

**Stephen J. Bailey, Paul Winyard, Anni Vanhatalo, Jamie R. Blackwell, Fred J. DiMenna, Daryl P. Wilkerson, Joanna Tarr, Nigel Benjamin and Andrew M. Jones**

*J Appl Physiol* 107:1144-1155, 2009. First published Aug 6, 2009; doi:10.1152/japphysiol.00722.2009

**You might find this additional information useful...**

---

This article cites 67 articles, 31 of which you can access free at:

<http://jap.physiology.org/cgi/content/full/107/4/1144#BIBL>

Updated information and services including high-resolution figures, can be found at:

<http://jap.physiology.org/cgi/content/full/107/4/1144>

Additional material and information about *Journal of Applied Physiology* can be found at:

<http://www.the-aps.org/publications/jappl>

---

This information is current as of March 16, 2010 .

## Dietary nitrate supplementation reduces the O<sub>2</sub> cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans

Stephen J. Bailey,<sup>1</sup> Paul Winyard,<sup>2</sup> Anni Vanhatalo,<sup>1</sup> Jamie R. Blackwell,<sup>1</sup> Fred J. DiMenna,<sup>1</sup> Daryl P. Wilkerson,<sup>1</sup> Joanna Tarr,<sup>2</sup> Nigel Benjamin,<sup>2</sup> and Andrew M. Jones<sup>1</sup>

<sup>1</sup>School of Sport and Health Sciences and <sup>2</sup>Peninsula College of Medicine and Dentistry, University of Exeter, Exeter, United Kingdom

Submitted 6 July 2009; accepted in final form 3 August 2009

**Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM.** Dietary nitrate supplementation reduces the O<sub>2</sub> cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107: 1144–1155, 2009. First published August 6, 2009; doi:10.1152/jappphysiol.00722.2009.—Pharmacological sodium nitrate supplementation has been reported to reduce the O<sub>2</sub> cost of submaximal exercise in humans. In this study, we hypothesized that dietary supplementation with inorganic nitrate in the form of beetroot juice (BR) would reduce the O<sub>2</sub> cost of submaximal exercise and enhance the tolerance to high-intensity exercise. In a double-blind, placebo (PL)-controlled, crossover study, eight men (aged 19–38 yr) consumed 500 ml/day of either BR (containing 11.2 ± 0.6 mM of nitrate) or blackcurrant cordial (as a PL, with negligible nitrate content) for 6 consecutive days and completed a series of “step” moderate-intensity and severe-intensity exercise tests on the last 3 days. On days 4–6, plasma nitrite concentration was significantly greater following dietary nitrate supplementation compared with PL (BR: 273 ± 44 vs. PL: 140 ± 50 nM; *P* < 0.05), and systolic blood pressure was significantly reduced (BR: 124 ± 2 vs. PL: 132 ± 5 mmHg; *P* < 0.01). During moderate exercise, nitrate supplementation reduced muscle fractional O<sub>2</sub> extraction (as estimated using near-infrared spectroscopy). The gain of the increase in pulmonary O<sub>2</sub> uptake following the onset of moderate exercise was reduced by 19% in the BR condition (BR: 8.6 ± 0.7 vs. PL: 10.8 ± 1.6 ml·min<sup>-1</sup>·W<sup>-1</sup>; *P* < 0.05). During severe exercise, the O<sub>2</sub> uptake slow component was reduced (BR: 0.57 ± 0.20 vs. PL: 0.74 ± 0.24 l/min; *P* < 0.05), and the time-to-exhaustion was extended (BR: 675 ± 203 vs. PL: 583 ± 145 s; *P* < 0.05). The reduced O<sub>2</sub> cost of exercise following increased dietary nitrate intake has important implications for our understanding of the factors that regulate mitochondrial respiration and muscle contractile energetics in humans.

exercise economy; muscle efficiency; O<sub>2</sub> uptake; exercise performance; fatigue

A FUNDAMENTAL TENET OF HUMAN exercise physiology is a predictable oxygen (O<sub>2</sub>) cost for a given submaximal work rate. Upon the initiation of moderate-intensity exercise [i.e., exercise performed at work rates below the gas exchange threshold (GET)], pulmonary O<sub>2</sub> uptake ( $\dot{V}_{O_2}$ ), which closely reflects O<sub>2</sub> consumption in the skeletal muscles (2, 29, 38), rises in an exponential fashion to attain a “steady state” within ~2–3 min in healthy humans (64). The steady-state increase in  $\dot{V}_{O_2}$  is linearly related to the increase in external work rate; is essentially independent of factors such as age, health status or aerobic fitness; and approximates 10 ml O<sub>2</sub>·min<sup>-1</sup>·W<sup>-1</sup> of external power output during cycle ergometry (i.e., 10

ml·min<sup>-1</sup>·W<sup>-1</sup>; Ref. 36). During supra-GET exercise,  $\dot{V}_{O_2}$  dynamics become more complex, owing, in part, to the development of a delayed-onset  $\dot{V}_{O_2}$  “slow component”, which elevates the O<sub>2</sub> cost of exercise above 10 ml·min<sup>-1</sup>·W<sup>-1</sup> (36, 64).

Whereas it is known that interventions such as training and the inspiration of hyperoxic gas can reduce the O<sub>2</sub> cost of heavy (above the GET but below critical power; Ref. 52) and severe (above critical power) exercise by reducing the amplitude of the  $\dot{V}_{O_2}$  slow component, the steady-state  $\dot{V}_{O_2}$  during moderate exercise is unaffected by these and other interventions in healthy humans (1, 15, 36, 51, 65). Surprisingly, however, it was recently reported that 6 days of dietary supplementation with pharmacological sodium nitrate reduced the O<sub>2</sub> cost of submaximal cycling at work rates expected to require 45–80% maximum  $\dot{V}_{O_2}$  ( $\dot{V}_{O_{2max}}$ ) (45). That this effect occurred without any increase in estimated nonoxidative energy production (as reflected by an unchanged blood [lactate]) (where brackets denote concentration) suggested that sodium nitrate ingestion improved the efficiency of muscle oxidative metabolism. It is known that tolerance to high-intensity exercise is, in certain respects, a function of  $\dot{V}_{O_{2max}}$  and submaximal exercise economy (20). Therefore, assuming that  $\dot{V}_{O_{2max}}$  is not altered, it is feasible that dietary nitrate supplementation might enhance exercise tolerance. However, this possibility has not been investigated.

The nitrate anion (NO<sub>3</sub><sup>-</sup>) is relatively inert, and thus any biological effects are likely conferred via its conversion to the bioactive nitrite anion (NO<sub>2</sub><sup>-</sup>). Inorganic nitrate is rapidly absorbed from the gut and is concentrated in saliva at least 10-fold. In the mouth, facultative anaerobic bacteria on the surface of the tongue reduce NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> (23). Nitrite can be converted to nitric oxide (NO) in the stomach (6, 47), but it is also clear that some is absorbed to increase circulating plasma [nitrite] (21, 46). We and several other groups have shown that NO<sub>2</sub><sup>-</sup> can be converted to NO under appropriate physiological conditions (6). The requisite one-electron reduction has variously been reported to be catalyzed via xanthine oxidoreductase, hemoglobin, myoglobin, endothelial NO synthase, and the mitochondrial electron transfer complexes (61).

There are at least two mechanisms by which NO derived from NO<sub>2</sub><sup>-</sup> (rather than from the much better known synthesis of NO from L-arginine by the NO synthase family of enzymes) might influence O<sub>2</sub> utilization by contracting skeletal muscle. First, as all of the known mechanisms for NO<sub>2</sub><sup>-</sup> reduction are facilitated by hypoxia, it may be that more NO (which is a potent vasodilator) is generated in parts of muscle that are receiving less or using more O<sub>2</sub>, and, therefore, this mechanism would help to match local blood flow to O<sub>2</sub> requirement, providing a more homogenous distribution of O<sub>2</sub> within skel-

Address for reprint requests and other correspondence: A. M. Jones Professor of Applied Physiology, Exeter Univ., Sport and Health Sciences, St. Luke's Campus, Heavitree Rd., Exeter, EX1 2LU UK (e-mail: a.m.jones@exeter.ac.uk).

etal muscle. However, while this might be beneficial in terms of muscle function, it would not explain a reduced  $O_2$  cost during exercise. A second possible mechanism involves the roles of  $NO_2^-$  and  $NO$  as regulators of cellular  $O_2$  utilization. For example,  $NO$  is known to be an important inhibitor of cytochrome oxidase activity (10). More recently, it has been suggested that  $NO$  might enhance the efficiency of oxidative phosphorylation by reducing "slippage" of the mitochondrial proton pumps (17). There is also evidence that  $NO_2^-$  can serve as an alternative electron acceptor, theoretically replacing the role of  $O_2$  in respiration (3).

The diet constitutes the main source of  $NO_3^-$  in humans, with vegetables accounting for 60–80% of daily  $NO_3^-$  intake in a Western diet (67). Given the reported ability of pharmacological sodium nitrate to reduce the  $O_2$  cost of exercise (45), we sought to determine whether similar effects are observed when the  $NO_3^-$  dose is administered in the form of nitrate-rich beetroot juice (BR). This is important because sodium nitrate is a pharmaceutical product, whereas BR is a natural food product that can be readily ingested as part of the normal diet. We, therefore, investigated the influence of BR ingestion on plasma [nitrite], blood pressure (BP), muscle oxygenation [assessed with near-infrared spectroscopy (NIRS)] and the  $\dot{V}O_2$  response to step transitions to moderate- and severe-intensity exercise. We hypothesized that dietary BR supplementation would reduce the  $O_2$  cost of moderate-intensity exercise and increase exercise tolerance (assessed as the time-to-task failure) during severe-intensity exercise.

## METHODS

### Subjects

Eight healthy men (mean  $\pm$  SD, age  $26 \pm 7$  yr, height  $180 \pm 3$  cm, body mass  $82 \pm 6$  kg;  $\dot{V}O_{2max}$   $49 \pm 5$  ml $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) who were recreationally active in sporting activities volunteered to participate in this study. None of the subjects was a tobacco smoker or user of dietary supplements. All subjects were fully familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. The procedures employed in this study were approved by the Institutional Research Ethics Committee. All subjects gave their written, informed consent before the commencement of the study, after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Each subject was also asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively. All tests were performed at the same time of day ( $\pm 2$  h).

### Procedures

The subjects were required to report to the laboratory on seven occasions, over a 4- to 5-wk period. During the first visit to the laboratory, subjects performed a ramp incremental exercise test for determination of the peak  $\dot{V}O_2$  ( $\dot{V}O_{2peak}$ ) and GET. All cycle tests were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). Initially, subjects completed 3 min of "unloaded" baseline cycling, after which the work rate was increased at a rate of 30 W/min until the subject was unable to continue. The participants cycled at a self-selected pedal rate (70–90 rpm), and this pedal rate, along with saddle and handle bar height and configuration, was recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas-exchange data were collected contin-

uously during the incremental tests and averaged over consecutive 10-s periods. The  $\dot{V}O_{2peak}$  was taken as the highest 30-s average value attained before the subject's volitional exhaustion. The GET was determined as described previously (1, 5). The work rates that would require 80% of the GET (moderate exercise) and 70%  $\Delta$  (70% of the difference between the power output at the GET and  $\dot{V}O_{2peak}$ , severe exercise) were subsequently calculated, with account taken of the mean response time for  $\dot{V}O_2$  during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the power output at GET and peak; Ref. 63).

Following completion of the ramp test, subjects were randomly assigned in a crossover design, to receive 6 days of dietary supplementation with either nitrate ( $NO_3^-$ ; 5.5 mmol/day; administered as 0.5 liter organic BR/day; Beet It, James White Drinks, Ipswich, UK) or "placebo" (PL; low-calorie black currant juice cordial with negligible nitrate content). The subjects were instructed to sip the beverages at regular intervals throughout the day. A 10-day washout separated the supplementation periods. The order between the nitrate and PL supplementation periods was balanced. The subjects were provided with a list of foods rich in nitrates and asked to abstain from consuming these foods for the duration of the study. 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 beverages. The personnel administering the exercise tests were not aware of the type of beverage being consumed by the subjects.

On days 4, 5, and 6 of the supplementation periods, the subjects completed "step" exercise tests from a 20-W baseline to moderate- and severe-intensity work rates for the determination of pulmonary  $\dot{V}O_2$  dynamics. On the 4th day of supplementation, subjects completed two bouts of moderate cycling, while on days 5 and 6 the subjects completed one bout of moderate cycling and one bout of severe cycling. The two bouts of exercise on each day were separated by 25 min of passive recovery. All exercise bouts were of 6-min duration, with the exception of the severe exercise bout on the final day, which was continued until task failure as a measure of exercise tolerance. The time to task failure was recorded when the pedal rate fell by  $>10$  rpm below the self-selected pedal rate. In these bouts, the subjects were verbally encouraged to continue for as long as possible. The  $\dot{V}O_2$  responses to the four moderate and two severe exercise bouts were averaged before analysis to reduce breath-to-breath noise and enhance confidence in the parameters derived from the modeling process (44). Before each exercise bout, BP was measured, and venous blood samples were collected for subsequent determination of plasma [nitrite] (see Measurements below).

### Measurements

During all tests, pulmonary gas exchange and ventilation were measured continuously using a portable metabolic cart (MetaMax 3B, Cortex Biophysik, Leipzig, Germany), as described previously (1). A turbine digital transducer measured inspired and expired airflow, while an electrochemical cell  $O_2$  analyzer and an infrared  $CO_2$  analyzer simultaneously measured expired gases. Subjects wore a nose clip and breathed through a low-dead-space, low-resistance mouthpiece that was securely attached to the volume transducer. The inspired and expired gas volume and gas concentration signals were continuously sampled via a capillary line connected to the mouthpiece and displayed breath by breath. Heart rate (HR) was measured during all tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). During one of the transitions to moderate and severe exercise, for both supplementation periods, a blood sample was collected from a fingertip into a capillary tube over the 20 s preceding the step transition in work rate and within the last 20 s of exercise. A capillary blood sample was also collected at the limit of tolerance for the severe bout performed on day 6 of each supplementation period.

These whole blood samples were subsequently analyzed to determine blood [lactate] (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH) within 30 s of collection. Blood lactate accumulation ( $\Delta$ blood [lactate]) was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline.

The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using a commercially available NIRS system (model NIRO 300, Hamamatsu Photonics KK, Hiugashi-ku, Japan). The system consisted of an emission probe that irradiates laser beams and a detection probe. Four different wavelength laser diodes provided the light source (776, 826, 845, and 905 nm), and the light returning from the tissue was detected by a photomultiplier tube in the spectrometer. The intensity of incident and transmitted light was recorded continuously at 2 Hz and used to estimate concentration changes from the resting baseline for oxygenated, deoxygenated, and total tissue hemoglobin/myoglobin. Therefore, the NIRS data represent a relative change based on the optical density measured in the first datum collected. The deoxyhemoglobin concentration ([HHb]) signal can be regarded as being essentially blood-volume insensitive during exercise and so was assumed to reflect the balance between local  $O_2$  supply and utilization and to provide an estimate of changes in  $O_2$  extraction in the field of interrogation (1, 22, 24, 30). The leg was initially cleaned and shaved around the belly of the muscle, and the probes were placed in the holder, which was secured to the skin with adhesive at 20 cm above the fibular head. To secure the holder and wires in place, an elastic bandage was wrapped around the subject's leg. The wrap helped to minimize the possibility that extraneous light could influence the signal and also ensured that the optodes did not move during exercise. Pen marks were made around the probes to enable precise reproduction of the placement in subsequent tests. The probe gain was set with the subject at rest in a seated position with the leg extended at down stroke on the cycle ergometer before the first exercise bout, and NIRS data were collected continuously throughout the exercise protocols. The data were subsequently downloaded onto a personal computer, and the resulting text files were stored on disk for later analysis.

BP of the brachial artery was measured with subjects in a rested, seated position before each exercise bout using an automated sphygmomanometer. Three measurements were taken at each sample point with the mean of the second and third BP measurements being recorded. Venous blood samples were also drawn into lithium-heparin tubes before each exercise bout and centrifuged at 4,000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was subsequently extracted and immediately frozen at  $-80^\circ\text{C}$  for later analysis of  $\text{NO}_2^-$  via chemiluminescence (4).

All glass wear, utensils, and surfaces were rinsed with deionized water to remove residual  $\text{NO}_2^-$  before analysis. After thawing at room temperature, plasma samples were initially deproteinized before analysis using the procedures of Higuchi and Motomizu (33). Initially, 100  $\mu\text{l}$  of sample were placed in a microcentrifuge tube, along with 200  $\mu\text{l}$  of deionized  $\text{H}_2\text{O}$  and 300  $\mu\text{l}$  of 0.3 N NaOH, and left to stand at room temperature for 5 min. Then 300  $\mu\text{l}$  of 5% by weight aqueous  $\text{ZnSO}_4$  was added to the mixture, after which the sample was vortexed and left to stand at room temperature for a further 10 min. Thereafter, samples were centrifuged at 4,000 rpm for 15 min, and the supernatant was removed for subsequent analysis. The  $[\text{NO}_2^-]$  of the deproteinized plasma samples was determined by its reduction to NO in the presence of 5-ml glacial acetic acid and 1% NaI under nitrogen at room temperature in a gas-sealed purging vessel. Samples were introduced to the vessel via injection into the septum at the top of the vessel. The NO content was quantified by a chemiluminescence NO analyzer (Sievers, 280i NO analyzer). The reaction of NO with ozone in the chemiluminescent reaction chamber yielded electronically excited  $\text{NO}_2$  (nitrogen dioxide), which emits light at the infrared region of the electromagnetic spectrum. Ozone was generated from an  $O_2$  supply via an electrostatic ozone generator and high-voltage transformer. To minimize the interference of the chemiluminescent reactions of sulfur-

containing compounds, an optical filter transmitted only red wavelengths ( $>600$  nm), since the light emitted by sulfur-containing compounds is of shorter wavelengths. The intensity of the filtered infrared light was quantified by a red-sensitive photomultiplier tube and amplified producing an analog millivolt output signal. The  $[\text{NO}_2^-]$  was derived from the integral of the NO-generated millivolt signal over time compared with those obtained for  $\text{NaNO}_2^-$  standards.

#### Data Analysis Procedures

The breath-by-breath  $\dot{V}O_2$  data from each test were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than 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 exercise bouts were of different duration (6 min for the first bout and  $>6$  min in the second bout, which was performed to task failure), and so, at this intensity, only the first 6 min of data were averaged together 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  $\dot{V}O_2$  responses to moderate exercise, and a biexponential model was used for severe exercise, as described in the following equations:

$$\dot{V}O_2(t) = \dot{V}O_{2\text{baseline}} + A_p [1 - e^{-(t-\text{TD}_p/\tau_p)}] \quad (\text{moderate}) \quad (1)$$

$$\dot{V}O_2(t) = \dot{V}O_{2\text{baseline}} + A_p [1 - e^{-(t-\text{TD}_p/\tau_p)}] + A_s [1 - e^{-(t-\text{TD}_s/\tau_s)}] \quad (\text{severe}) \quad (2)$$

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

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values.  $\dot{V}O_{2\text{baseline}}$  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. Because the asymptotic value ( $A_s$ ) of the exponential term describing the  $\dot{V}O_2$  slow component may represent a higher value than is reached at the end of the exercise, the actual amplitude of the  $\dot{V}O_2$  slow component at the end of exercise was defined as  $A'_s$ . The  $A'_s$  parameter was compared at the same iso-time (360 s) under both supplementation periods. The amplitude of the slow component was also described relative to the entire  $\dot{V}O_2$  response. In addition, the functional "gain" of the fundamental  $\dot{V}O_2$  response was computed by dividing  $A_p$  by the  $\Delta$ work rate. The functional gain of the entire response (i.e., end-exercise gain) was calculated in a similar manner. To determine the overall kinetics of the  $\dot{V}O_2$  response to both moderate- and severe-intensity exercise, data were fit with a monoexponential model from 0 s to end exercise, without TD.

To provide information on muscle oxygenation, we also modeled the [HHb] response to exercise. Mono- and biexponential models, similar to those described above, were applied to the ensemble-averaged data, with the exception that the fitting window commenced at the time at which the [HHb] signal increased 1 SD above the baseline mean (22). The [HHb] kinetics for moderate exercise were determined by constraining the fitting window to the point at which monoexponentiality became distorted, consequent to a gradual fall in [HHb] (1), as determined by visual inspection of the residual plots. The [HHb] kinetics for severe exercise were determined by fitting a biexponential model from the first data point, which was 1 SD above

the baseline mean through the entire response. The [HHb] TD and time constant values were summed to provide information on the overall [HHb] response dynamics in the fundamental phase of the response. The oxyhemoglobin concentration ([HbO<sub>2</sub>]) and total hemoglobin concentration ([Hb<sub>tot</sub>]) responses do not approximate an exponential (22) and were, therefore, not modeled. Rather, we assessed changes in these parameters by determining the HbO<sub>2</sub> and [Hb<sub>tot</sub>] at baseline (90-s preceding step transition), 120 s, and end exercise (average response over the final 30 s of exercise).

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 monoexponential and biexponential models without TD were used to fit the data to moderate- and severe-intensity exercise, respectively, with the fitting window commencing at  $t = 0$  s. The HR  $\tau_p$  so derived provides information on the overall HR response dynamics.

### Statistics

Differences in the cardiorespiratory and NIRS-derived variables between conditions were analyzed with two-tailed, paired-samples  $t$ -tests. Alterations in BP and plasma [NO<sub>2</sub><sup>-</sup>] were determined via a two-tailed, two-way (supplement  $\times$  time) repeated-measures ANOVA. Significant effects were further explored using simple contrasts, with the  $\alpha$ -level adjusted via a Bonferroni correction. Correlations were assessed via Pearson's product-moment correlation coefficient. Data are presented as means  $\pm$  SD, unless otherwise stated. Statistical significance was accepted when  $P < 0.05$ .

## RESULTS

The BR supplementation regimen implemented in this study was well tolerated with no deleterious side effects. Subjects did, however, report beeturia (red urine) and red stools, consistent with a previous study (62).

### Plasma [NO<sub>2</sub><sup>-</sup>] and BP

The group mean plasma [NO<sub>2</sub><sup>-</sup>] values obtained at the two sample points on each of *days 4, 5, and 6* of the BR and PL supplementation periods are illustrated in Fig. 1. Participants showed elevations in plasma [NO<sub>2</sub><sup>-</sup>] during the BR supplementation compared with PL at all sample points (Fig. 1). On average, across the six sample points, BR ingestion increased plasma [NO<sub>2</sub><sup>-</sup>] by 96%. The BR-induced elevations in plasma [NO<sub>2</sub><sup>-</sup>] were not different across *days 4–6*.

The group mean systolic BP values measured at the six BR and PL sample points are shown in Fig. 2. The ingestion of BR significantly reduced systolic BP at five of the six sample points, relative to PL. Overall, systolic BP was reduced by 6 mmHg across the six samples points (Fig. 2); however, similar to plasma [NO<sub>2</sub><sup>-</sup>], the BR-induced reductions in systolic BP were not significantly different among *days 4–6*. The systolic BP was significantly related to the plasma [NO<sub>2</sub><sup>-</sup>] on *day 5* ( $r = -0.71$ ,  $P < 0.05$ ), but no relationships were detected between systolic BP and plasma [NO<sub>2</sub><sup>-</sup>] on *days 4 or 6*. Diastolic BP ( $\sim 72 \pm 8$  mmHg) and mean arterial pressure ( $\sim 91 \pm 5$  mmHg) were not significantly affected by BR ingestion.

### NIRS Measurements

**Moderate exercise.** The [HbO<sub>2</sub>], [HHb], and [Hb<sub>tot</sub>] values measured during moderate exercise are shown in Table 1, and the group mean responses are shown in Fig. 3. A 13% reduc-

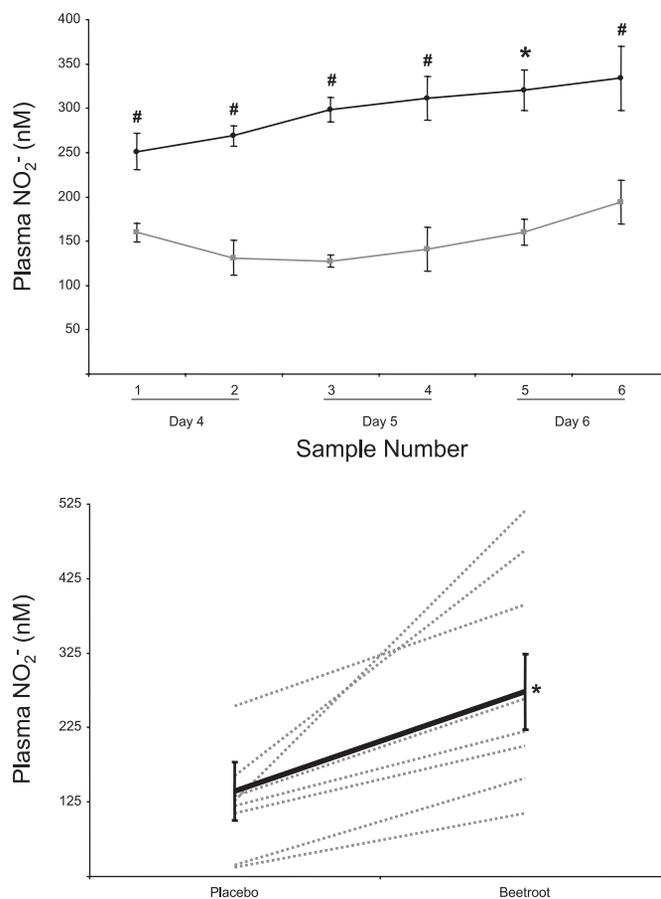


Fig. 1. Plasma nitrite concentration ([NO<sub>2</sub><sup>-</sup>]) following 4–6 days of dietary nitrate or placebo supplementation. *Top*: group mean ( $\pm$ SE) values of plasma [NO<sub>2</sub><sup>-</sup>] on *days 4, 5, and 6* of supplementation, with either nitrate (solid circles) or placebo (shaded squares). The blood samples for [NO<sub>2</sub><sup>-</sup>] determination were taken before each of the six exercise bouts that were completed in each condition (*bouts 1, 2, 3, and 5* were moderate, and *bouts 4 and 6* were severe; see text for further details). Note the significantly greater plasma [NO<sub>2</sub><sup>-</sup>] following dietary nitrate supplementation. Significant difference from placebo at corresponding time point at the #5% and \*1% levels of significance are given. *Bottom*: individual (dotted shaded lines) and mean  $\pm$  SE (solid line) values for plasma [NO<sub>2</sub><sup>-</sup>] measured over *days 4, 5, and 6*. \* $P < 0.01$ .

tion in the [HHb] amplitude was observed following BR ingestion, indicating that fractional O<sub>2</sub> extraction was reduced (PL:  $88 \pm 38$  vs. BR:  $78 \pm 34$  AU;  $P < 0.05$ ; Fig. 3). The [HbO<sub>2</sub>] within the microvasculature was increased at baseline and at 2 min into exercise, but was not significantly different at the end of exercise (Table 1). BR ingestion elevated [Hb<sub>tot</sub>] (an index of vascular red blood cell content) at baseline; however, this effect was not maintained during exercise (Table 1).

**Severe exercise.** The [HbO<sub>2</sub>], [HHb], and [Hb<sub>tot</sub>] values measured during severe exercise are shown in Table 1, and the group mean response is shown in Fig. 4. In contrast to the BR-induced changes in indexes of muscle oxygenation during moderate exercise, the [HHb], [HbO<sub>2</sub>], and [Hb<sub>tot</sub>] parameters were unaffected by BR ingestion during severe exercise.

### $\dot{V}O_2$ Dynamics and Exercise Tolerance

**Moderate exercise.** The pulmonary  $\dot{V}O_2$  response during moderate exercise is illustrated in Fig. 5, and the parameters derived from the model fit are presented in Table 2. Dietary BR

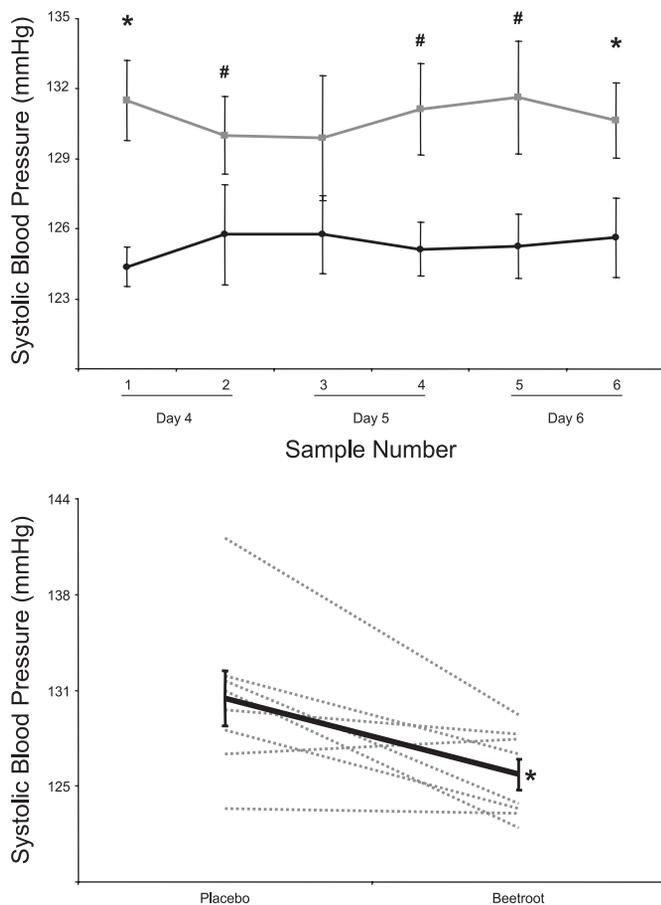


Fig. 2. Systolic blood pressure (SBP) following 4–6 days of dietary nitrate or placebo supplementation. *Top*: group mean ( $\pm$ SE) values of SBP on days 4, 5, and 6 of supplementation with either nitrate (solid circles) or placebo (shaded squares). Note the significantly lower SBP following dietary nitrate supplementation. Significant difference from placebo at corresponding time point at the #5% and \*1% levels of significance are given. *Bottom*: individual (dotted shaded lines) and mean  $\pm$  SE (solid line) values for SBP measured over days 4, 5, and 6. \* $P < 0.01$ .

supplementation resulted in a 19% reduction in the amplitude of the pulmonary  $\dot{V}O_2$  response, relative to PL, following a step increment to the same absolute moderate-intensity cycling work rate (PL:  $640 \pm 146$  vs. BR:  $521 \pm 153$  ml/min;  $P < 0.01$ ; Fig. 5), with there being no difference in  $\dot{V}O_2$  during the baseline period of very-low-intensity (20 W) cycling. Accordingly, the functional gain (i.e., the ratio of the increase in  $O_2$  consumed per minute to the increase of external power output) was reduced from  $10.8 \text{ ml} \cdot \text{min}^{-1} \cdot \text{W}^{-1}$  following PL supplementation to  $8.6 \text{ ml} \cdot \text{min}^{-1} \cdot \text{W}^{-1}$  following BR supplementation. The absolute  $\dot{V}O_2$  value over the final 30 s of moderate exercise was also significantly lower following BR ingestion (PL:  $1,517 \pm 123$  vs. BR:  $1,448 \pm 129$  ml/min;  $P < 0.01$ ; Fig. 5). The phase II time constant was not significantly altered by BR supplementation (PL:  $26 \pm 7$  vs. BR:  $29 \pm 6$  s;  $P > 0.05$ ). The 95% confidence interval for the estimation of the phase II time constant was  $3 \pm 1$  s for both conditions. The baseline and end-exercise values of  $\text{CO}_2$  production, minute ventilation ( $\dot{V}E$ ), respiratory exchange ratio (RER), HR, and blood [lactate] were not significantly between the conditions (Tables 2 and 3).

*Severe exercise.* The pulmonary  $\dot{V}O_2$  response during severe exercise is shown in Fig. 6, and the parameters derived from the bi-exponential fit are presented in Table 2. In contrast to the effects observed for moderate exercise, the primary  $\dot{V}O_2$  amplitude during severe exercise was significantly elevated following BR supplementation (PL:  $2,158 \pm 168$  vs. BR:  $2,345 \pm 179$  ml/min;  $P < 0.05$ ). Additionally, the phase II time constant was significantly greater following BR supplementation relative to PL (PL:  $33 \pm 11$  vs. BR:  $40 \pm 13$  s;  $P < 0.05$ ; Table 2). The 95% confidence intervals for the estimation of the phase II time constant were  $5 \pm 2$  and  $6 \pm 3$  s for the PL and BR conditions, respectively. The amplitude of the  $\dot{V}O_2$  slow component was significantly smaller following BR supplementation (PL:  $739 \pm 242$  vs. BR:  $568 \pm 195$  ml/min;  $P < 0.05$ ), and, therefore, represented a smaller proportion of the overall  $\dot{V}O_2$  response (PL:  $25 \pm 6$  vs. BR:  $19 \pm 6\%$ ;  $P < 0.05$ ). The  $\dot{V}O_2$  attained at task failure was not different, either between the conditions or from the  $\dot{V}O_{2\text{max}}$  recorded during the initial ramp incremental test. Exercise tolerance was enhanced following BR supplementation, as demonstrated by the increased time to task failure (PL:  $583 \pm 145$  vs. BR:  $675 \pm 203$  s;  $P < 0.05$ ; Table 2). However, the

Table 1. Near-infrared spectroscopy-derived HHb, HbO<sub>2</sub>, and Hb<sub>tot</sub> dynamics during moderate- and severe-intensity exercise following supplementation with nitrate and placebo

	Placebo	Nitrate
<i>Moderate-intensity exercise</i>		
[HHb]		
Baseline, AU	$-132 \pm 84$	$-131 \pm 96$
120 s, AU	$-41 \pm 56$	$-54 \pm 74$
End, AU	$-51 \pm 55$	$-55 \pm 83$
Mean response time, s	$32 \pm 8$	$29 \pm 9$
Amplitude, AU	$88 \pm 38$	$78 \pm 34^*$
[HbO <sub>2</sub> ]		
Baseline, AU	$-29 \pm 74$	$21 \pm 51^*$
120 s, AU	$-80 \pm 72$	$-15 \pm 30^*$
End, AU	$-5 \pm 67$	$25 \pm 39$
[Hb <sub>tot</sub> ]		
Baseline, AU	$-160 \pm 129$	$-110 \pm 89^*$
120 s, AU	$-47 \pm 89$	$-29 \pm 70$
End, AU	$-57 \pm 92$	$-30 \pm 81$
<i>Severe-intensity exercise</i>		
[HHb]		
Baseline, AU	$-142 \pm 95$	$-104 \pm 89$
120 s, AU	$176 \pm 107$	$202 \pm 125$
End, AU	$215 \pm 110$	$246 \pm 126$
Primary time constant, s	$9 \pm 2$	$11 \pm 2$
Primary time delay, s	$8 \pm 1$	$8 \pm 2$
Primary amplitude, AU	$300 \pm 70$	$287 \pm 103$
Slow-phase amplitude, AU	$63 \pm 27$	$67 \pm 16$
[HbO <sub>2</sub> ]		
Baseline, AU	$96 \pm 119$	$57 \pm 91$
120 s, AU	$-147 \pm 66$	$-176 \pm 68$
End, AU	$-133 \pm 59$	$-166 \pm 65$
[Hb <sub>tot</sub> ]		
Baseline, AU	$-46 \pm 116$	$-47 \pm 69$
120 s, AU	$29 \pm 121$	$26 \pm 105$
End, AU	$82 \pm 110$	$80 \pm 104$

Values are means  $\pm$  SD. [HHb], deoxygenated hemoglobin concentration; [HbO<sub>2</sub>], oxygenated hemoglobin concentration; [Hb<sub>tot</sub>], total hemoglobin concentration; AU, arbitrary units. \*Significantly different from placebo,  $P < 0.05$ .

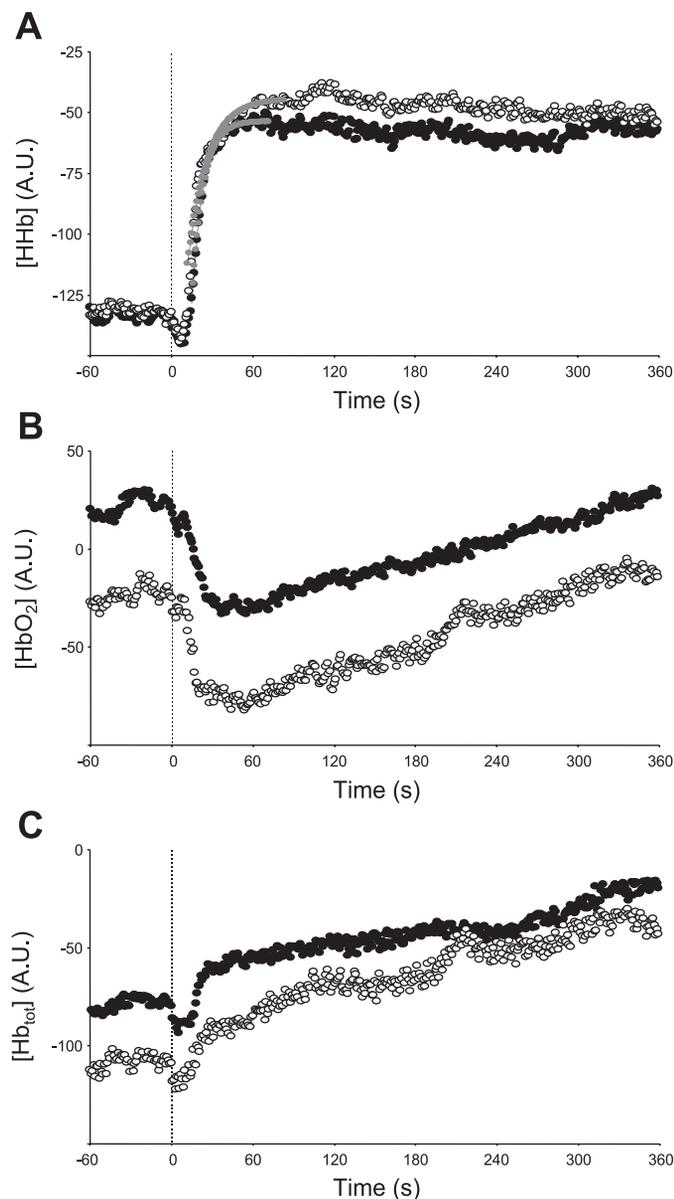


Fig. 3. Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a moderate-intensity cycle work rate. Responses following nitrate supplementation are shown as solid circles, while the placebo responses are shown as open circles. The dotted vertical line represents the abrupt imposition of a moderate work rate from a baseline of “unloaded” cycling. *A*: deoxyhemoglobin concentration ([HHb]). *B*: oxyhemoglobin concentration ([HbO<sub>2</sub>]). *C*: total hemoglobin concentration ([Hb<sub>tot</sub>]). For each individual, the responses to four like-transitions were averaged together before analysis. Note the reduction in the amplitude of the [HHb] response and the greater [HbO<sub>2</sub>] and [Hb<sub>tot</sub>] before and during moderate exercise following dietary nitrate supplementation. Error bars are not shown for clarity, but see Table 1 for further details. AU, arbitrary units.

increased time to task failure was not correlated with the reduction of the  $\dot{V}O_2$  slow component ( $r = -0.14$ ;  $P = 0.70$ ). The baseline and end-exercise values of CO<sub>2</sub> production,  $\dot{V}_E$ , RER, and HR were not significantly different between the conditions (Tables 2 and 3). Blood [lactate] at 6 min of exercise and at task failure was also not significantly different between the conditions (Table 3).

## DISCUSSION

The principal original finding of this investigation is that 3 days of dietary supplementation with nitrate-rich BR (which doubled the plasma [nitrite]) significantly reduced the O<sub>2</sub> cost of cycling at a fixed submaximal work rate and increased the time to task failure during severe exercise. These findings were consistent with our experimental hypotheses. The O<sub>2</sub> cost of cycling at a fixed moderate work rate is known to be highly consistent in human populations, irrespective of factors such as age and training status (36, 49). That an acute nutritional

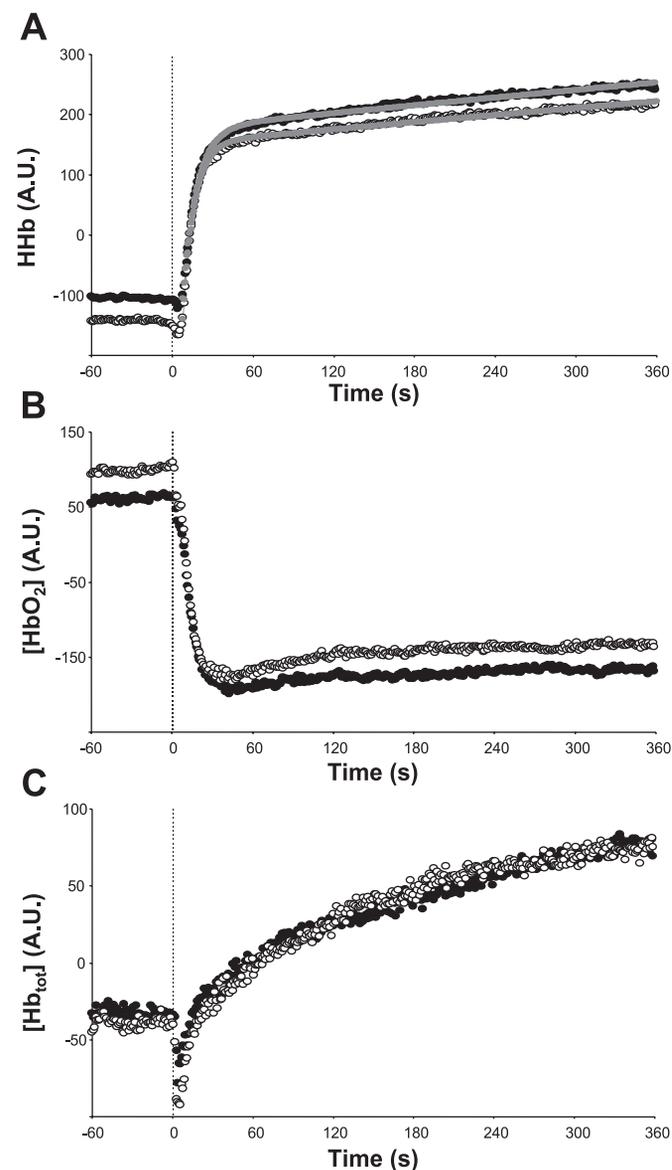


Fig. 4. Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a severe-intensity cycle work rate. Responses following nitrate supplementation are shown as solid circles, while the placebo responses are shown as open circles. The dotted vertical line represents the abrupt imposition of a severe work rate from a baseline of “unloaded” cycling. *A*: [HHb]. *B*: [HbO<sub>2</sub>]. *C*: [Hb<sub>tot</sub>]. For each individual, the responses to two like-transitions were averaged together before analysis. The near-infrared spectroscopy-derived parameters were not appreciably different before or during severe exercise following dietary nitrate supplementation. Error bars are not shown for clarity, but see Table 1 for further details.

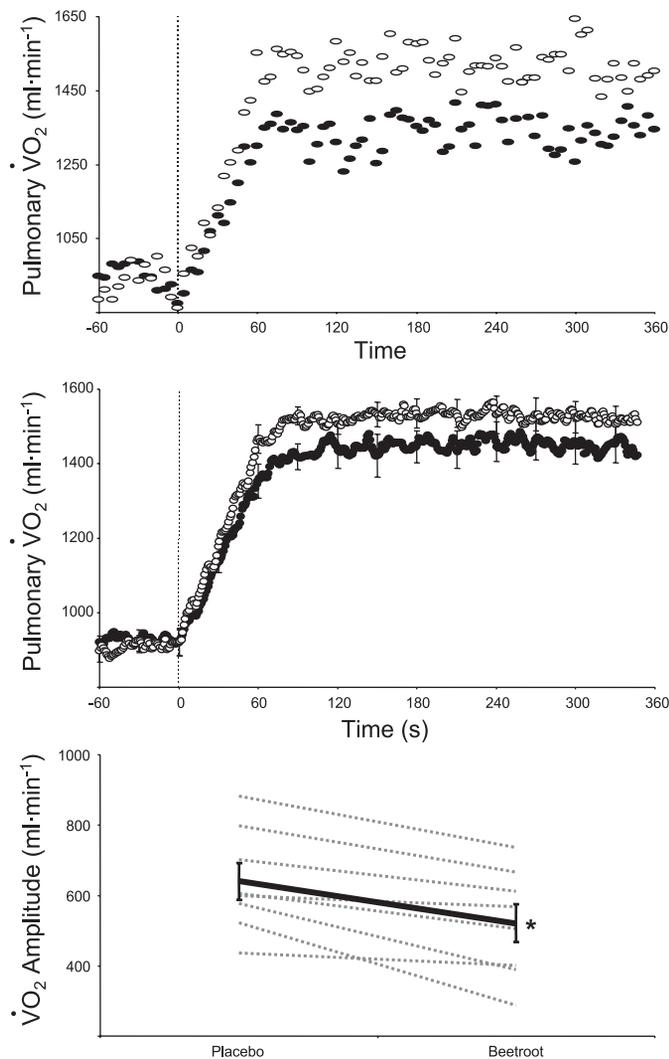


Fig. 5. Pulmonary oxygen uptake ( $\dot{V}O_2$ ) response following nitrate and placebo supplementation during a step increment to a moderate-intensity work rate. Responses following nitrate supplementation are shown as solid circles, while the placebo responses are shown as open circles. The dotted vertical line represents the abrupt imposition of the moderate work rate from a baseline of “unloaded” cycling. *Top*:  $\dot{V}O_2$  response of a representative individual (data are shown at 5-s intervals). *Middle*: group mean  $\dot{V}O_2$  response with error bars shown every 30 s for clarity. The oxygen cost of moderate exercise was significantly reduced following beetroot supplementation. *Bottom*: individual changes in the amplitude of the  $\dot{V}O_2$  response to moderate exercise following nitrate supplementation (dotted shaded lines), along with the group mean change (solid line). For each individual, the responses to four like-transitions were averaged together before analysis. Note that the effect was observed in all participants. \* $P < 0.01$ .

intervention (i.e., dietary supplementation with a natural food product that is rich in nitrate) can reduce the  $O_2$  cost of a given increment in work rate by  $\sim 20\%$  is, therefore, remarkable.

Short-term (i.e., 4–6 days) dietary supplementation with BR increased plasma  $[NO_2^-]$  by  $\sim 96\%$  in this investigation. Consistent with these findings, dietary  $NO_3^-$  supplementation has previously been shown to increase plasma  $[NO_2^-]$  when administered as either sodium nitrate (45) or BR (62). Importantly, interrupting the entero-salivary circulation by spitting out saliva thwarted the rise in plasma  $[NO_2^-]$  (62), while administration of antibacterial mouthwash before sodium nitrate ingestion also prevented the rise in plasma  $[NO_2^-]$  by

decreasing the  $NO_3^-$ -reducing bacteria counts in the oral cavity (28). Collectively, these data highlight the dependence of the  $NO_3^-$ -to- $NO_2^-$  conversion pathway on the commensal bacterial nitrate reductases present in the human oral cavity. The bacterially derived  $NO_2^-$  can increase circulating plasma  $NO_2^-$  and undergo reduction to yield NO in hypoxia or acidosis (21, 46).

*Effects of dietary nitrate on BP.* In the present study, dietary supplementation with BR reduced systolic BP by an average of 6 mmHg, but without altering diastolic BP or mean arterial pressure. In contrast, both systolic and diastolic BP were reduced following 3 days of dietary  $NO_3^-$  supplementation in the study of Larsen et al. (45). Recent data indicate that, following BR ingestion, peak reductions in systolic and diastolic BP are observed 2.5 and 3 h post-ingestion, respectively (62). Furthermore, the BR-induced reduction in systolic BP persisted for 24 h post-ingestion, while diastolic BP had returned toward baseline (62). Collectively, these data suggest that systolic BP is more amenable to nitrate-induced change than is diastolic BP. The reduced BP observed with a diet rich in nitrates

Table 2. Mean  $\pm$  SD ventilatory and gas exchange dynamics during moderate- and severe-intensity exercise following supplementation with nitrate and placebo

	Placebo	Nitrate
<i>Moderate-intensity exercise</i>		
$\dot{V}O_2$		
Baseline, l/min	0.91 $\pm$ 0.09	0.93 $\pm$ 0.05
End-exercise, l/min	1.52 $\pm$ 0.12	1.45 $\pm$ 0.13*
Phase II time constant, s	26 $\pm$ 7	29 $\pm$ 6
Mean response time, s	39 $\pm$ 8	45 $\pm$ 4
Primary amplitude, l/min	0.64 $\pm$ 0.15	0.52 $\pm$ 0.15*
Primary gain, ml·min <sup>-1</sup> ·W <sup>-1</sup>	10.8 $\pm$ 1.6	8.6 $\pm$ 0.7†
$\dot{V}CO_2$		
Baseline, l/min	0.85 $\pm$ 0.08	0.84 $\pm$ 0.05
End-exercise, l/min	1.31 $\pm$ 0.15	1.32 $\pm$ 0.15
$\dot{V}E$		
Baseline, l/min	25 $\pm$ 2	24 $\pm$ 1
End-exercise, l/min	36 $\pm$ 4	34 $\pm$ 3
Respiratory exchange ratio		
Baseline	0.93 $\pm$ 0.07	0.90 $\pm$ 0.07
End-exercise	0.90 $\pm$ 0.05	0.91 $\pm$ 0.02
<i>Severe-intensity exercise</i>		
$\dot{V}O_2$		
Baseline, l/min	0.99 $\pm$ 0.10	0.96 $\pm$ 0.07
End-exercise, l/min	3.87 $\pm$ 0.29	3.82 $\pm$ 0.28
Phase II time constant, s	33 $\pm$ 11	40 $\pm$ 13†
Primary amplitude, l/min	2.19 $\pm$ 0.17	2.35 $\pm$ 0.18†
Primary gain, ml·min <sup>-1</sup> ·W <sup>-1</sup>	9.0 $\pm$ 0.7	9.4 $\pm$ 0.6
Slow-phase amplitude, l/min	0.74 $\pm$ 0.24	0.57 $\pm$ 0.20†
Slow-component amplitude, %	25 $\pm$ 6	19 $\pm$ 6†
Overall gain, ml·min <sup>-1</sup> ·W <sup>-1</sup>	11.6 $\pm$ 0.9	10.8 $\pm$ 0.8*
Overall mean response time, s	75 $\pm$ 16	71 $\pm$ 16
$\dot{V}CO_2$		
Baseline, l/min	0.85 $\pm$ 0.23	0.86 $\pm$ 0.10
End-exercise, l/min	3.99 $\pm$ 0.32	4.03 $\pm$ 0.43
$\dot{V}E$		
Baseline, l/min	24 $\pm$ 7	25 $\pm$ 3
End-exercise, l/min	140 $\pm$ 14	139 $\pm$ 21
Respiratory exchange ratio		
Baseline	0.86 $\pm$ 0.17	0.89 $\pm$ 0.09
End-exercise	1.04 $\pm$ 0.05	1.05 $\pm$ 0.05

Values are means  $\pm$  SD.  $\dot{V}O_2$ , oxygen uptake;  $\dot{V}CO_2$ , expired carbon dioxide;  $\dot{V}E$ , minute ventilation. Significantly different from placebo: \* $P < 0.01$ , † $P < 0.05$ .

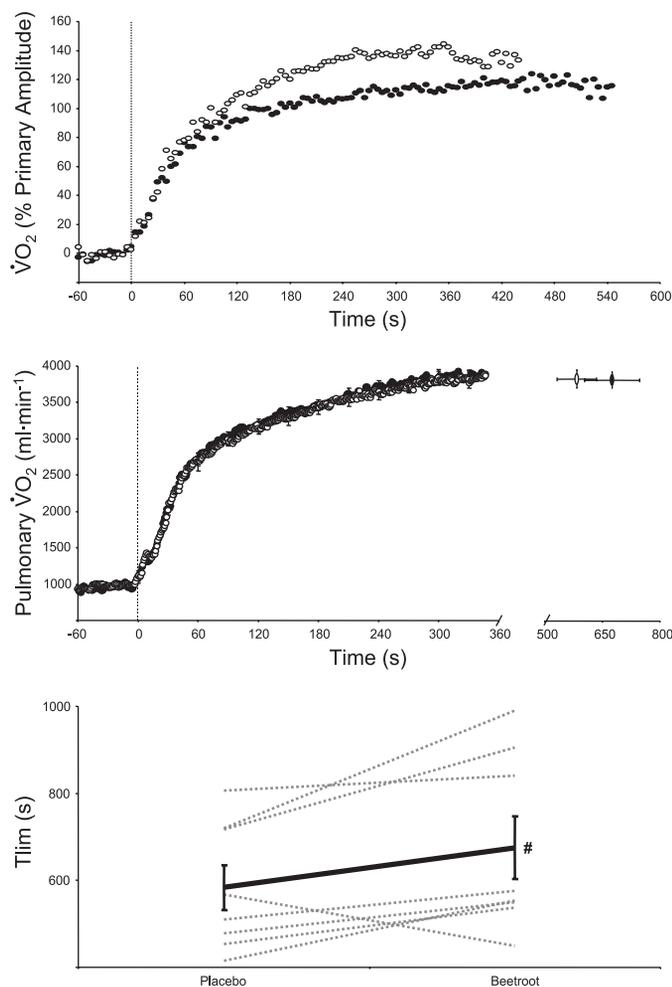


Fig. 6. Pulmonary  $\dot{V}O_2$  response following nitrate and placebo supplementation during a step increment to a severe-intensity work rate. Responses following nitrate supplementation are shown as solid circles, while the placebo responses are shown as open circles. The dotted vertical line represents the abrupt imposition of the severe work rate from a baseline of “unloaded” cycling. *Top*:  $\dot{V}O_2$  response of a representative individual (data are shown at 5-s intervals). The data are plotted as a fraction of the  $\dot{V}O_2$  fundamental component amplitude to more clearly illustrate the slower phase II  $\dot{V}O_2$  kinetics and reduced  $\dot{V}O_2$  slow component following nitrate supplementation. *Middle*: group mean  $\dot{V}O_2$  response with error bars shown every 30 s for clarity. The group mean  $\pm$  SE  $\dot{V}O_2$  at task failure is also shown. *Bottom*: individual changes in the tolerance of severe exercise following nitrate supplementation (dotted shaded lines) along with the group mean change (solid line). # $P < 0.05$ .

suggests that this “natural” approach has the potential to maintain or enhance aspects of human cardiovascular health.

The  $NO_3^-$ -induced reduction in systolic BP is likely mediated via its conversion to  $NO_2^-$  and thence NO. NO is known to be an important endothelial relaxing factor, through its role as a secondary messenger in cyclic guanosine monophosphate synthesis, culminating in smooth muscle relaxation (31). Conventionally, NO production was considered to be derived, universally, via the NO synthase family of enzymes in humans. These enzymes produce NO through catalyzing the five-electron oxidation of L-arginine in a reaction requiring  $O_2$  and NADPH (9). More recent research has identified an  $O_2$ -independent pathway for the generation of NO, via the reduction of  $NO_2^-$  to NO in an acidic milieu (6). Importantly, this pathway

preserves blood and tissue NO production during hypoxia when the enzymatic activity of the NO synthases are rate limited by the lack of  $O_2$  availability. As such, a considerable body of evidence now supports a biological role for  $NO_2^-$  in hypoxic vasodilation (26), which serves to protect tissues from ischemia and reperfusion injury (27).

*Effects of dietary nitrate on the physiological responses to moderate exercise.* Increased dietary  $NO_3^-$  consumption altered indexes of muscle oxygenation as investigated using NIRS. During moderate exercise,  $[Hb_{tot}]$  was elevated at baseline, and  $[HbO_2]$  was elevated both at baseline and over the first 120 s of exercise following BR ingestion. The increased blood volume in the region of interrogation at baseline following BR ingestion is presumably a consequence of enhanced muscle vasodilatation, resulting from increased NO production from  $NO_2^-$ . The NIRS-derived  $[HHb]$  response reflects the balance between local  $O_2$  delivery and utilization and has been used previously as an index of muscle fractional  $O_2$  extraction (1, 22, 24, 30). In the present study, the amplitude of the  $[HHb]$  response was reduced by 13% following BR ingestion. Conversely, inhibition of NO synthesis via  $N^G$ -nitro-L-arginine methyl ester (L-NAME) administration has been shown to increase muscle  $O_2$  extraction in the exercising horse (40). By the Fick equation, for the same  $\dot{V}O_2$ , an increased muscle  $O_2$  delivery would be expected to enable a reduced muscle fractional  $O_2$  extraction. However, in the present study, in which  $\dot{V}O_2$  was reduced by dietary BR supplementation, an alternative interpretation is that less  $O_2$  extraction was required consequent to a reduced aerobic energy turnover or muscle energy utilization.

Perhaps the most striking finding of the present investigation was the significant reduction in the  $O_2$  cost of submaximal exercise following increased dietary  $NO_3^-$  intake. While the  $\dot{V}O_2$  response during the unloaded baseline cycling period was unaffected, a 19% reduction in the amplitude of the pulmonary

Table 3. Heart rate and blood lactate responses to moderate- and severe-intensity exercise following supplementation with nitrate and placebo

	Placebo	Nitrate
<i>Moderate-intensity exercise</i>		
Heart rate		
Baseline, beats/min	81 $\pm$ 9	81 $\pm$ 8
End, beats/min	98 $\pm$ 12	98 $\pm$ 13
Time constant, s	28 $\pm$ 12	31 $\pm$ 16
Blood [lactate]		
Baseline, mM	1.0 $\pm$ 0.5	0.9 $\pm$ 0.3
End, mM	1.2 $\pm$ 0.7	1.1 $\pm$ 0.2
$\Delta$ , mM	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2
<i>Severe-intensity exercise</i>		
Heart rate		
Baseline, beats/min	85 $\pm$ 8	88 $\pm$ 8
End, beats/min	170 $\pm$ 8	170 $\pm$ 8
Time constant, s	16 $\pm$ 10	17 $\pm$ 5
Blood [lactate]		
Baseline, mM	1.0 $\pm$ 0.2	1.1 $\pm$ 0.5
End, mM	6.9 $\pm$ 1.6	6.9 $\pm$ 1.2
$\Delta$ , mM	5.9 $\pm$ 1.6	5.7 $\pm$ 1.0
Exhaustion, mM	10.0 $\pm$ 1.9	10.0 $\pm$ 1.7

Values are means  $\pm$  SD. [Lactate], lactate concentration;  $\Delta$ , change.

$\dot{V}O_2$  response, relative to PL, was evident following  $NO_3^-$  supplementation during a step increment to the same absolute moderate-intensity cycling work rate. Accordingly, the functional gain (i.e., the ratio of the increase in  $O_2$  consumed per minute to the increase of external power output and the reciprocal of delta efficiency; Ref. 64) was reduced from  $10.8 \text{ ml}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$  following PL supplementation to  $8.6 \text{ ml}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$  following  $NO_3^-$  supplementation. Moreover, the gross  $O_2$  cost of exercise (comprising resting metabolic rate, the  $O_2$  cost of moving the limbs during baseline pedaling, and the  $O_2$  cost of muscle contraction to meet the imposed work rate) was reduced by  $\sim 5\%$ . The magnitude of effect was similar to that reported by Larsen et al. (45) using sodium nitrate supplementation. While we therefore suggest that the reduced  $O_2$  cost of submaximal exercise in our study was consequent to the increased dietary nitrate content, we cannot presently exclude the possibility that other substances contained in BR contributed to the results we obtained. Importantly, the reduction in  $\dot{V}O_2$ , and thus of ATP resynthesis through oxidative phosphorylation, was not compensated by elevations in glycolytic ATP provision, as inferred, albeit crudely, from the similar blood [lactate] values between the BR and PL conditions. These findings extend those of Larsen et al. (45) by demonstrating that the  $O_2$  cost of submaximal exercise is reduced following  $NO_3^-$  ingestion in the form of a natural food product. That HR and  $\dot{V}E$  were not significantly different between treatments suggests that the reduction in  $\dot{V}O_2$  originated from the skeletal muscles and not from alterations in the energetic cost of cardiorespiratory support processes. Moreover, the similar RER between the conditions indicates that substrate utilization (which can influence the  $O_2$  cost of exercise) was not altered by the intervention.

The mechanistic bases for the reduced  $O_2$  cost of submaximal exercise following increased  $NO_3^-$  intake, either by pharmacological (45) or natural dietary means (present study), are unclear. The inhibition of NO synthesis has previously been shown to increase steady-state  $\dot{V}O_2$  in dogs (56), but not humans (38) or horses (41). It is widely accepted, however, that NO is involved in the regulation of mitochondrial  $O_2$  consumption. In particular, it has been established that NO has a strong affinity for cytochrome-c oxidase (CytOX; Ref. 10), but there is also evidence that NO has the potential to modulate other aspects of mitochondrial and muscle contractile function (11, 57, 58).

A reduction in the  $O_2$  cost of mitochondrial ATP resynthesis would require either more protons pumped per  $O_2$  molecule reduced, or the use of an alternative terminal electron acceptor in place of  $O_2$ . It has been proposed that mitochondrial efficiency is intimately linked to the process of uncoupled respiration in which mitochondrial proton leak results in energy dissipation as heat instead of conversion to ATP (8). In this regard, the improved  $O_2$  efficiency noted in the present study following BR ingestion might be related to a reduction of mitochondrial proton leak or proton pump slippage. There is evidence to suggest that NO increases the efficiency of oxidative phosphorylation in isolated mitochondria by reducing slipping of the proton pumps (17). Another possibility is that  $NO_2^-$  could be acting in place of  $O_2$  as the final electron acceptor in the respiratory chain, thereby reducing the requirement for  $O_2$  consumption (3). An intra-mitochondrial  $NO_2^-$  regeneration pathway would be critical in this scenario, given

the limited  $NO_2^-$  concentration within the mitochondria. Under conditions of low electron flux, NO can inhibit CytOX by binding to the  $Cu^{2+}$  active site yielding nitrosonium ( $NO^+$ ), which is subsequently hydrated to  $NO_2^-$  (18). One possible scenario is that the  $NO_2^-$  so produced could be reduced to NO by accepting an electron from CytOX, and this NO could subsequently bind to the  $Cu^{2+}$  active site, completing the cyclical process to regenerate  $NO_2^-$ . This possibility is intriguing, as the hydration of  $NO^+$  to  $NO_2^-$  yields an electron that can be redistributed within CytOX (19, 60). Subsequently, this electron may be accepted by  $NO_2^-$ , potentially utilizing the electron derived from its synthesis, and could contribute to proton pumping and ATP synthesis, in an efficiently coupled process.

The reduction in  $O_2$  consumption with increased dietary  $NO_3^-$  intake could also be attributed, in part, to a reduced ATP cost of force production, requiring less flux through oxidative phosphorylation. One of the most energetically costly processes during skeletal muscle contraction is sarcoplasmic reticulum  $Ca^{2+}$  pumping, which may account for up to 50% of the total ATP turnover (7). The presence of reactive oxygen species increases the opening probability of the sarcoplasmic reticulum  $Ca^{2+}$  release channels (48), and the active reuptake of the elevated cytosolic  $Ca^{2+}$  would present a considerable energetic challenge (7). NO donors that invoke small elevations in NO might protect the channel against oxidation-induced  $Ca^{2+}$  release, without significantly altering channel function (53). Therefore, the BR-induced elevations in NO may have prevented an excess of  $Ca^{2+}$  release and subsequently reduced the considerable energetic cost of its re-sequestration. These suggestions are naturally speculative at the present time and await further investigation.

*Effects of dietary nitrate on the physiological responses to severe exercise.* The physiological responses during severe exercise were different from those observed during moderate exercise following BR supplementation, suggesting that the influence of  $NO_2^-$  and/or NO on muscle function is specific to the exercise intensity domain being investigated. First, there were no significant differences in NIRS-derived indexes of muscle oxygenation between the  $NO_3^-$  and PL conditions during severe exercise, although this might be a function of the fact that severe exercise always followed moderate exercise in our experiments. Moreover, in contrast to the reduced steady-state  $\dot{V}O_2$  observed during moderate exercise following increased  $NO_3^-$  consumption, the amplitude of the primary or fundamental component  $\dot{V}O_2$  response during severe exercise was increased (by  $\sim 7\%$ ), and the amplitude of the subsequent  $\dot{V}O_2$  slow component was reduced (by  $\sim 23\%$ ), with the  $\dot{V}O_2$  at the point of task failure being not significantly different between the conditions. These changes in  $\dot{V}O_2$  kinetics during high-intensity exercise following dietary  $NO_3^-$  supplementation resemble those that are observed following an initial "priming" bout of high-intensity exercise (15, 35, 45), effects that have been attributed, either separately or in combination, to increased muscle  $O_2$  delivery, increased oxidative metabolic enzyme activity and carbon substrate availability, and altered motor unit recruitment patterns (15, 35, 45). We have previously reported that NO synthase inhibition with L-NAME significantly increased the amplitude of the  $\dot{V}O_2$  slow component and speculated that this might be related to changes in muscle  $O_2$  delivery or its distribution and/or to (related)

changes in motor unit recruitment patterns (39). It is presently unclear which, if any, of the above-named mechanisms contributed to the altered  $\dot{V}_{O_2}$  kinetics during severe exercise following dietary  $\text{NO}_3^-$  supplementation. Myocytes in close proximity to a capillary are advantaged with respect to muscle  $\text{O}_2$  availability, whereas myocytes situated further away are increasingly less well supplied with  $\text{O}_2$ , creating an  $\text{O}_2$  pressure gradient within the contracting muscles. The hypoxic and acidic milieu within and surrounding the distal myocytes might stimulate  $\text{NO}_2^-$  reduction to NO facilitating vasodilatation and thus delivery of  $\text{O}_2$ . The NO so produced is capable of diffusion and may inhibit mitochondrial  $\text{O}_2$  consumption in the myocytes proximal to the capillary bed, promoting deeper diffusion of the available  $\text{O}_2$  (32, 59) and, therefore, enabling a more appropriate matching of local  $\text{O}_2$  delivery to  $\text{O}_2$  requirement. Another possibility is that the increased NO availability following increased dietary  $\text{NO}_3^-$  intake promotes mitochondrial biogenesis (16, 50). Greater mitochondrial volume would enable the same rate of mitochondrial respiration to be accomplished with a reduced perturbation of adenine nucleotides. However, irrespective of the mechanism(s) involved, the return of  $\dot{V}_{O_2}$  kinetics toward first-order linear system dynamics during severe exercise will likely limit the rate at which metabolites that have been associated with the fatigue process (e.g.,  $\text{H}^+$ ,  $\text{P}_i$ , ADP) accumulate in skeletal muscle (14), effects that would be expected, in turn, to portend enhanced exercise tolerance.

The  $\tau_p$  was significantly longer (i.e., phase II pulmonary  $\dot{V}_{O_2}$  kinetics was slower) following  $\text{NO}_3^-$  supplementation relative to PL for severe exercise (group mean  $\tau_p$  40 vs. 33 s for BR and PL). This is consistent with our laboratory's previous reports that the  $\tau_p$  was significantly faster when NOS activity was inhibited by L-NAME (38, 39, 66). The  $\tau_p$  was also slightly longer during moderate exercise (group mean  $\tau_p$  29 vs. 26 s for BR and PL), but this difference was not statistically significant. While it is acknowledged that changes in muscle blood flow, which might occur with greater or lesser NO availability, have the potential to dissociate the normally close relationship between muscle and pulmonary  $\dot{V}_{O_2}$  kinetics (2), our data suggest that NO might have an important regulatory influence on the inertia of  $\dot{V}_{O_2}$  dynamics following the onset of exercise (36, 51). It is of interest that  $\text{NO}_3^-$  supplementation resulted in changes in the amplitudes of the  $\dot{V}_{O_2}$  response to exercise (i.e., lower steady-state  $\dot{V}_{O_2}$  during moderate exercise and higher  $\dot{V}_{O_2}$  fundamental component and reduced  $\dot{V}_{O_2}$  slow component during severe exercise), while also slowing the phase II  $\dot{V}_{O_2}$  kinetics. While the mechanistic bases of this effect is not clear, it is possible that increased NO availability results, simultaneously, in a slowing of the rate at which  $\dot{V}_{O_2}$  rises following the onset of exercise (via competitive inhibition of CytOX; Refs. 10, 38, 39, 41, 56) and changes in the amplitudes of the fundamental and slow components of  $\dot{V}_{O_2}$  (through effects on the efficiency of muscle oxidative metabolism and/or contractile function; Refs. 3, 11, 17, 57).

The kinetics of  $\dot{V}_{O_2}$  are considered to be an important determinant of exercise tolerance (14, 36). However, in this study, we observed a 16% improvement in the time to task failure during severe exercise in the  $\text{NO}_3^-$  condition, despite the slower phase II  $\dot{V}_{O_2}$  kinetics. Another parameter of  $\dot{V}_{O_2}$  dynamics considered to influence exercise tolerance is the slow component rise in  $\dot{V}_{O_2}$  observed during supra-GET exercise,

since this parameter is associated with greater utilization of the finite phosphocreatine (55) and glycogen (42) reserves. Indeed, a reduction in the  $\dot{V}_{O_2}$  slow-component amplitude has been associated with improved exercise tolerance (1, 14). However, in the present study, the improvement in severe exercise tolerance with  $\text{NO}_3^-$  supplementation was not significantly correlated with the reduction of the  $\dot{V}_{O_2}$  slow-component amplitude. The  $\dot{V}_{O_2}$  at task failure (which was equivalent to the preestablished  $\dot{V}_{O_{2\max}}$ ) was not different between conditions, suggesting that the  $\dot{V}_{O_{2\max}}$  was reached more slowly and/or could be sustained for longer following  $\text{NO}_3^-$  supplementation. It is noteworthy that the  $\dot{V}_{O_{2\max}}$  during severe exercise was not impaired with  $\text{NO}_3^-$  supplementation, although the steady-state  $\dot{V}_{O_2}$  was reduced during submaximal exercise. These results differ from those obtained with NO synthase inhibition. With the latter,  $\dot{V}_{O_{2\max}}$  and exercise tolerance are impaired during both ramp incremental exercise (37) and supramaximal step exercise (66). Although the mechanism for the enhanced performance observed in the present study is uncertain, an interesting possibility is that an elevation of tissue s-nitrosothiols with increased dietary nitrate intake (13) prevents those nitrosylated thiols undergoing irreversible oxidative modification as a consequence of the production of reactive oxygen species (12, 54), an effect that is known to compromise skeletal muscle force production (25). We wish to stress here that, while the 16% improvement in the time to task failure during severe constant work rate exercise is impressive, the magnitude of effect would be expected to be much smaller during time trial exercise tasks in which a given distance is completed in the shortest possible time (34). Nevertheless, it is possible that the effect might still be meaningful in terms of performance enhancement.

*Applications and conclusions.* A short period of dietary  $\text{NO}_3^-$  supplementation through a natural food product resulted in increased plasma [nitrite] and reduced systolic BP in the normotensive young adult men who participated in our study. During exercise at a fixed moderate work rate, increased  $\text{NO}_3^-$  intake resulted in improvements in NIRS-derived indexes of muscle oxygenation and a significant reduction in pulmonary  $\dot{V}_{O_2}$ . It should be stressed that the remarkable reduction in the  $\text{O}_2$  cost of submaximal cycle exercise following dietary supplementation with inorganic nitrate in the form of a natural food product cannot be achieved by any other known means, including long-term endurance exercise training (1, 15, 49, 65). Although not directly tested in the present study, the results suggest that increased dietary  $\text{NO}_3^-$  intake has the potential to enhance exercise tolerance during longer term endurance exercise. Moreover, in certain human populations (including the senescent and those with cardiovascular, respiratory, or metabolic diseases), the activities of daily living are physically difficult because they have an energy requirement that represents a high fraction of the  $\dot{V}_{O_{2\max}}$ . A reduction in the  $\dot{V}_{O_2}$  associated with such activities following dietary nitrate supplementation, therefore, has the potential to improve exercise tolerance and the quality of life in these groups. During exercise at a fixed severe work rate, BR ingestion reduced the amplitude of the  $\dot{V}_{O_2}$  slow component and increased the time to task failure by  $\sim 16\%$ , suggesting that dietary nitrate supplementation might enhance high-intensity exercise performance. Further research is required to investigate the mechanistic bases for the reduced  $\text{O}_2$  cost of submaximal exercise

observed with increased dietary nitrate intake in this study and previously (Ref. 45; i.e., reduced ATP cost of force production and/or increased mitochondrial P/O ratio; i.e., ratio of phosphate radicals esterified to atoms of oxygen consumed). Finally, the possible ergogenicity of dietary nitrate supplementation during different types of exercise in humans is likely to be a fertile area for further research.

## REFERENCES

- Bailey SJ, Wilkerson DP, DiMenna FJ, Jones AM. Influence of repeated sprint training on pulmonary  $O_2$  uptake and muscle deoxygenation kinetics in humans. *J Appl Physiol* 106: 1875–1887, 2009.
- Barstow TJ, Lamarra N, Whipp BJ. Modulation of muscle and pulmonary  $O_2$  uptakes by circulatory dynamics during exercise. *J Appl Physiol* 68: 979–89, 1990.
- Basu S, Azarova NA, Font MD, King SB, Hogg N, Gladwin MT, Shiva S, Kim-Shapiro DB. Nitrite reductase activity of cytochrome c. *J Biol Chem* 283: 32590–32597, 2008.
- Bateman RM, Ellis CG, Freeman DJ. Optimization of nitric oxide chemiluminescence operating conditions for measurement of plasma nitrite and nitrate. *Clin Chem* 48: 570–573, 2002.
- Beaver WL, Wasserman K, Whipp BJ. A new method for detecting the anaerobic threshold by gas exchange. *J Appl Physiol* 60: 2020–2027, 1986.
- Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. *Nature* 368: 502–503, 1994.
- Bergstrom M, Hultman E. Energy cost and fatigue during intermittent electrical stimulation of human skeletal muscle. *J Appl Physiol* 65: 1500–1505, 1988.
- Brand MD. The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans* 33: 897–904, 2005.
- Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351: 714–718, 1991.
- Brown GC, Cooper CE. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* 356: 295–298, 1994.
- Brunori M, Giuffrè A, Sarti P, Stubauer G, Wilson MT. Nitric oxide and cellular respiration. *Cell Mol Life Sci* 56: 549–557, 1999.
- Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 104: 19144–19149, 2007.
- Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol* 1: 290–297, 2005.
- Burnley M, Jones AM. Oxygen uptake kinetics as a determinant of sports performance. *Eur J Sports Sci* 7: 63–79, 2007.
- Burnley M, Jones AM, Carter H, Doust JH. Effects of prior heavy exercise on phase II pulmonary oxygen uptake kinetics during heavy exercise. *J Appl Physiol* 89: 1387–1396, 2000.
- Clementi E, Nisoli E. Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism. *Comp Biochem Physiol A Mol Integr Physiol* 142: 102–110, 2005.
- Clerc P, Rigoulet M, Leverve X, Fontaine E. Nitric oxide increases oxidative phosphorylation efficiency. *J Bioenerg Biomembr* 39: 158–166, 2007.
- Cooper CE, Giulivi C. Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology. *Am J Physiol Cell Physiol* 292: C1993–C2003, 2007.
- Cooper CE, Torres J, Sharpe MA, Wilson MT. Nitric oxide ejects electrons from the binuclear centre of cytochrome c oxidase by reacting with oxidized copper: a general mechanism for the interaction of copper proteins with nitric oxide? *FEBS Lett* 414: 281–284, 1997.
- Coyle EF. Integration of the physiological factors determining endurance performance ability. *Exerc Sport Sci Rev* 23: 25–63, 1995.
- Dejam A, Hunter CJ, Schechter AN, Gladwin MT. Emerging role of nitrite in human biology. *Blood Cells Mol Dis* 32: 423–429, 2004.
- DeLorey DS, Kowalchuk JM, Heenan AP, duManoir GR, Paterson DH. Prior exercise speeds pulmonary  $O_2$  uptake kinetics by increases in both local muscle  $O_2$  availability and  $O_2$  utilization. *J Appl Physiol* 103: 771–778, 2007.
- Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, Benjamin N. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* 1: 546–551, 1995.
- Ferreira LF, Koga S, Barstow TJ. Dynamics of noninvasively estimated microvascular  $O_2$  extraction during ramp exercise. *J Appl Physiol* 103: 1999–2004, 2007.
- Ferreira LF, Reid MB. Muscle-derived ROS and thiol regulation in muscle fatigue. *J Appl Physiol* 104: 853–860, 2008.
- Gladwin MT, Raat NJ, Shiva S, DeZfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol* 291: H2026–H2035, 2006.
- Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR Jr, Feelisch M, Lundberg JO. The emerging biology of the nitrite anion. *Nat Chem Biol* 1: 308–314, 2005.
- Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19: 333–337, 2008.
- Grassi B, Poole DC, Richardson RS, Knight DR, Erickson BK, Wagner PD. Muscle  $O_2$  uptake kinetics in humans: implications for metabolic control. *J Appl Physiol* 80: 988–998, 1996.
- Grassi B, Pogliaghi S, Rampichini S, Quaresima V, Ferrari M, Marconi C, Cerretelli P. Muscle oxygenation and pulmonary gas exchange kinetics during cycle exercise on-transitions in humans. *J Appl Physiol* 95: 149–158, 2003.
- Gruetter CA, Barry BK, McNamara DB, Gruetter DY, Kadowitz PJ, Ignarro L. Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosoamine. *J Cyclic Nucleotide Res* 5: 211–224, 1979.
- Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 alpha. *Science* 302: 1975–1978, 2003.
- Higuchi K, Motomizu S. Flow-injection spectrophotometric determination of nitrite and nitrate in biological samples. *Anal Sci* 15: 129–134, 1999.
- Hopkins WG, Hawley JA, Burke LM. Design and analysis of research on sport performance enhancement. *Med Sci Sports Exerc* 31: 472–485, 1999.
- Jones AM, Koppo K, Burnley M. Effects of prior exercise on metabolic and gas exchange responses to exercise. *Sports Med* 33: 949–71, 2003.
- Jones AM, Poole DC. Oxygen uptake dynamics: from muscle to mouth—an introduction to the symposium. *Med Sci Sports Exerc* 37: 1542–1550, 2005.
- Jones AM, Wilkerson DP, Campbell IT. Nitric oxide synthase inhibition with L-NAME reduces maximal oxygen uptake but not gas exchange threshold during incremental cycle exercise in man. *J Physiol* 560: 329–338, 2004.
- Jones AM, Wilkerson DP, Koppo K, Wilmschurst S, Campbell IT. Inhibition of nitric oxide synthase by L-NAME speeds phase II pulmonary  $\dot{V}O_2$  kinetics in the transition to moderate-intensity exercise in man. *J Physiol* 552: 265–272, 2003.
- Jones AM, Wilkerson DP, Wilmschurst S, Campbell IT. Influence of L-NAME on pulmonary  $O_2$  uptake kinetics during heavy-intensity cycle exercise. *J Appl Physiol* 96: 1033–1038, 2004.
- Kindig CA, Gallatin LL, Erickson HH, Fedde MR, Poole DC. Cardio-respiratory impact of the nitric oxide synthase inhibitor L-NAME in the exercising horse. *Respir Physiol* 120: 151–166, 2000.
- Kindig CA, McDonough P, Erickson HH, Poole DC. Nitric oxide synthase inhibition speeds oxygen uptake kinetics in horses during moderate domain running. *Respir Physiol Neurobiol* 132: 169–178, 2002.
- Krustrup P, Hellsten YH, Bangsbo J. Intense interval training enhances human skeletal muscle oxygen uptake in the initial phases of dynamic exercise at high but not at low intensities. *J Physiol* 559: 335–345, 2004.
- Krustrup P, Jones AM, Wilkerson DP, Calbet JA, Bangsbo J. Muscular and pulmonary  $O_2$  uptake kinetics during moderate- and heavy-intensity sub-maximal knee-extensor exercise in humans. *J Physiol* 587: 1843–1856, 2009.
- Lamarra N, Whipp BJ, Ward SA, Wasserman K. Effect of interbreath fluctuations on characterising exercise gas exchange kinetics. *J Appl Physiol* 62: 2003–2012, 1987.

45. **Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E.** Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf)* 191: 59–66, 2007.
46. **Lundberg JO, Govoni M.** Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37: 395–400, 2004.
47. **Lundberg JO, Weitzberg E, Cole JA, Benjamin N.** Nitrate, bacteria and human health. *Nat Rev Microbiol* 2: 593–602, 2004.
48. **Morris TE, Sulakhe PV.** Sarcoplasmic reticulum  $Ca^{2+}$ -pump dysfunction in rat cardiomyocytes briefly exposed hydroxyl radical. *Free Radic Biol Med* 22: 37–47, 1997.
49. **Moseley L, Achten J, Martin JC, Jeukendrup AE.** No differences in cycling efficiency between world-class and recreational cyclists. *Int J Sports Med* 25: 374–379, 2004.
50. **Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E.** Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proc Natl Acad Sci USA* 101: 16507–16512, 2004.
51. **Poole DC, Barstow TJ, McDonough P, Jones AM.** Control of oxygen uptake during exercise. *Med Sci Sports Exerc* 40: 462–474, 2008.
52. **Poole DC, Ward SA, Gardner GW, Whipp BJ.** Metabolic and respiratory profile of the upper limit for prolonged exercise in man. *Ergonomics* 31: 1265–1279, 1988.
53. **Reid MB.** Nitric oxide, reactive oxygen species, and skeletal muscle contraction. *Med Sci Sports Exerc* 33: 371–376, 2001.
54. **Reid MB.** Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand* 162: 401–409, 1998.
55. **Rossiter HB, Ward SA, Howe FA, Kowalchuk JM, Griffiths JR, Whipp BJ.** Dynamics of intramuscular  $^{31}P$ -MRS  $P_i$  peak splitting and the slow components of PCr and  $O_2$  uptake during exercise. *J Appl Physiol* 93: 2059–2069, 2002.
56. **Shen W, Xu X, Ochoa M, Zhao G, Wolin MS, Hintze TH.** Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ Res* 75: 1086–1095, 1994.
57. **Smith MA, Reid MB.** Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respir Physiol Neurobiol* 151: 229–241, 2006.
58. **Stamler JS, Meissner G.** Physiology of nitric oxide in skeletal muscle. *Physiol Rev* 81: 209–237, 2001.
59. **Thomas DD, Liu X, Kantrow SP, Lancaster JR Jr.** The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and  $O_2$ . *Proc Natl Acad Sci USA* 98: 355–360, 2001.
60. **Torres J, Sharpe MA, Rosquist A, Cooper CE, Wilson MT.** Cytochrome c oxidase rapidly metabolizes nitric oxide to nitrite. *FEBS Lett* 475: 263–266, 2000.
61. **van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J, Gladwin MT.** Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev* 29: 683–741, 2009.
62. **Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A.** Acute blood pressure lowering, vasoprotective, and anti-platelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 51: 784–790, 2008.
63. **Whipp BJ, Davis JA, Torres F, Wasserman K.** A test to determine parameters of aerobic function during exercise. *J Appl Physiol* 50: 217–221, 1981.
64. **Whipp BJ, Wasserman K.** Oxygen uptake kinetics for various intensities of constant-load work. *J Appl Physiol* 33: 351–356, 1972.
65. **Wilkerson DP, Berger NJ, Jones AM.** Influence of hyperoxia on pulmonary  $O_2$  uptake kinetics following the onset of exercise in humans. *Respir Physiol Neurobiol* 153: 92–106, 2006.
66. **Wilkerson DP, Campbell IT, Jones AM.** Influence of nitric oxide synthase inhibition on pulmonary  $O_2$  uptake kinetics during supra-maximal exercise in humans. *J Physiol* 561: 623–635, 2004.
67. **Ysart G, Miller P, Barrett G, Farrington D, Lawrance P, Harrison N.** Dietary exposures to nitrate in the UK. *Food Addit Contam* 16: 521–532, 1999.

