplementations with a 10-day washout period separating the treatments. Blood pressure (BP) and plasma nitrite [NO₂⁻] were measured on *days 1, 2, 5, 8, 12, and 15*. GET, gas exchange threshold.

Measurements. Pulmonary gas exchange and ventilation were measured breath by breath during all exercise tests with subjects wearing a nose clip and breathing through a low dead space (90 ml), low-resistance (0.75 mmHg·l⁻¹·s⁻¹ at 15 l/s⁻¹) 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 using paramagnetic (O₂) and infrared (CO₂) analyzers (Oxycon Pro; Jaeger, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-liter syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in capillary gas transit and analyzer rise time relative to the volume signal. Oxygen uptake, carbon dioxide output, and minute ventilation were calculated using standard formulas (6) and displayed breath by breath. Heart rate (HR) was measured using short-range radiotelemetry (model S610; Polar Electro Oy, Kempele, Finland).

Capillary blood samples were collected from a fingertip into a capillary tube during the baseline preceding each step transition in work rate, during the final 30 s of each moderate bout, and following exhaustion in the ramp test. These samples were analyzed immediately to determine blood [lactate] (model YSI 1500; Yellow Springs Instruments, Yellow Springs, OH).

The BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL) during all visits to the laboratory with the subjects in a seated position. After arrival at the laboratory and following 10 min of rest, four measurements were recorded, and the mean of the final three measurements was used for data analysis. MAP was calculated as 1/3·systolic pressure + 2/3·diastolic pressure.

Venous blood samples were drawn into lithium-heparin tubes (7.5 ml Monovette lithium heparin; Sarstedt, Leicester, UK), which have very low levels of nitrate (0.89 ± 0.35 μM) and nitrite (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 subsequently extracted and immediately frozen at -80°C for later analysis of [NO₂⁻] using a modification of the chemiluminescence technique that we have used

previously (1, 2, see 4). Equipment and surfaces were regularly rinsed with ionized water to minimize contamination of samples by extraneous sources of nitrite and nitrate. Before samples were analyzed for NO₂⁻ content, they were thawed at room temperature and deproteinized using zinc sulfate precipitation (17). The deproteinized samples were then refluxed in 0.3 M sodium iodide and glacial acetic acid at room temperature and analyzed for [NO₂⁻] using an NO analyzer (Sievers NOA 280i, Analytix, Durham, UK).

Data analyses. Data collected during the ramp incremental tests at presupplementation baseline and on *days 1, 5, and 15* were used to establish the $\dot{V}_{O_{2max}}$ and GET. The $\dot{V}_{O_{2max}}$, $\dot{V}_{CO_{2max}}$, and $\dot{V}_{E_{max}}$ were calculated as mean values over the final 30 s of exercise. The GET was established from data averaged in 10-s time bins using the following criteria: 1) the first disproportionate increase in CO₂ 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) the first increase in end-tidal O₂ tension with no fall in end-tidal CO₂ tension. The GET was estimated independently by five assessors using code-labeled data files such that the assessors were blind to the subject and condition being assessed. The GET was determined as the majority agreement; in all cases, at least three assessors identified the same GET. Linear regression was used to determine the slope of the relationship between the change (Δ) in \dot{V}_{O_2} and the change in work rate ($\Delta\dot{V}_{O_2}/\Delta WR$) over two regions of the ramp test(s): S₁ (from ~1 min after the start of the ramp forcing function up to the GET) and S₂ (from the GET up to the point at which \dot{V}_{O_2} reached its maximum value or just before any visible plateau in \dot{V}_{O_2}) (20, 47). The data from the initial ramp test were used to normalize the work rate for the moderate-intensity tests (90% GET), taking into account the delay in \dot{V}_{O_2} increase during incremental exercise by deducting two-thirds of the ramp rate from the work rate at GET (47).

The breath-by-breath \dot{V}_{O_2} data collected at presupplementation baseline and on *days 1, 5, and 15* for constant-work-rate moderate-intensity bouts were initially examined to exclude errant breaths, and values 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 the two identical

repetitions performed on each visit 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 (48), and a nonlinear least-square algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the kinetics of the overall $\dot{V}O_2$ response to the criterion exercise bouts as described in the following equation: $\dot{V}O_2(t) = \dot{V}O_{2\text{baseline}} + A_p [1 - e^{-(t - TD_p)/\tau_p}]$, where $\dot{V}O_2(t)$ is the absolute $\dot{V}O_2$ at a given time (t); $\dot{V}O_{2\text{baseline}}$ is the mean $\dot{V}O_2$ in the baseline period; and A_p , TD_p , and τ_p represent the amplitude, time delay, and time constant, respectively, describing the fundamental or *phase II* increase in $\dot{V}O_2$ above baseline.

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. $\dot{V}O_{2\text{baseline}}$ was defined as the mean $\dot{V}O_2$ measured over the final 90 s of baseline pedaling. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 s of exercise. The functional gain of the entire response (i.e., end-exercise gain) was calculated by dividing A_p by the Δ work rate.

The baseline $\dot{V}CO_2$, respiratory exchange ratio, and $\dot{V}E$ were calculated as mean values over the final 60 s preceding the start of exercise, and the end-exercise values were calculated as mean values over the final 30 s.

Statistical analyses. Two-way repeated-measures ANOVA were used to assess differences across treatments (PL and BR) and across time (2.5 h, *days 5 and 15* for exercise test variables; or 2.5 h, *days 2, 5, 8, 12, and 15* for plasma $[NO_2^-]$ and BP variables). Time \times treatment interactions were followed up with changes relative to presupplementation baseline within the BR and PL conditions assessed using one-way repeated-measures analysis with Bonferroni corrected pairwise comparisons as appropriate. Paired *t*-tests were used to compare between BR and PL treatment differences at individual time points. Specific differences were reported using 95% confidence intervals and *P* values. Effect sizes for the primary outcome variables were established using Pearson's correlation coefficient (*r*), with a large effect defined as $r > 0.5$ ($0 \leq r \leq 1$), where effect accounts for >25% of the variance (15). The possible existence of an order effect of trials was assessed by paired samples *t*-tests for variables measured first and second at *day 15*. Relationships between

variables were assessed using Pearson's product moment correlation coefficients. All data analyses were performed using the SPSS (version 15.0; SPSS, Chicago, IL) statistical package, with statistical significance accepted at $P < 0.05$. Data are presented as means \pm SD unless stated otherwise.

RESULTS

Subjects' self-reported adherence to supplementation was 100% for both supplementation periods, and subjects reported that their habitual physical activity patterns and dietary intake were similar in the first and second supplementation periods. Subjects experienced beeturia during the BR supplementation. However, the supplementation regimen was well tolerated and no harmful side effects were reported.

Plasma $[NO_2^-]$ and BP. The group mean plasma $[NO_2^-]$ was 454 ± 81 nM at baseline (mean of all presupplementation measurements). Responses in plasma $[NO_2^-]$ across the supplementation periods are presented in Fig. 2A. The plasma $[NO_2^-]$ was elevated by BR supplementation as indicated by the response across time differing between groups ($P = 0.022$; effect size, 0.25). Follow-up tests show the plasma $[NO_2^-]$ was significantly elevated in BR from presupplementation baseline at all time points except *day 8* (95% CI range 35.3 to 431.4; $P < 0.02$). Compared with PL, BR was significantly higher at all follow-up time points (95% CI range 3.2 to 433.7; $P < 0.02$). The mean difference between BR and PL was 185 nM. The group mean plasma $[NO_2^-]$ was elevated by 36% at 2.5 h postingestion with the highest values attained at 12 (59%) and 15 (46%) days. No significant changes in plasma $[NO_2^-]$ were observed during PL supplementation.

At baseline prior to any dietary supplementation, the group mean systolic BP was 127 ± 6 mmHg, diastolic BP was 72 ± 5 mmHg, and MAP was 90 ± 4 mmHg. Systolic BP (Fig. 2B) was lowered by BR supplementation as indicated by the

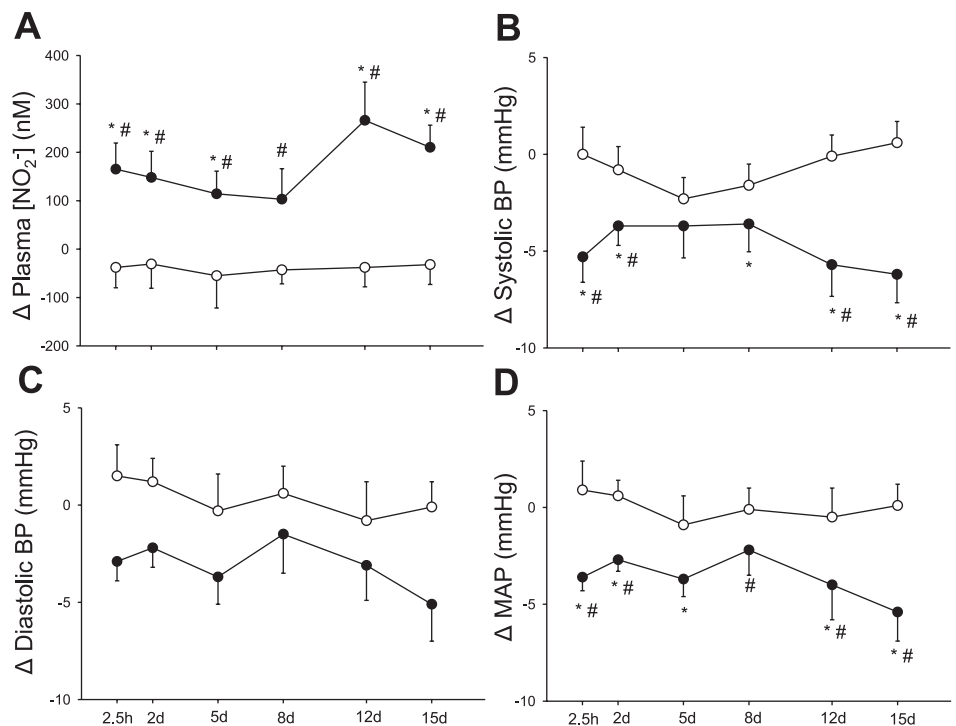


Fig. 2. Change (Δ) relative to presupplementation baseline in plasma $[NO_2^-]$ (A), systolic blood pressure (BP; B), diastolic BP (C), and mean arterial pressure (MAP; D), during the 15-day supplementation period with BR (●) and PL (○) (group means \pm SE). *Difference from presupplementation baseline ($P < 0.05$); #difference from PL ($P < 0.05$).

response across time differing between groups ($P = 0.000$; effect size, 0.34). Follow-up tests show systolic BP was significantly lowered in BR from presupplementation baseline at all time points except *day 5* (95% CI range -10.9 to -0.82 ; $P < 0.05$). Compared with PL, BR was significantly lower at 2.5 h postingestion, 2, 12, and 15 days (95% CI range -12.4 to -1.1 ; $P < 0.05$). The mean diastolic BP response across time points differed between groups ($P = 0.003$; effect size, 0.86, Fig. 2C), with a decrease for BR compared with PL (95% CI -4.3 to -1.3 ; $P < 0.01$). The MAP (Fig. 2D) was lowered by BR supplementation as indicated by the response across time differing between groups ($P = 0.014$; effect size, 0.26). Follow-up tests show MAP was significantly lowered in BR from presupplementation baseline at all time points except *day 8* (95% CI range -9.2 to -0.59 ; $P < 0.05$). Compared with PL, BR was significantly lower at all follow-up time points except *day 5* (95% CI range -9.8 to -0.94 ; $P < 0.05$). No significant changes in BP were observed during PL supplementation.

Moderate-intensity exercise. The pulmonary gas exchange and ventilatory responses to moderate exercise across PL and BR supplementation periods are summarized in Table 1. The \dot{V}_{O_2} measured during the period of baseline cycling at 20 W, which preceded the transition to moderate-intensity exercise was not affected by either supplement. The end-exercise \dot{V}_{O_2} (Fig. 3A) was reduced by BR supplementation, as indicated by the response across time differing between groups ($P = 0.001$; effect size, 0.52). Follow-up tests show the end-exercise \dot{V}_{O_2} was significantly reduced in BR from presupplementation baseline at all time points (2.5 h postingestion, 95% CI -128.7

to -3.5 ; *day 5*, 95% CI -99.4 to -7.1 ; and *day 15*, 95% CI -105.7 to -11.8 ; $P < 0.05$). Compared with PL, BR was significantly lower at 5 (95% CI -103.6 to -55.1 , $P < 0.001$) and 15 days (95% CI -101.7 to -37.1 , $P < 0.001$).

The amplitude of the \dot{V}_{O_2} response (end-exercise minus baseline \dot{V}_{O_2} ; Fig. 3B) was reduced by $\sim 10\%$ from baseline during the BR supplementation, with the response across time differing between groups ($P = 0.001$; effect size, 0.54). Follow-up tests show the amplitude of the \dot{V}_{O_2} response was significantly reduced in BR from presupplementation baseline at all time points (2.5 h postingestion, 95% CI -129.9 to -9.8 ; *day 5*, 95% CI -95.0 to -9.0 ; and *day 15*, 95% CI -115.6 to -13.6 ; $P < 0.05$). Compared with PL, BR was significantly lower at all time points (2.5 h postingestion, 95% CI -103.3 to -11.7 ; *day 5*, 95% CI -111.7 to -34.3 ; and *day 15*, 95% CI -104.8 to -39.7 ; $P < 0.05$).

Similarly, there was a reduction for the O_2 cost of exercise expressed as the functional gain (the increase in \dot{V}_{O_2} relative to the increase in external work rate; Fig. 3C), with the response across time differing between groups ($P = 0.002$; effect size, 0.51). Follow-up tests show functional gain was significantly lower in BR from presupplementation baseline at all time points (2.5 h postingestion, 95% CI -2.1 to -0.15 ; *day 5*, 95% CI -1.5 to -0.10 ; and *day 15*, 95% CI -1.9 to -0.10 ; $P < 0.05$). Compared with PL, BR was significantly lower at all time points (2.5 h postingestion, 95% CI -1.7 to -0.17 ; *day 5*, 95% CI -1.8 to -0.49 ; and *day 15*, 95% CI -1.7 to -0.54 ; $P < 0.05$). No significant differences from presupplementation values were observed in the \dot{V}_{O_2} variables during the PL supplementation.

Table 1. Ventilatory and gas exchange dynamics and blood lactate concentration ([lac]) measured during moderate-intensity exercise at presupplementation baseline and across 15 days of beetroot juice (BR) and placebo (PL) supplementation

	Presupplementation		2.5 h Postingestion	Day 5	Day 15
\dot{V}_{O_2}					
Baseline, l/min	0.81 \pm 0.12	PL	0.80 \pm 0.13	0.82 \pm 0.11	0.82 \pm 0.11
		BR	0.81 \pm 0.11	0.83 \pm 0.13	0.82 \pm 0.13
End exercise, l/min	1.42 \pm 0.21	PL	1.40 \pm 0.20	1.45 \pm 0.21	1.43 \pm 0.23
		BR	1.35 \pm 0.17*	1.38 \pm 0.20*†	1.37 \pm 0.23*†
MRT, s	36 \pm 7	PL	35 \pm 10	34 \pm 7	36 \pm 8
		BR	34 \pm 9	32 \pm 8	35 \pm 6
Amplitude, l/min	0.61 \pm 0.11	PL	0.59 \pm 0.10	0.63 \pm 0.12	0.61 \pm 0.12
		BR	0.54 \pm 0.10*†	0.56 \pm 0.09*†	0.56 \pm 0.11*†
Gain, ml·min ⁻¹ ·W ⁻¹)	9.8 \pm 0.8	PL	9.6 \pm 0.9	10.1 \pm 1.1	9.9 \pm 0.9
		BR	8.7 \pm 0.7*†	8.9 \pm 0.5*†	8.9 \pm 0.5*†
\dot{V}_{CO_2}					
Baseline, l/min	0.75 \pm 0.13	PL	0.74 \pm 0.15	0.72 \pm 0.12	0.74 \pm 0.14
		BR	0.73 \pm 0.13	0.74 \pm 0.10	0.76 \pm 0.14
End, l/min	1.30 \pm 0.20	PL	1.30 \pm 0.19	1.29 \pm 0.21	1.34 \pm 0.22
		BR	1.28 \pm 0.17	1.32 \pm 0.16	1.32 \pm 0.20
RER					
Baseline	0.93 \pm 0.04	PL	0.92 \pm 0.09	0.88 \pm 0.05	0.90 \pm 0.07
		BR	0.90 \pm 0.11	0.89 \pm 0.05	0.92 \pm 0.05
End	0.91 \pm 0.05	PL	0.93 \pm 0.05	0.89 \pm 0.06	0.94 \pm 0.04
		BR	0.95 \pm 0.06	0.93 \pm 0.04	0.96 \pm 0.03
\dot{V}_E					
Baseline, l/min	21 \pm 3	PL	22 \pm 4	21 \pm 3	22 \pm 3.0
		BR	21 \pm 3	22 \pm 3	22 \pm 3
End, l/min	34 \pm 5	PL	35 \pm 5	34 \pm 4	36 \pm 5
		BR	34 \pm 4	35 \pm 4	34 \pm 4
End blood [lac], mM	1.1 \pm 0.3	PL	1.1 \pm 0.3	1.0 \pm 0.4	1.1 \pm 0.4
		BR	1.0 \pm 0.3	1.1 \pm 0.2	1.0 \pm 0.3

Values are means \pm SD. MRT, mean response time; RER, respiratory exchange ratio. *Different from presupplementation baseline, $P < 0.05$; †different from PL, $P < 0.05$.

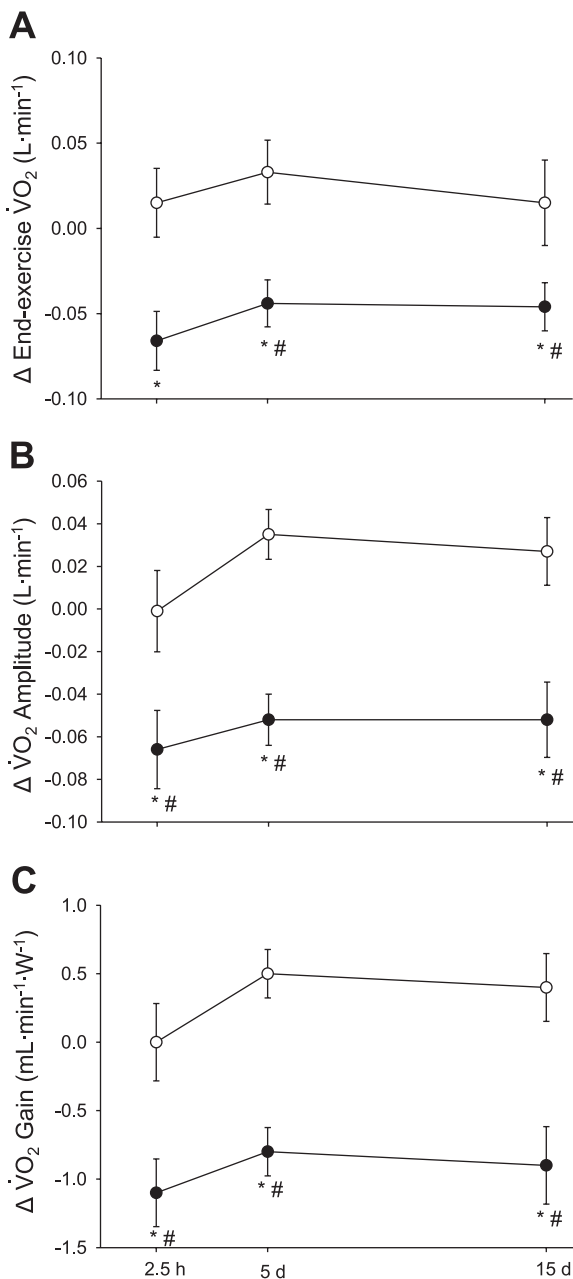


Fig. 3. Change (Δ) relative to presupplementation baseline in end-exercise $\dot{V}O_2$ (A), $\dot{V}O_2$ response amplitude (B), and functional gain (C) measured during moderate-intensity exercise during the 15-day supplementation period with BR (●) and PL (○) (group means \pm SE). $\dot{V}O_2$, oxygen uptake. *Difference from presupplementation baseline ($P < 0.05$); #difference from PL ($P < 0.05$).

The group mean $\dot{V}O_2$ response profiles at all time points for the BR and PL conditions, relative to presupplementation, are shown in Fig. 4. The baseline and end-exercise $\dot{V}CO_2$, respiratory exchange ratio, $\dot{V}E$, and blood [lactate] were not significantly altered by either BR or PL supplementation (Table 1).

Ramp incremental exercise. The effects of BR and PL supplementation on the ramp test parameters are summarized in Table 2 and Fig. 5. $\dot{V}O_{2max}$ showed only the combined group response changed over time ($P = 0.000$; effect size, 0.56), with a significant increase at 5 (95% CI 33.5 to 172.4, $P < 0.01$) and 15 days (95% CI 26.8 to 177.7, $P < 0.01$). Although the

unspecific F -test for interaction effect over all four time-points did not attain significance at the 95% level, it should be noted that the specific test for a difference between the baseline and 15 days of BR was significant ($P = 0.003$), whereas the comparison between baseline and 15 days of PL was not ($P = 0.12$).

The peak power output (Fig. 5B) was increased by BR supplementation, as indicated by the response across time differing between groups ($P = 0.008$; effect size, 0.44). Follow-up tests show the peak power output was significantly increased in BR at 15 days compared with presupplementation baseline (95% CI 2.9 to 15.3; $P < 0.01$) and 2.5 h postingestion (95% CI 0.84 to 11.9; $P < 0.05$). The peak power output in BR was significantly higher compared to PL at 15 days (95% CI 5.4 to 11, $P < 0.001$). The PL supplementation did not significantly affect the peak power output.

There were no significant effects on the $\dot{V}O_2$ at GET with BR or PL supplementation (Fig. 5C). An increase by BR supplementation on the work rate associated with the GET (Fig. 5D) is indicated by the response across time differing between groups ($P < 0.009$; effect size, 0.44). Follow-up tests show the work rate associated with the GET was significantly increased in BR at 15 days compared with presupplementation baseline (95% CI 1.3 to 34.9; $P < 0.05$) and 2.5 h postingestion (95% CI 4.3 to 33.2; $P < 0.05$). The work rate at GET was significantly higher in BR compared to PL at 15 days (95% CI 9.6 to 34.1, $P < 0.001$). No changes were observed in the work rate at GET during the PL supplementation.

The S_1 slope of the $\Delta\dot{V}O_2/\Delta$ work rate relationship was significantly reduced after 15 days of BR (8.9 ± 0.4 ml·min⁻¹·W⁻¹) compared with baseline (9.9 ± 0.9 ml·min⁻¹·W⁻¹) and 15 days of PL (9.8 ± 0.8 ml·min⁻¹·W⁻¹; $P = 0.03$). The S_2 slope was significantly increased after 15 days of BR (9.7 ± 0.8 ml·min⁻¹·W⁻¹) compared with baseline (9.1 ± 0.7 ml·min⁻¹·W⁻¹) and 15 days of PL (9.3 ± 0.8 ml·min⁻¹·W⁻¹; $P = 0.000$) (Fig. 6).

No order effect on ramp test parameters was detected when the results of the condition performed first were compared with that performed second: $\dot{V}O_{2max}$ (1st vs. 2nd), 3.46 ± 0.85 vs. 3.46 ± 0.82 l/min ($P = 0.99$); peak power, 327 ± 69 vs. 328 ± 67 W ($P = 0.77$); GET, 1.52 ± 0.37 vs. 1.52 ± 0.36 l/min ($P = 0.96$); GET, 98 ± 24 vs. 91 ± 27 W ($P = 0.48$).

DISCUSSION

The principal novel finding of this study was that the lower O_2 cost of moderate-intensity exercise measured 2.5 h after the first ingestion of organic BR was maintained when supplementation was continued for 15 days. The significantly elevated plasma $[NO_2^-]$ throughout the 15-day dietary nitrate supplementation period was accompanied by reduced systolic and diastolic BP, and these effects tended to be particularly pronounced after ≥ 12 days of supplementation. The $\dot{V}O_{2max}$, the peak power output in the ramp incremental test, and the GET were not affected 2.5 h postingestion or after 5 days of supplementation. However, after 15 days of BR intake, the peak power output and the work rate associated with the GET were higher than the baseline and the placebo condition and the $\dot{V}O_{2max}$ was also elevated above baseline. These findings support our hypotheses in demonstrating that dietary nitrate intake

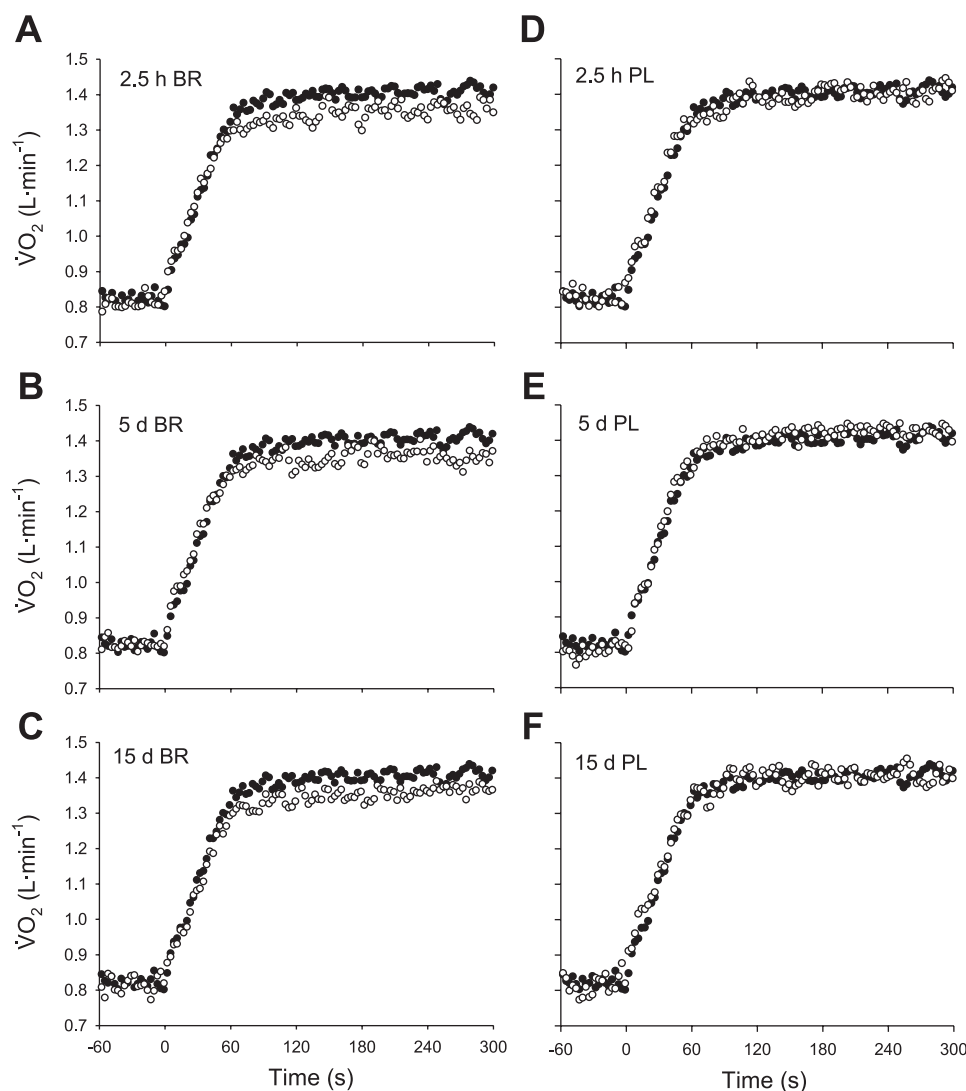


Fig. 4. Group mean $\dot{V}O_2$ profiles during moderate-intensity exercise across the 15-day supplementation periods with BR and PL compared with presupplementation baseline (●). Open symbols (○) indicate BR supplemented trials in A–C and PL supplemented trials in D–F. Error bars are omitted for clarity.

appears to have distinct acute and chronic effects on the physiological responses to exercise.

Effects of dietary nitrate supplementation on BP. In recent years, dietary nitrate has emerged as a promising therapeutic agent for the treatment of hypertension (8, 16, 45). Vasodilation is mediated through NO, which is generated by two known pathways: by oxidation of L-arginine by NO synthase (NOS) requiring the presence of O_2 and a number of essential cofactors and, alternatively, by the reduction of nitrate-derived nitrite to NO (7, 8, 13). NOS enzyme dysfunction is associated with a number of vascular and metabolic pathologies, and therefore, the nitrate-nitrite-NO pathway, which is amenable to dietary manipulation, could offer a natural and cost-effective preventative intervention and an alternative to pharmaceutical treatment (16, 30). The present data indicate that the addition of nitrate-rich vegetable juice to the normal diet acutely reduced BP in normotensive subjects with this response being sustained over 15 days of continued supplementation. The reductions in the systolic (−4 mmHg; −3%) and diastolic (−4 mmHg; −5%) pressures were similar to those reported previously in healthy volunteers (1, 2, 27, 40, 45). Some of these studies reported a reduction in systolic pressure only (1), with

another study (40) reporting a reduction in diastolic pressure only, and the rest (27, 45) observing a reduction in both systolic and diastolic pressure. Although such changes in the BP of normotensive young adults might appear small in absolute terms, they could become meaningful when considered, for example, as reduced shear stress on the vascular endothelium over the entire lifespan of the individual. Indeed, it has been suggested that, in moderately hypertensive subjects, a 5 mmHg reduction in blood pressure might reduce the incidence of stroke by 22% and coronary heart disease by 16% and prevent up to 75,000 deaths per year in the United Kingdom alone (39a).

Effects of dietary nitrate supplementation on the physiological responses to submaximal exercise. To our knowledge, this is the first study to assess the physiological consequences of dietary nitrate supplementation in humans over a time period beyond 4–6 days. We assessed the acute response to nitrate supplementation at 2.5 h postingestion based upon the established peak values in plasma $[NO_2^-]$ and associated reduction in BP following BR ingestion (45). The steady-state $\dot{V}O_2$ during moderate-intensity cycle exercise was reduced by ~5% compared with the presupplementation baseline at 2.5 h after

Table 2. Parameters measured in the ramp incremental test at presupplementation baseline and across 15 days of BR and PL supplementation

	Presupplementation		2.5 h Postingestion	Day 5	Day 15
$\dot{V}O_{2\max}$, l/min	3.36 ± 0.84	PL	3.38 ± 0.81	3.43 ± 0.85	3.42 ± 0.88
		BR	3.42 ± 0.82	3.49 ± 0.83	3.50 ± 0.82*
Peak power, W	322 ± 67	PL	322 ± 68	323 ± 67	323 ± 68
		BR	325 ± 71	328 ± 68	331 ± 68*†
GET, l/min	1.46 ± 0.29	PL	1.48 ± 0.28	1.51 ± 0.40	1.45 ± 0.31
		BR	1.44 ± 0.28	1.57 ± 0.50	1.60 ± 0.40
GET, W	89 ± 15	PL	93 ± 16	88 ± 19	84 ± 18
		BR	88 ± 21	100 ± 36	105 ± 28*†
$\dot{V}CO_{2\max}$, l/min	4.33 ± 1.05	PL	4.43 ± 1.04	4.37 ± 1.12	4.43 ± 1.10
		BR	4.32 ± 1.04	4.51 ± 1.04	4.50 ± 1.11
RER max	1.29 ± 0.03	PL	1.31 ± 0.05	1.27 ± 0.05	1.30 ± 0.06
		BR	1.26 ± 0.05	1.29 ± 0.04	1.28 ± 0.05
$\dot{V}E_{\max}$, l/min	150 ± 52	PL	155 ± 53	150 ± 46	152 ± 42
		BR	153 ± 48	155 ± 50	150 ± 42
HR peak, beats/min	178 ± 9	PL	180 ± 11	178 ± 9	179 ± 9
		BR	179 ± 12	181 ± 9	183 ± 10
End blood [lac], mM	7.8 ± 1.4	PL	7.9 ± 0.9	7.4 ± 1.5	7.4 ± 1.2
		BR	8.2 ± 2.1	8.1 ± 1.0	7.9 ± 1.8
End blood [glu], mM	4.1 ± 0.7	PL	4.2 ± 0.4	3.9 ± 0.5	3.9 ± 0.6
		BR	4.1 ± 0.4	4.2 ± 0.6	3.7 ± 0.8

Values are means ± SD. GET, gas exchange ratio; [glu], glucose concentration. *Different from presupplementation baseline, $P < 0.05$; †different from PL, $P < 0.05$.

the first BR ingestion and remained reduced by 3–4% at days 5 and 15. Moreover, the functional gain of the response, the reciprocal of delta efficiency, was reduced by ~10% (from 9.8 ml·min⁻¹·W⁻¹ at baseline to 8.7–8.9 ml·min⁻¹·W⁻¹) during the nitrate supplementation period. The present findings are similar to the responses that have been reported after 4–6 days (1, 27) and are consistent with a recent report showing a 6% reduction in the steady-state $\dot{V}O_2$ during submaximal exercise 1 h postingestion of pharmacological sodium nitrate (0.033 mmol/kg) (28). The present study shows that these acute

effects on the $\dot{V}O_2$ response to moderate-intensity exercise are maintained over 15 days of supplementation, with no indication of either a reduced sensitivity to supplementation or an increasing effect with time.

The changes in $\dot{V}O_2$ during submaximal exercise were less pronounced in the present data set than in our previous study, where 4–6 days of supplementation with BR (with identical nitrate concentration) resulted in a ~20% reduction in the functional gain of the $\dot{V}O_2$ response to moderate-intensity exercise (1). However, it is important to note that, in the

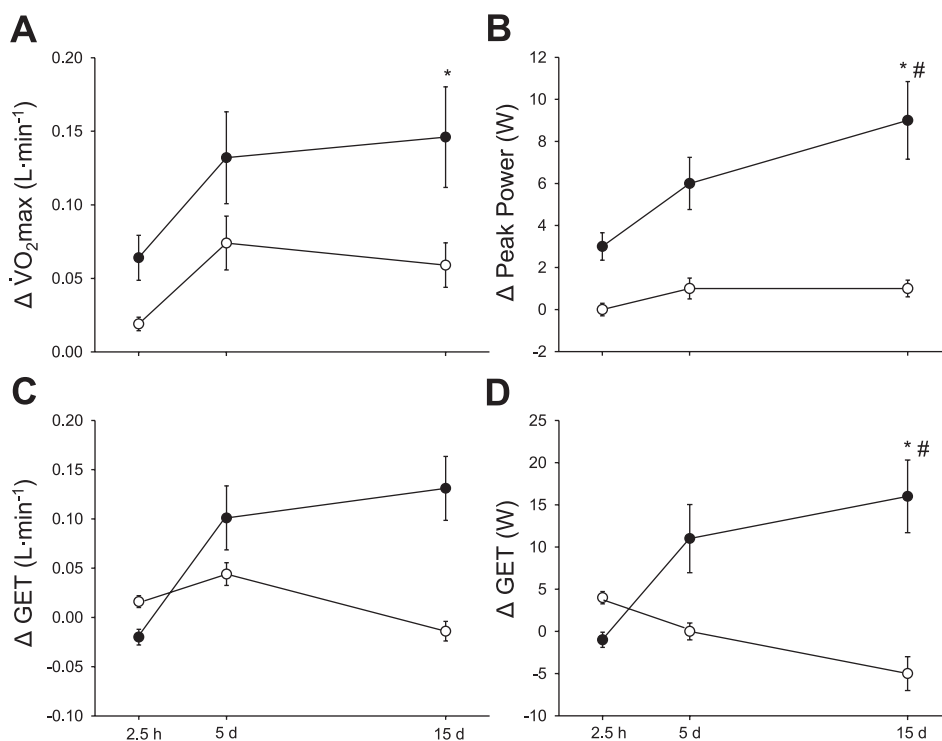


Fig. 5. The change (Δ) relative to presupplementation baseline in $\dot{V}O_{2\max}$ (A), peak power output (B), GET (C), and work rate at the GET (D) measured in the ramp incremental test during the 15-day supplementation period with BR (●) and PL (○) (group means ± SE). *Difference from presupplementation baseline ($P < 0.05$); #difference from PL ($P < 0.05$).

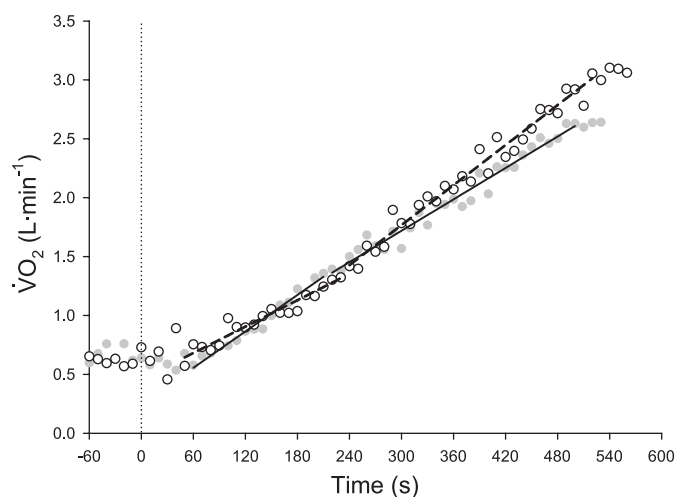


Fig. 6. The S_1 and S_2 slopes of the $\Delta\dot{V}O_2/\Delta\text{work rate}$ relationship in the incremental ramp test at presupplementation baseline (grey symbols) and after 15 days of BR (white symbols) in a representative individual. The S_1 and S_2 slopes are indicated by solid lines for presupplementation and by dashed lines for the 15-day BR trial. Notice also the higher $\dot{V}O_{2\max}$ in the BR condition and that the GET occurs at the same $\dot{V}O_2$ but at a higher work rate after BR.

present study, subjects' nitrate intake was intentionally not restricted at any time during the study period. Previously we have instructed participants to avoid the consumption of certain nitrate-rich vegetables and cured meats (1) and others have excluded all vegetables, some fruits, nicotine, tea, cured meats, and fish (27, 28). It was of interest to establish whether the observed effects of nitrate supplementation on exercise efficiency would still be evident compared with a normal diet control condition, rather than a state of reduced nitrate intake. An original finding in the present study is therefore that additional dietary nitrate had beneficial effects on BP, exercise efficiency, and indices of aerobic exercise performance (see next section) of physically active young adults who consumed a self-selected mixed diet throughout the study.

The mechanistic bases for the reduced O_2 cost of exercise observed following dietary nitrate intake remain unclear. However, we have recently reported that dietary nitrate supplementation reduced the muscle ATP turnover rate for the same external work rate (2). These results suggest that the improved efficiency following nitrate supplementation might be linked to a reduced ATP expenditure on the maintenance of sarcoplasmic Ca^{2+} homeostasis and/or effects of NO on the actomyosin ATPase (2, 39). In our previous study (2), nitrate supplementation resulted in reduced phosphocreatine degradation and reduced accumulation of adenosine diphosphate and inorganic phosphate for the same work rate. These changes would be expected to reduce the stimuli to oxidative phosphorylation (consistent with the reduction in steady-state $\dot{V}O_2$) (9, 31, 37), and also to reduce the rate of muscle fatigue development (46).

Effects of dietary nitrate supplementation on the physiological responses to ramp incremental exercise. We used a ramp incremental exercise test to establish key parameters of aerobic fitness across the supplementation periods (47). The peak power output achieved at the limit of tolerance in the incremental test was not significantly altered at 2.5 h postingestion or after 5 days, but was elevated after 15 days. We have previously reported that the end-exercise $\dot{V}O_2$ measured in a

constant-work-rate severe-intensity cycle trial performed to the limit of tolerance was not significantly altered, but the time to exhaustion was extended by 16% following 4–6 days of dietary nitrate supplementation (1). The improvements in exercise tolerance demonstrated during high-intensity constant-work-rate exercise tests following a given intervention are consistently greater than those demonstrated during incremental exercise, such that a 20% increase in time to exhaustion during constant-work-rate exercise would be equivalent to no greater than a 5% increase in time to exhaustion in an incremental test (38, 43, 49). According to this proportionality and the 16% improvement in time to exhaustion during constant-work-rate exercise reported previously (1), the expected improvement in time to exhaustion in a 30 W/min ramp protocol can be estimated to be 5–10 W, which is similar to the effects shown after 5 days (+6 W) and 15 days (+9 W) of nitrate supplementation in the present study.

Relative to baseline, the $\dot{V}O_{2\max}$ was increased by ~ 140 ml/min after 15 days of nitrate supplementation. This result is in contrast to a recent report that the $\dot{V}O_{2\max}$ during incremental exercise was reduced following 2 days of sodium nitrate intake (28). Considering that a reduction in NO availability via acute NOS inhibition has been shown to reduce the $\dot{V}O_{2\max}$ (by 200 ml/min) during ramp incremental exercise (23), it is unclear how the 100 ml/min reduction in $\dot{V}O_{2\max}$ reported by Larsen et al. (28) could also be attributed to an increase in NO availability. We have observed no reduction in $\dot{V}O_{2\max}$ following dietary nitrate supplementation either during exhaustive constant-work-rate exercise (1, 2) or during ramp incremental exercise (present data). However, in agreement with Larsen et al. (28), we have shown that the improved exercise efficiency following nitrate supplementation is associated with enhanced exercise tolerance.

The possible mechanisms by which $\dot{V}O_{2\max}$ may be increased following 15 days of nitrate supplementation in the present study may include NO-mediated changes in local perfusion in skeletal muscle (42) and possible effects on cardiac output (23). Alternatively, the gradual increase in $\dot{V}O_{2\max}$ over the 15-day nitrate supplementation period could be linked to increased mitochondrial mass as a consequence of elevated NO availability (10, 35). Chronic exposure of mammalian cells to NO has been shown to result in cGMP-mediated activation of regulatory protein sirtuin (SIRT1), which upregulates transcriptional factors and nuclear respiratory factors involved in the coordination of mitochondrial fusion and fission events (24, 35, 36). It is important to note that in addition to nitrate, beetroot is also rich in several polyphenols, of which quercetin has been linked to increased activation of SIRT1 and also to increased exercise tolerance (12). However, whether NO-cGMP-induced mitochondrial biogenesis is manifested in human skeletal muscle in vivo following dietary nitrate intervention remains to be determined. It should also be noted that the increased $\dot{V}O_{2\max}$ measured in the present study following longer-term nitrate supplementation was relatively small, and further studies are required to confirm this observation.

This is the first investigation to assess the effects of dietary nitrate supplementation on the GET. Although the $\dot{V}O_2$ at which GET occurred during incremental exercise was not significantly affected by nitrate ingestion, the work rate associated with the GET was elevated above baseline and placebo after 15 days of supplementation. The unchanged $\dot{V}O_2$ but

higher work rate at the GET following 15 days of nitrate supplementation is explained by the significantly reduced S_1 slope of the $\Delta\dot{V}O_2/\Delta WR$ relationship during incremental exercise. The reduced S_1 slope is consistent with the reduced steady-state $\dot{V}O_2$ measured during moderate-intensity exercise following nitrate supplementation (in both cases, the response gain was reduced from ~ 10 to ~ 9 $\text{ml}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$). The S_2 slope was significantly steeper after 15 days of nitrate supplementation compared with the presupplementation baseline. The mechanism responsible for this effect is unclear but might be related to alterations in aerobic fitness, muscle O_2 delivery, and/or changes in motor unit recruitment (3, 20, 21, 23) following nitrate supplementation. The lower S_1 slope but steeper S_2 slope during incremental exercise is consistent with the differential changes in the fundamental phase $\dot{V}O_2$ amplitude during moderate-intensity and severe-intensity exercise we have reported previously (1).

Perspectives and Significance

Supplementing the diet of normotensive young adults consuming a mixed diet with ~ 0.07 mmol/kg of nitrate resulted in a sustained reduction in systolic and diastolic BP. The O_2 cost of moderate-intensity exercise was significantly reduced 2.5 h after the ingestion of one 0.5-liter dose of nitrate-rich BR, and these effects were maintained after 5 and 15 days of continued supplementation with 0.5 l/day BR. While nitrate supplementation had no acute effects on $\dot{V}O_{2\text{max}}$ and the GET, these parameters of aerobic fitness were elevated after 15 days of supplementation. Unlike previous studies using BR or pharmacological sodium nitrate supplements, subjects' normal dietary nitrate intake was not restricted at any time during the study period. While the mechanistic bases of the acute and chronic effects of nitrate supplementation on $\dot{V}O_2$ dynamics remain to be ascertained, these results provide intriguing new directions for the potential use of dietary nitrate in enhancing training adaptations in athletic populations and improving cardiovascular health within the wider population.

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DISCLOSURES

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

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