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Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans

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Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N, Jones AM. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* 109: 135–148, 2010. First published May 13, 2010; doi:10.1152/japplphysiol.00046.2010.—The purpose of this study was to elucidate the mechanistic bases for the reported reduction in the O_2 cost of exercise following short-term dietary nitrate (NO_3^-) supplementation. In a randomized, double-blind, crossover study, seven men (aged 19–38 yr) consumed 500 ml/day of either nitrate-rich beetroot juice (BR, 5.1 mmol of NO_3^-/day) or placebo (PL, with negligible nitrate content) for 6 consecutive days, and completed a series of low-intensity and high-intensity "step" exercise tests on the last 3 days for the determination of the muscle metabolic (using ^{31}P-MRS) and pulmonary oxygen uptake ($\dot{V}O_2$) responses to exercise. On *days 4–6*, BR resulted in a significant increase in plasma [nitrite] (mean ± SE, PL 231 ± 76 vs. BR 547 ± 55 nM; $P < 0.05$). During low-intensity exercise, BR attenuated the reduction in muscle phosphocreatine concentration ([PCr]; PL 8.1 ± 1.2 vs. BR 5.2 ± 0.8 mM; $P < 0.05$) and the increase in $\dot{V}O_2$ (PL 484 ± 41 vs. BR 362 ± 30 ml/min; $P < 0.05$). During high-intensity exercise, BR reduced the amplitudes of the [PCr] (PL 3.9 ± 1.1 vs. BR 1.6 ± 0.7 mM; $P < 0.05$) and $\dot{V}O_2$ (PL 209 ± 30 vs. BR 100 ± 26 ml/min; $P < 0.05$) slow components and improved time to exhaustion (PL 586 ± 80 vs. BR 734 ± 109 s; $P < 0.01$). The total ATP turnover rate was estimated to be less for both low-intensity (PL 296 ± 58 vs. BR 192 ± 38 μM/s; $P < 0.05$) and high-intensity (PL 607 ± 65 vs. BR 436 ± 43 μM/s; $P < 0.05$) exercise. Thus the reduced O_2 cost of exercise following dietary NO_3^- supplementation appears to be due to a reduced ATP cost of muscle force production. The reduced muscle metabolic perturbation with NO_3^- supplementation allowed high-intensity exercise to be tolerated for a greater period of time.

bioenergetics; muscle metabolism; ^{31}P-MRS; fatigue; efficiency; respiratory control

AT THE ONSET of moderate-intensity exercise (that is, exercise performed at work rates below the gas exchange threshold, GET), pulmonary O_2 uptake ($\dot{V}O_2$) rises in an exponential fashion to attain a steady state within approximately 2–3 min in healthy humans (76, 77). In the steady state, the rate of ATP catabolism is in equilibrium with the rate of ATP resynthesis through oxidative phosphorylation. For moderate-intensity cycle exercise, the steady-state $\dot{V}O_2$ is linearly related to the external work rate with the functional "gain" (increase in $\dot{V}O_2$ per unit increment in external work rate) approximating 10 $ml \cdot min^{-1} \cdot W^{-1}$ (38, 76). During supra-GET exercise, however, $\dot{V}O_2$ dynamics become more complex due to the development of a delayed-onset $\dot{V}O_2$ "slow component" which elevates the O_2 cost of exercise above that predicted from linear extrapolation of the moderate-intensity $\dot{V}O_2$-work rate relationship (38, 76, 77).

The steady-state $\dot{V}O_2$ for a given moderate-intensity work rate during cycle ergometry has been considered to be intransigent to a variety of acute exercise and pharmacological interventions and to be essentially unaltered with age and training (2, 10, 16, 40, 78). However, recent research has shown that 3–6 days of either pharmacological (sodium nitrate; 52) or dietary (beetroot juice; 3) nitrate (NO_3^-) administration can reduce the steady-state $\dot{V}O_2$ during submaximal cycle exercise in young healthy participants. During severe-intensity exercise, dietary NO_3^- supplementation reduced the $\dot{V}O_2$ slow component, delaying the attainment of the maximal $\dot{V}O_2$ ($\dot{V}O_{2\,max}$) and increasing the tolerable duration of exercise (3).

The mechanism(s) by which NO_3^- administration reduces the O_2 cost of submaximal exercise and enhances exercise tolerance is/are presently unclear. Given that NO_3^- is relatively inert, the effect is unlikely to be mediated by an elevated [NO_3^-], per se, but rather, through the action of its bioactive nitrogen derivative, nitrite (NO_2^-), or the subsequent bioconversion of NO_2^- to nitric oxide (NO). Ingested inorganic NO_3^- is rapidly absorbed from the gut and concentrated in the saliva; facultative anaerobic bacteria on the surface of the tongue then reduce NO_3^- to NO_2^- (21). Swallowed NO_2^- can be converted to NO in the stomach (9, 55) but some is absorbed to increase the circulating plasma [NO_2^-] (19, 54). We (9) and others (56) have shown that NO_2^- can be converted to NO, with the requisite one-electron reduction being catalyzed via xanthine oxidoreductase, hemoglobin, myoglobin, endothelial nitric oxide synthase, and the mitochondrial electron transfer complexes (see 15 for review).

To reduce the O_2 cost of moderate-intensity exercise, NO_2^-/NO would be required to *1*) increase the mitochondrial P/O ratio (i.e., reduce the O_2 cost of mitochondrial ATP resynthesis); *2*) improve the coupling between ATP hydrolysis and muscle force production (i.e., reduce the ATP cost of force production); or *3*) inhibit mitochondrial ATP production, in which case a compensatory increase in energy provision through substrate-level phosphorylation would be required. An elevation in NO_2^-/NO may increase the P/O ratio through a reduction in "slippage" at the mitochondrial proton pumps (18) or through the potential role for NO_2^- as an alternative electron acceptor (7). The ATP cost of contraction in skeletal myocytes is essentially the sum of ATP consumption via the interaction between actin and myosin (actomyosin-ATPase) and calcium (Ca^{2+}) pumping in the sarcoplasmic reticulum (Ca^{2+}-ATPase), with membrane depolarization (Na^+-K^+-ATPase) making a further small contribution to the total ATP turn-

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over (6). NO has been demonstrated to slow cross-bridge cycling kinetics (25, 34), reduce ryanodine activity, and therefore Ca^{2+} release (33) and inhibit Ca^{2+}-ATPase activity (71), and NO may therefore have a regulatory influence on the ATP cost of force production. Previous studies have suggested that the reduced O_2 cost of exercise with dietary NO_3^- supplementation was not compensated by an elevation in anaerobic metabolism, as inferred from an unchanged blood [lactate] (3, 52). However, it is acknowledged that blood [lactate] represents a very indirect and incomplete assessment of muscle anaerobic energy turnover. Therefore, it is presently unclear which of these potential mechanisms underpins the reduced O_2 cost of exercise and improved exercise tolerance following dietary NO_3^- supplementation.

Phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) facilitates the assessment of human muscle metabolism noninvasively, in vivo, and with a high temporal and spatial resolution (39, 44, 61, 79). Utilization of ^{31}P-MRS, to provide information on tissue changes in [phosphocreatine] ([PCr]), [ADP], and pH, in conjunction with pulmonary $\dot{V}O_2$ dynamics, which closely reflects skeletal muscle O_2 consumption (31, 47), enables estimation of changes in total ATP turnover rate and the proportional contribution of PCr hydrolysis, glycolysis, and oxidative phosphorylation during exercise following dietary NO_3^- supplementation (42, 43, 51, 53). A reduction in the steady-state $\dot{V}O_2$ amplitude following NO_3^- administration, as observed previously (3, 52), with no change in energy derived through the PCr and glycolytic pathways, would be indicative of an increased mitochondrial P/O ratio. Alternatively, a reduction in the steady-state $\dot{V}O_2$ amplitude with accompanying reductions in energy derived through one or both of the PCr or glycolytic pathways might suggest a reduced ATP cost of force production following NO_3^- administration, i.e., a reduction in ATP turnover for the same external work rate rather than an improvement in the efficiency of mitochondrial oxidative ATP synthesis, per se. However, a greater fall in pH or greater [PCr] degradation following NO_3^- administration would indicate that the reduction in aerobic ATP yield was compensated through an increased anaerobic ATP yield.

The purpose of the present study was to elucidate the mechanism by which NO_3^- administration resulted in a reduced O_2 cost of submaximal exercise and an improved tolerance of high-intensity exercise (3). We hypothesized that NO_3^-, administered as nitrate-rich beetroot juice (BR), would reduce the O_2 cost of both low-intensity and high-intensity exercise and that this would be accompanied by reductions in the extent of intramuscular PCr degradation of a similar magnitude (reflecting a reduced high-energy phosphate cost of force production), with no change in pH. We used quantitative ^{31}P-MRS (42) to investigate changes in the rates of ATP resynthesis deriving from PCr hydrolysis, glycolysis, and oxidative phosphorylation, and the total muscle ATP turnover rate (51, 53), with and without dietary NO_3^- supplementation. Given that the termination of high-intensity, constant-work-rate exercise is associated with the attainment of a consistent muscle metabolic milieu (i.e., low [PCr] and pH and high P_i concentration, [P_i]; 70), we also hypothesized that exercise tolerance would be enhanced following dietary NO_3^- supplementation due to a sparing of the finite [PCr] reserves and reduced muscle metabolic perturbation.

METHODS

Subjects

Seven healthy, recreationally active males (mean ± SD, age 28 ± 7 yr, height 1.80 ± 0.02 m, body mass 81 ± 7 kg) volunteered to participate in this study. None of the subjects were tobacco smokers or users of dietary supplements, and all were familiar with the experimental procedures used in this study. 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 6 and 24 h before each test, respectively, and to abstain from the consumption of foods rich in nitrates for the duration of the study. All tests were performed at the same time of day (±2 h).

Procedures

The subjects were required to report to the laboratory on seven occasions over a 4-wk period. During the first visit to the laboratory, subjects provided a venous blood sample for determination of plasma [nitrite], and resting blood pressure (BP) was measured. The subjects then completed three maximum voluntary isometric contractions (MVC) of the quadriceps; each MVC was 3 s in duration and was separated by 1 min of passive recovery. Five minutes after completion of the final MVC, subjects commenced an incremental test using a two-legged knee-extension ergometer to establish the peak work rate (WR_{peak}). These and subsequent exercise tests were conducted in the prone position, with subjects secured to the ergometer bed via Velcro straps at the thigh, buttocks, lower back, and middle back to minimize extraneous movement. The custom-designed ergometer consisted of a nylon frame that fitted onto the bed in alignment with the subject's feet and a base unit that was positioned behind the bed. Cuffs with Velcro straps were secured to the subject's feet, and ropes were attached to the cuffs. These ropes passed around pulleys housed within the frame to points of attachment on chains that meshed with a cassette of sprockets on the base unit. The sprocket arrangement was such that when a bucket containing nonmagnetic weights was attached to each chain it provided a concentric-only resistive load for each leg. This allowed for the performance of rhythmic two-legged knee-extension exercise in a contralateral alternating manner over a distance of ~0.22 m. Subjects lifted the weight in accordance with a visual cue at a frequency of 40/min. The load for the first step increment was 4 kg per leg and the load was increased by 1 kg for each subsequent increment until the limit of tolerance.

Throughout the MVCs and incremental test, muscle activity (integrated electromyography, iEMG) of the right vastus lateralis muscle was recorded. The leg was initially shaved and cleaned with alcohol around the belly of the muscle, and graphite snap electrodes (Unilect 40713, Unomedical, Stonehouse, UK) were then adhered to the prepared area in a bipolar arrangement (interelectrode distance: 40 mm). A ground electrode was positioned on the rectus femoris muscle equidistant from the active electrodes. To secure electrodes and wires in place and to minimize movement, an elastic bandage was wrapped around the subject's leg. Indelible pen marks were made around the electrodes to enable reproduction of the placement in subsequent tests. The EMG signal was recorded using a ME3000PB Muscle Tester (Mega Electronics). EMG measurements at a sampling frequency of 1,000 Hz were recorded throughout all exercise tests. The bipolar signal was amplified (amplifier input impedance > 1 MΩ), and data were collected in raw form and stored on a personal computer using MegaWin software (Mega Electronics). The work rates that would require 15% and 30% of the MVC iEMG signal were calculated and

applied as the low- and high-intensity work rates, respectively, in subsequent tests.

Following completion of the step incremental test, subjects were randomly assigned, in a double-blind, crossover fashion, to receive 6 days of dietary supplementation with nitrate [NO_3^-; 5.1 mmol/day; administered as 0.5 liter of organic beetroot juice (BR) per day; Beet It, James White Drinks, Ipswich, UK] and placebo (PL; low-calorie black-currant juice cordial with negligible NO_3^- content) with a 10-day washout separating the supplementation periods. The concentration of NO_3^- in the beetroot juice was determined by its reduction to NO in the presence of VCl_3 at 90°C using chemiluminescence (8). 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. This study was completed before the publication of our initial study (3) such that the subjects were not aware that beetroot juice might be ergogenic.

On *days 4* and *5* of the supplementation periods, the subjects completed "step" exercise tests from a resting baseline to low-intensity and high-intensity work rates for the determination of breath-by-breath pulmonary $\dot{V}O_2$ dynamics and muscle activity. Pulmonary gas exchange and ventilation ($\dot{V}E$) were measured breath by breath with subjects wearing a nose clip and breathing through a low-dead-space, low-resistance mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O_2) and infrared (CO_2) analyzers (Jaeger Oxycon Pro, Hoechberg, Germany) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated with a 3-liter syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in the capillary gas transit and the analyzer rise time relative to the volume signal. Pulmonary gas exchange and $\dot{V}E$ were calculated and displayed breath by breath. Heart rate (HR) was measured during all tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). Subjects completed two 4-min bouts of low-intensity exercise and one bout of high-intensity exercise with 6 min passive recovery separating each exercise bout. On *day 4*, the high-intensity work rate was continued until task failure as a measure of exercise tolerance whereas on *day 5*, the high-intensity work rate was discontinued after 6 min. The time to task failure was recorded when the subjects were unable to sustain the required contraction frequency. The coefficient of variation for time to task failure in this mode of exercise in our laboratory is 5–7%. 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 (50). EMG data were also recorded during testing on *days 4* and *5* using the setup as previously described.

On *day 6* of the nitrate and placebo supplementation periods, subjects repeated the testing protocol completed on *day 4*, but on this occasion, the step exercise tests were performed with the ergometer placed in the bore of a 1.5-T superconducting magnet (Intera, Philips, The Netherlands) at the Peninsula Magnetic Resonance Research Centre (Exeter, UK) while simultaneously undertaking ^{31}P-MRS for the determination of in vivo skeletal muscle energetics. The time to task failure was therefore calculated as the mean of the times recorded on *days 4* and *6*, for both experimental conditions. Before the exercise protocol commenced, absolute baseline concentrations of metabolites were established via a technique similar to that described by Kemp et al. (42) using a 6-cm ^{31}P transmit/receive surface coil. First, spatially localized spectroscopy was undertaken to determine the relative signal intensities obtained from a phosphoric acid source within the scanner bed and P_i from the subject's right quadriceps muscle, which was centered over the coil. A subsequent scan was obtained comparing the signals obtained from the phosphoric acid standard and an external P_i solution, where the localized voxel sampled within the external solution was of the same dimensions and distance from the coil as the muscle, allowing the calculation of muscle P_i concentration following corrections for relative coil loading. Absolute values of PCr and ATP concentrations were subsequently calculated via the ratio of P_i:PCr and P_i:ATP.

For the exercise protocol, the knee-extension rate was set in unison with the magnetic pulse sequence to ensure the quadriceps muscles were positioned in the same phase of contraction during each MR pulse acquisition. The subjects were visually cued via a display consisting of two vertical bars, one that moved at a constant frequency of 0.67 Hz and one that monitored foot movement via a sensor in the ergometer pulley system. Initially, fast-field echo images were acquired to determine whether the muscle was positioned correctly relative to the coil. This was aided by placing cod liver oil capsules, which yield high-intensity signal points within the image, adjacent to the coil, allowing its orientation relative to the muscle volume under examination to be assessed. A number of preacquisition steps were carried out to optimize the signal from the muscle under investigation. Matching and tuning of the coil was performed and an automatic shimming protocol was then undertaken within a volume that defined the quadriceps muscle. Before and during exercise, data were acquired every 1.5 s, with a spectral width of 1,500 Hz and 1 K data points. Phase cycling with four phase cycles was employed, leading to a spectrum being acquired every 6 s. The subsequent spectra were quantified via peak fitting, assuming prior knowledge, using the jMRUI (version 3) software package employing the AMARES fitting algorithm (69). Spectra were fitted assuming the presence of the following peaks: P_i, phosphodiester, PCr, α-ATP (2 peaks, amplitude ratio 1:1), γ-ATP (2 peaks, amplitude ratio 1:1), and β-ATP (3 peaks, amplitude ratio 1:2:1). In all cases, relative amplitudes recorded during exercise were corrected for partial saturation by obtaining a baseline spectrum before exercise with long repetition time (TR = 20 s) in which the relative unsaturated peak amplitudes could be determined. Intracellular pH was calculated using the chemical shift of the P_i spectral peak relative to the PCr peak (67). Resting and end-exercise values of [PCr], [P_i], and pH were calculated over the last 30 s of the rest or exercise period. ADP was calculated via knowledge of P_i, PCr, and pH values, as described by Kemp et al. (43) taking into account the dependency of rate constants on pH.

The total ATP turnover rate (ATP_{total}) was calculated as the sum of the total ATP turnover deriving from PCr hydrolysis, glycolysis, and oxidative phosphorylation (ATP_{PCr}, ATP_{Gly}, and ATP_{Ox}, respectively) using the methods of Lanza et al. (51) and Layec et al. (53). ATP_{PCr} was obtained from the rate of change in [PCr] from the modeling of [PCr] values acquired at each time point during the exercise protocol. ATP_{Gly} was determined by proton flux, assuming that the production of 1 mol of H^+ yields 1.5 mol of ATP. Proton flux calculations were based on determining the protons consumed by the creatine kinase (CK) reaction and buffering capacity and those produced via oxidative phosphorylation and cellular efflux. ATP_{Ox} was determined based on the hyperbolic relationship between ATP production rate and free cytosolic ADP concentration, requiring the calculation of the first-order PCr recovery rate constant determined from fitting PCr to a single-exponential function.

Before exercise on *days 4, 5,* and *6*, resting BP was measured and a venous blood sample was collected for subsequent determination of plasma [nitrite]. BP of the brachial artery was measured with subjects in a rested, seated position before each exercise bout using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, FL). Following 10 min of rest, four measurements were taken with the mean of the 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°C, for later analysis of [nitrite] (NO_2^-) via chemiluminescence (8) as described previously (3).

Data Analysis Procedures

Oxygen uptake. The breath-by-breath $\dot{V}O_2$ data from each test on *days 4* and *5* were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than four 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 first 20 s of data after the onset of exercise (i.e., the phase I response) was 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:

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

$$\dot{V}O_2(t) = \dot{V}O_{2\,\text{baseline}} + A_p(1 - e^{-(t-TD_p/\tau_p)}) + A_s(1 - e^{-(t-TD_s/\tau_s)}) \quad \text{(severe)} \quad (2)$$

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

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. $\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the final 90 s of the resting baseline period. The 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 actually 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) for both supplementation periods. To determine the overall kinetics of the $\dot{V}O_2$ response to both moderate- and severe-intensity exercise, the data were also fit with a monoexponential model from 0 s to end exercise without time delay.

Energy expenditure (EE) at rest and in the "steady state" was calculated from $\dot{V}O_2$ taking into account the energetic value of oxygen based on the respiratory exchange ratio (RER). Work efficiency (WE) was subsequently calculated by dividing the exercise work rate by the difference between the exercise EE and the baseline EE.

Phosphorous metabolites. To enhance the signal-to-noise properties and therefore the underlying features of the [PCr], [P_i], [ADP], and pH response profiles before kinetic parameter estimation, each subject's low-intensity exercise transitions were time-aligned to the onset of exercise ($t = 0$ s), averaged, and interpolated yielding a single second-by-second response. The [PCr] responses were subsequently modeled using nonlinear least-squares regression techniques. We used a procedure similar to that of Rossiter et al. (60) as described in the following equation:

$$\Delta PCr(t) = [PCr]ss(1 - e^{-t/\tau}) \quad (3)$$

where (t) represents the absolute metabolite concentration at a given time, ss is the projected asymptotic value, and τ is the time constant of the response. The low-intensity data manifested a monoexponential time course and were thus fit from $t = 0$ through the entire 240 s of the response. Data analysis became more complex during the high-intensity exercise bouts owing to the existence of a delayed-onset, secondary component response. Therefore, the fitting window was constrained to an initial start point of 60 s and increased iteratively thereafter until there was a clear departure of the measured data from the model fit, as judged from visual inspection of a plot of the residuals. In this way, the best-fit exponential for the fundamental component of the response was established. The magnitude of the [PCr] slow component was then calculated as the difference between the asymptotic amplitude of the fundamental response and the mean value measured over the last 30 s of exercise for that condition. Metabolite concentrations (for PCr, ADP, P_i, and also pH) at end exercise (low intensity) and task failure (high intensity) were taken as the mean values measured over the final 30 s of exercise.

EMG. Raw EMG data were exported as an ASCII file and digitally filtered using a custom-designed filter developed through Labview 8.2 (National Instruments, Newbury, UK). Initially, the signals were filtered with a 20-Hz high-pass, second-order Butterworth filter to remove contamination from movement artifacts. The signal was then rectified and low-pass filtered at a frequency of 500 Hz to produce a linear envelope. The average iEMG was calculated at 1-s intervals during the MVCs while the average iEMG was calculated at 15-s intervals throughout the low- and high-intensity exercise bouts, with these values normalized to the highest 1-s average value attained in the three MVCs. Therefore, all iEMG data are presented as a percentage of the MVC iEMG response attained before supplementation. Data from repeat trials were averaged.

Statistics

Differences in BP and plasma [NO_2^-] were assessed using one-way repeated-measures ANOVA. Significant effects were further explored using simple contrasts with the α-level adjusted via a Fisher's LSD. Differences in the cardiorespiratory, muscle activity, and muscle metabolic responses between conditions were analyzed with two-tailed, paired-samples t-tests. Correlations were assessed via Pearson's product-moment correlation coefficient. Data are presented as means ± SE. Statistical significance was accepted when $P < 0.05$, while a tendency was noted when $P < 0.10$.

RESULTS

The NO_3^- supplementation regimen employed in this study was well tolerated with no deleterious side effects. Subjects did, however, report beeturia (red urine) and red stools, consistent with previous studies (3, 74).

Plasma [NO_2^-] and BP

The group mean plasma [NO_2^-] values obtained before exercise in control (CON) and on each of *days 4*, *5*, and *6* of the NO_3^- and PL supplementation periods are shown in Table 1. Plasma [NO_2^-] was elevated during the NO_3^- supplementation compared with CON and to PL at all sample points (Table 1). The mean plasma [NO_2^-] obtained over the three samples was 137% greater following NO_3^- compared with PL supplementation (Table 1). However, the elevations in plasma [NO_2^-] with NO_3^- supplementation were not different across *days 4–6*.

The mean blood pressure parameters measured at CON and at the three NO_3^- and PL sample points are shown in Table 1. NO_3^- supplementation significantly reduced the systolic BP on *day 6* and the mean arterial pressure on *day 4* relative to PL (Table 1). Over the three sample points, NO_3^- supplementation resulted in significant reductions in systolic BP (−5 mmHg), diastolic BP (−2 mmHg), and mean arterial pressure (−2 mmHg) relative to PL, with systolic BP and mean arterial pressure also being reduced below CON values (Table 1). Similar to plasma [NO_2^-], the reductions in systolic and dia-

Table 1. *Blood pressure and plasma [nitrite] before exercise in the control condition and following dietary supplementation with nitrate (beetroot juice) or placebo*

		Placebo				Nitrate			
	Control	*Day 4*	*Day 5*	*Day 6*	Mean	*Day 4*	*Day 5*	*Day 6*	Mean
Plasma nitrite, nM	219 ± 65	257 ± 76	231 ± 80	206 ± 93	231 ± 76	591 ± 41*†	406 ± 57*†	643 ± 110*†	547 ± 55*†
Systolic blood pressure, mmHg	125 ± 2	123 ± 2	124 ± 2	124 ± 2	124 ± 2	119 ± 2	120 ± 2	118 ± 2*†	119 ± 2*†
Diastolic blood pressure, mmHg	73 ± 4	70 ± 3	68 ± 2	67 ± 1	68 ± 2	66 ± 2	66 ± 2	66 ± 2	66 ± 2†
Mean arterial pressure, mmHg	91 ± 2	90 ± 2	89 ± 1	89 ± 1	89 ± 1	87 ± 1†	87 ± 1	87 ± 1	87 ± 1*†

Values are means ± SE. *Significantly different from control ($P < 0.05$). †Significantly different from placebo ($P < 0.05$).

stolic BP with NO_3^- supplementation were not significantly different across *days 4–6*.

Pulmonary $\dot{V}O_2$ Dynamics

Low-intensity exercise. There was no significant difference between the NO_3^- and PL conditions for muscle activity as assessed by iEMG (PL 17 ± 3 vs. BR 16 ± 2% MVC). End-exercise HR was not significantly different between conditions (PL 85 ± 4 vs. BR 86 ± 4 beats/min).

The group mean pulmonary $\dot{V}O_2$ responses during low-intensity exercise following both NO_3^- and PL supplementation are shown in Fig. 1*A*, and the response parameters are reported in Table 2. The increase in pulmonary $\dot{V}O_2$ from rest to low-intensity exercise was reduced by 25% following dietary NO_3^- supplementation (PL 484 ± 41 vs. BR 362 ± 30 ml/min; $P < 0.05$) and the end-exercise $\dot{V}O_2$ was also reduced (PL 870 ± 42 vs. BR 778 ± 38 ml/min; $P < 0.05$). However, neither the resting baseline $\dot{V}O_2$ nor the τ, which characterizes

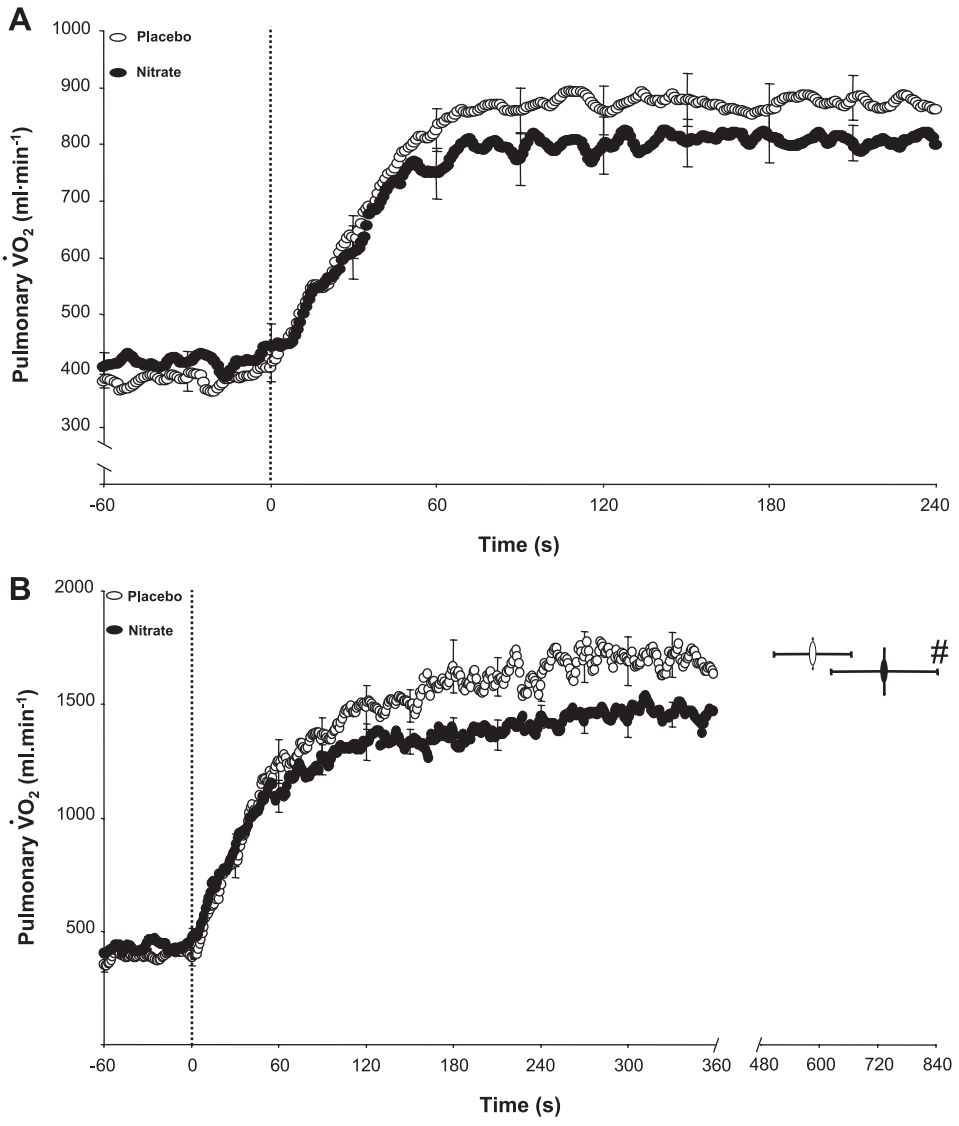

Fig. 1. Group mean ± SE pulmonary oxygen uptake ($\dot{V}O_2$) response during low-intensity (*A*) and high-intensity (*B*) exercise following dietary nitrate and placebo supplementation. The dashed vertical line represents the abrupt imposition of the work rate from a resting baseline. Note that the oxygen cost of both low-intensity and high-intensity exercise was substantially spared following nitrate supplementation (see Table 2) and that the time to task failure was extended in the latter. #Time to task failure significantly different from placebo ($P < 0.01$).

Table 2. *Pulmonary $\dot{V}O_2$ dynamics during low-intensity and high-intensity exercise following dietary supplementation with nitrate (beetroot juice) or placebo*

	Placebo	Nitrate
Low-intensity exercise		
$\dot{V}O_2$		
Baseline, ml/min	389 ± 16	429 ± 16
Primary amplitude, ml/min	484 ± 41	362 ± 30*
End exercise, ml/min	870 ± 42	778 ± 38*
Phase II time constant, s	22 ± 2	21 ± 5
Mean response time, s	33 ± 3	32 ± 2
High-intensity exercise		
$\dot{V}O_2$		
Baseline, ml/min	391 ± 8	426 ± 15
Primary amplitude, ml/min	1,116 ± 83	947 ± 53
At 360 s of exercise, ml/min	1,692 ± 70	1,460 ± 54*
At task failure, ml/min	1,726 ± 65	1,647 ± 100
Phase II time constant, s	37 ± 6	40 ± 8
Slow component amplitude, ml/min	209 ± 30	100 ± 26*
Mean response time, s	69 ± 8	58 ± 13

Values are means ± SE; $n = 6$ for values at task failure. $\dot{V}O_2$, oxygen uptake. *Significantly different from placebo ($P < 0.05$).

the rate of $\dot{V}O_2$ adjustment toward the required steady state, was significantly altered by dietary NO_3^- supplementation (Table 2). The end-exercise values of CO_2 production ($\dot{V}CO_2$) (PL 726 ± 21 vs. BR 682 ± 25 ml/min), $\dot{V}E$ (PL 23 ± 2 vs. BR 22 ± 2 l/min), and RER (PL 0.84 ± 0.03 vs. BR 0.87 ± 0.02) were not significantly different between the conditions. WE was significantly greater following NO_3^- supplementation (PL 10.1 ± 0.6 vs. BR 13.0 ± 1.0%; $P < 0.05$).

High-intensity exercise. There was no significant difference between the NO_3^- and PL conditions for muscle activity as assessed by iEMG (PL 36 ± 3 vs. BR 33 ± 3% MVC). End-exercise HR was not significantly different between conditions (PL 117 ± 3 vs. BR 113 ± 3 beats/min). One subject exercised for 21–22 minutes during high-intensity exercise following NO_3^- supplementation, exceeding the 20 min available for data storage during this bout in the magnet. Therefore, the "end-exercise" data for this subject were excluded from analysis.

The group mean pulmonary $\dot{V}O_2$ responses during high-intensity exercise following both NO_3^- and PL supplementation are shown in Fig. 1*B*, and the parameters derived from the biexponential model fits are presented in Table 2. The primary component $\dot{V}O_2$ amplitude tended to be lower following dietary NO_3^- supplementation (PL 1,116 ± 83 vs. BR 947 ± 53 ml/min; $P = 0.08$; Fig. 1). The baseline $\dot{V}O_2$ and $\dot{V}O_2$ τ were not significantly different between the conditions (Table 2). The amplitude of the $\dot{V}O_2$ slow component was significantly reduced (by 50%) following NO_3^- supplementation (PL 209 ± 30 vs. BR 100 ± 26 ml/min; $P < 0.05$; Fig. 1), resulting in a significantly reduced $\dot{V}O_2$ at 360 s (PL 1,692 ± 70 vs. BR: 1,460 ± 54 ml/min; $P < 0.05$; Fig. 1), but there was no significant difference in $\dot{V}O_2$ at the limit of tolerance (Table 2). The end-exercise values of $\dot{V}CO_2$ (PL 1,529 ± 28 vs. BR 1,452 ± 51 ml/min) and $\dot{V}E$ (PL 48 ± 3. vs. BR 44 ± 3 l/min) were not significantly different between the conditions. WE was significantly greater following NO_3^- supplementation (PL 6.3 ± 0.3 vs. BR 7.7 ± 0.3%; $P < 0.05$).

Muscle Metabolic Measurements In Vivo

Low-intensity exercise. The muscle metabolic effects of dietary NO_3^- supplementation during low-intensity exercise are reported in Table 3 and illustrated in Fig. 2. NO_3^- supplementation resulted in a 36% reduction in the amplitude of PCr degradation during low-intensity exercise (PL 8.1 ± 1.2 vs. BR 5.2 ± 0.8 mM; $P < 0.05$). This effect was similar in magnitude to the reduction in the $\dot{V}O_2$ amplitude with NO_3^- supplementation (Fig. 3). Consistent with the low-intensity $\dot{V}O_2$ response, the baseline [PCr] and [PCr] τ were not different following NO_3^- and PL supplementation (Table 3). A 21% reduction in the accumulation of [P_i] was observed with NO_3^- supplementation (PL 4.4 ± 0.8 vs. BR 3.5 ± 0.8 mM; $P < 0.05$; Fig. 2). The estimated [ADP] amplitude was significantly reduced following NO_3^- supplementation (PL 17.0 ± 2.8 vs. BR 10.3 ± 1.7 μM; $P < 0.05$; Fig. 2). Muscle pH at baseline and throughout exercise was not different between NO_3^- and PL supplementation (Table 3).

The estimated mean ATP_{total}, ATP_{Ox}, ATP_{PCr} and ATP_{Gly} for low-intensity exercise are shown in Fig. 4. The estimated total ATP turnover rate (ATP_{total}) at resting baseline was not significantly different between conditions (PL 75 ± 9 vs. BR 57 ± 4 μM/s). However, the ATP_{total} averaged over the entire 4-min exercise bout was significantly reduced by NO_3^- supplementation (PL 296 ± 58 vs. BR 192 ± 38 μM/s; $P < 0.05$). The oxidative ATP turnover rate (ATP_{Ox}) averaged over 4 min was also reduced with NO_3^- supplementation (PL 199 ± 37 vs. BR 120 ± 15 μM/s; $P < 0.05$). The amplitude of ATP_{Ox} from rest to steady-state exercise was lower following NO_3^- supplementation (PL 127 ± 35 vs. BR 65 ± 14 μM/s; $P < 0.05$), an effect which was consistent with the reduction in the amplitude of the pulmonary $\dot{V}O_2$ response (Fig. 1*A*). The mean ATP turnover rate from PCr hydrolysis (ATP_{PCr}) over the entire low-intensity bout was lower following NO_3^- supplementation

Table 3. *Muscle metabolic response during low-intensity exercise following dietary supplementation with nitrate (beetroot juice) and placebo*

	Placebo	Nitrate
[PCr]		
Baseline, mM	35.8 ± 1.5	34.9 ± 1.7
120 s, mM	27.9 ± 1.9	29.9 ± 2.4
240 s, mM	27.7 ± 2.0	29.7 ± 2.5
Time constant, s	24 ± 3	30 ± 4
Amplitude, mM	8.1 ± 1.2	5.2 ± 0.8*
[P_i]		
Baseline, mM	4.0 ± 0.4	4.0 ± 0.2
120 s, mM	8.2 ± 0.7	7.5 ± 0.7
240 s, mM	8.2 ± 0.7	7.3 ± 0.7
Amplitude, mM	4.4 ± 0.8	3.5 ± 0.8*
[ADP]		
Baseline, μM	6.1 ± 0.7	6.1 ± 0.7
120 s, μM	23.4 ± 2.5	16.3 ± 1.4*
240 s, μM	22.4 ± 2.2	15.8 ± 1.3*
Amplitude, μM	17.0 ± 2.8	10.3 ± 1.7*
pH		
Baseline	7.05 ± 0.01	7.03 ± 0.01
120 s	7.06 ± 0.02	7.05 ± 0.01
240 s	7.04 ± 0.02	7.03 ± 0.01
ΔBaseline − 240 s	−0.01 ± 0.02	0.00 ± 0.01

Values are mean ± SE. PCr, phosphocreatine. *Significantly different from placebo ($P < 0.05$).

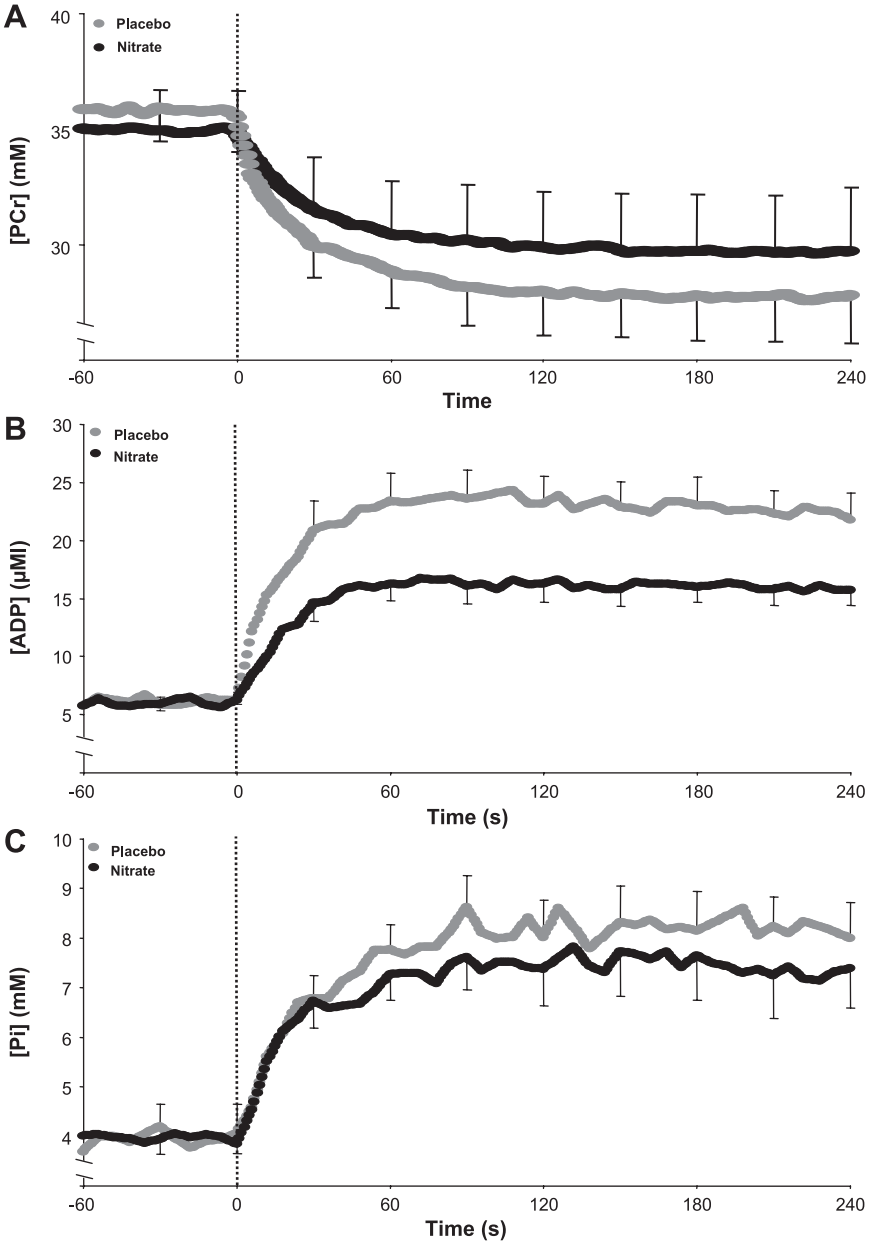

Fig. 2. Group mean ± SE muscle metabolic responses to low-intensity exercise following dietary nitrate and placebo supplementation. The change in muscle phosphocreatine concentration ([PCr]) (*A*), [ADP] (*B*), and [P_i] (*C*) from rest to the steady state was significantly reduced following nitrate supplementation (see Table 3). The dashed vertical line represents the abrupt imposition of the work rate from a resting baseline.

(PL 34 ± 5 vs. BR 22 ± 3 μM/s; $P < 0.05$), but the mean glycolytic rate (ATP_{Gly}) was not different between conditions (Fig. 4). The relative contribution of ATP_{Ox} (PL 70 ± 3 vs. BR 69 ± 6%), ATP_{PCr} (PL 13 ± 2 vs. BR 13 ± 2%), and ATP_{Gly} (PL 17 ± 4 vs. BR 18 ± 6%) to ATP_{total} over the 4-min bout of low-intensity exercise was not significantly different between conditions.

High-intensity exercise. The muscle metabolic effects of dietary NO_3^- supplementation during high-intensity exercise are reported in Table 4 and illustrated in Fig. 5. The primary [PCr] amplitude, [PCr] baseline, and τ values were not different between the NO_3^- and PL conditions. However, the [PCr] slow component amplitude was significantly reduced (by 59%) following NO_3^- supplementation (PL 3.9 ± 1.1 vs. BR 1.6 ± 0.7 mM; $P < 0.05$; Fig. 5*A*), consistent with the reduced $\dot{V}O_2$ slow component (Fig. 3). The relative magnitude of the changes in [PCr] with NO_3^- supplementation was not different from the relative magnitude of the changes in $\dot{V}O_2$ either in the fundamental or slow phases of the response. There were no significant differences in any of the dynamic parameters of [P_i] and [ADP] between conditions (Table 4). Muscle pH was not significantly different between the NO_3^- and PL conditions either at baseline or during exercise (Table 4).

The estimated mean ATP_{total}, ATP_{Ox}, ATP_{PCr}, and ATP_{Gly} for high-intensity exercise are shown in Fig. 6. The estimated ATP_{total} averaged over the entire exercise bout was significantly reduced by NO_3^- supplementation (PL 607 ± 65 vs. BR 436 ± 43 μM/s; $P < 0.05$). The mean ATP_{Ox} (PL 468 ± 49 vs. BR 329 ± 18 μM/s; $P < 0.05$) and ATP_{PCr} (PL 44 ± 8 vs. BR 25 ± 7 μM/s; $P < 0.05$) were also lower following NO_3^- supplementation. The mean ATP_{Gly} was not significantly affected (PL 95 ± 21 vs. BR 82 ± 35 μM/s). The amplitude of change in ATP_{total} from resting baseline to end exercise (averaged over the final 30 s) was lower following NO_3^- supple-

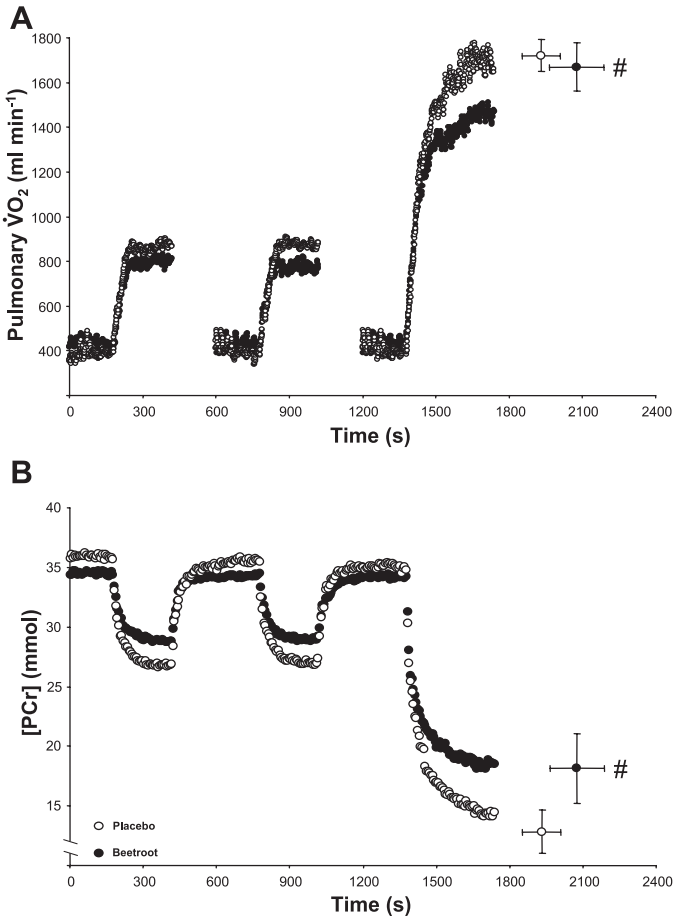

Fig. 3. Pulmonary $\dot{V}O_2$ (*A*) and intramuscular [PCr] (*B*) responses to the experimental protocol which involved 2 bouts of low-intensity exercise and 1 bout of high-intensity exercise. Note the proportionately similar sparing in $\dot{V}O_2$ and [PCr] across the 3 exercise bouts. Models of respiratory control predict that a reduced decrement in [PCr] (presumably as a consequence of a reduced muscle ATP turnover requirement for the same work rate) would result in a reduced stimulation of oxidative phosphorylation. See text for further details. #Time to task failure significantly different from placebo ($P < 0.01$).

mentation (PL 491 ± 71 vs. BR 334 ± 39 μM/s; $P < 0.05$), an effect that was also evident in the ATP_{Ox} response (PL 414 ± 47 vs. BR 275 ± 20 μM/s; $P < 0.05$). The relative contribution of ATP_{Ox} (PL 77 ± 3 vs. BR 78 ± 5%) and ATP_{Gly} (PL 15 ± 2 vs. BR 16 ± 5%) to ATP_{total} over the 6-min bout of high-intensity exercise was not significantly different between conditions but the relative contribution from ATP_{PCr} was smaller following NO_3^- supplementation (PL 8 ± 2 vs. BR 6 ± 2%; $P < 0.05$).

Exercise Tolerance

Exercise tolerance was enhanced following NO_3^- supplementation as demonstrated by the 25% increased time to task failure (PL 586 ± 80 vs. BR 734 ± 109 s; $P < 0.01$). All seven subjects had a longer time to task failure in the BR condition. Following NO_3^- supplementation, the time to task failure was correlated with the average plasma [NO_2^-] over *days 4–6*, although this relationship did not attain statistical significance ($r = 0.73$, $P = 0.06$). The time to task failure with NO_3^- supplementation tended to be related to the [PCr]

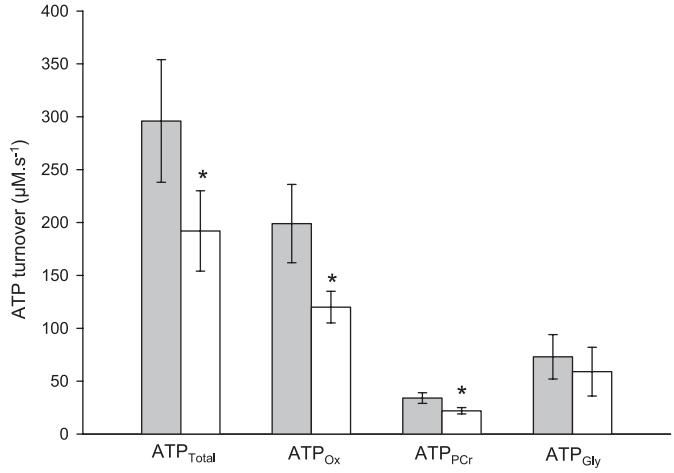

Fig. 4. Group mean ± SE ATP resynthesis rate averaged over the entire exercise bout during low-intensity knee-extension exercise following dietary nitrate (white bars) and placebo (gray bars). Total ATP turnover (ATP_{Total}), ATP derived from oxidative phosphorylation (ATP_{Ox}), ATP derived from PCr splitting (ATP_{PCr}), and ATP derived from glycolysis (ATP_{Gly}) are shown. The reductions in ATP_{Total}, ATP_{Ox}, and ATP_{PCr} were statistically significant. See text for further details. *Significantly different from placebo ($P < 0.05$).

remaining at 120 s ($r = 0.72$, $P = 0.07$) and 360 s ($r = 0.71$, $P = 0.07$) of exercise. The intramuscular [PCr] and pulmonary $\dot{V}O_2$ at task failure were not significantly different between the placebo and NO_3^--supplemented conditions ($n = 6$).

Table 4. *Muscle metabolic response during high-intensity exercise following dietary supplementation with nitrate (beetroot juice) and placebo*

	Placebo	Nitrate
[PCr]		
Baseline, mM	35.2 ± 1.5	35.0 ± 1.8
120 s, mM	18.0 ± 2.2	21.6 ± 2.4
360 s, mM	15.3 ± 2.2	20.0 ± 3.0
At task failure, mM	12.8 ± 1.8	18.1 ± 2.9
Time constant, s	22 ± 2	26 ± 5
Primary amplitude, mM	15.8 ± 2.0	13.3 ± 0.1
SC amplitude, mM	3.9 ± 1.1	1.6 ± 0.7*
[P_i]		
Baseline, mM	3.7 ± 0.5	3.9 ± 0.3
120 s, mM	14.9 ± 1.5	12.1 ± 1.7
360 s, mM	16.3 ± 2.2	13.7 ± 2.7
At task failure, mM	17.1 ± 2.4	13.7 ± 2.7
Primary amplitude, mM	11.1 ± 1.5	8.5 ± 2.0
SC amplitude, mM	1.7 ± 0.7	1.7 ± 1.0
[ADP]		
Baseline, μM	7.1 ± 0.6	6.2 ± 0.5
120 s, μM	74.7 ± 18.4	42.9 ± 5.9
360 s, μM	97.7 ± 31.8	42.6 ± 28.5
At task failure, μM	100.2 ± 31.7	46.2 ± 6.3
Primary amplitude, μM	96.0 ± 41.6	36.7 ± 6.6
SC amplitude, μM	2.8 ± 2.8	1.2 ± 1.2
pH		
Baseline	7.04 ± 0.01	7.01 ± 0.01
120 s	7.04 ± 0.03	7.02 ± 0.03
360 s	6.95 ± 0.05	6.92 ± 0.04
At task failure	6.84 ± 0.08	6.85 ± 0.08
ΔBaseline − 360 s	−0.09 ± 0.05	−0.09 ± 0.04
Δ 360 s − Tlim	−0.11 ± 0.07	−0.07 ± 0.05

Values are mean ± SE; $n = 6$ for values at task failure. SC, slow component. Tlim = time to task failure. *Significantly different from placebo ($P < 0.05$).

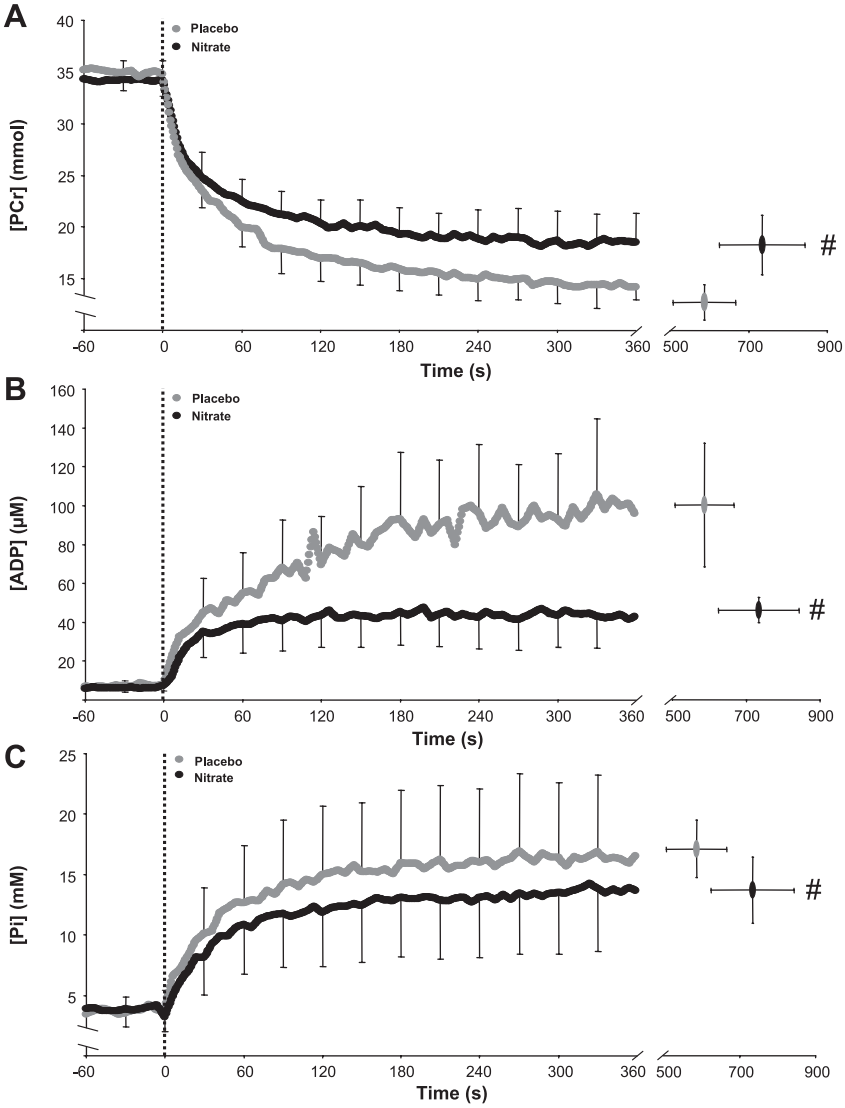

Fig. 5. Group mean ± SE muscle metabolic responses to high-intensity exercise following dietary nitrate and placebo supplementation. The change in muscle [PCr] (*A*), [ADP] (*B*), and [P_i] (*C*) are illustrated. The dashed vertical line represents the abrupt imposition of the work rate from a resting baseline. #Time to task failure significantly different from placebo ($P < 0.01$).

DISCUSSION

The major novel finding of this study was that dietary supplementation with nitrate-rich BR (which more than doubled plasma [NO_2^-]) reduced both the O_2 cost and the degree of PCr degradation during both low- and high-intensity exercise, without affecting muscle pH. Moreover, NO_3^- supplementation significantly reduced the estimated ATP_{total} during both low- and high-intensity exercise. These findings are consistent with our experimental hypotheses and suggest that NO_3^- supplementation predominantly reduces the O_2 cost of exercise (improves exercise efficiency) through reducing the total ATP cost of muscle force production rather than by increasing the mitochondrial P/O ratio. Moreover, dietary NO_3^- supplementation appears to improve the tolerance of high-intensity exercise by reducing muscle metabolic perturbation as reflected, for example, in the extent to which the finite muscle PCr reserve is depleted over time. These findings are important as they provide the first step in elucidating the intramuscular mechanism(s) by which dietary NO_3^- supplementation improves exercise efficiency and exercise tolerance in young healthy humans.

Effects of Dietary Nitrate on Plasma NO_2^- and BP

Dietary NO_3^- supplementation elevated plasma [NO_2^-] on *days 4, 5*, and *6* of the supplementation regime, with a mean 137% increase in the mean plasma [NO_2^-] over the 3 days. This finding corroborates previous observations that NO_3^- administration substantially elevates plasma [NO_2^-] in humans (3, 52, 74). The differences in absolute plasma [NO_2^-] that have been reported in this and in previous studies likely reflect the relative complexity and technical challenges associated with its measurement. It is clear that NO_2^- can be converted to NO under appropriate physiological conditions (9, 56) and therefore the elevated plasma [NO_2^-] would be expected to increase NO bioavailability during exercise. An elevation in extracellular NO activates guanylate cyclase, which synthesizes cyclic guanosine monophosphate (cGMP) from guanosine triphosphate, leading to smooth muscle relaxation (32). As such, reductions in both systolic and diastolic BP have been noted following NO_3^- administration (52, 74). In our previous study, we observed a reduction in systolic but not diastolic BP following BR ingestion (3). In the present study, we observed

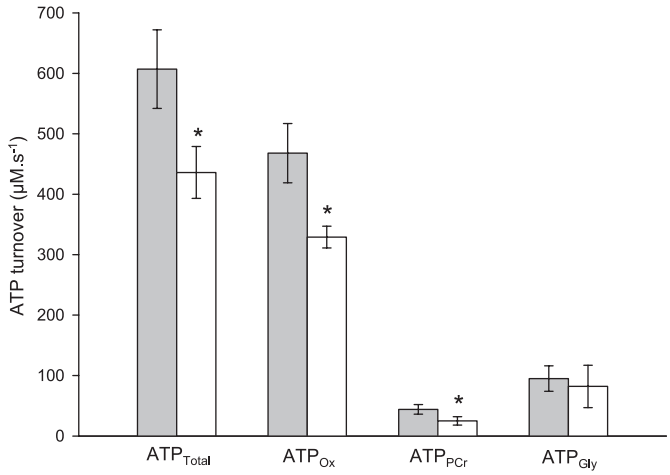

Fig. 6. Group mean ± SE ATP resynthesis rate averaged over the entire exercise bout during high-intensity knee-extension exercise following dietary nitrate (white bars) and placebo (gray bars) supplementation. Total ATP turnover (ATP_{Total}), ATP derived from oxidative phosphorylation (ATP_{Ox}), ATP derived from PCr splitting (ATP_{PCr}), and ATP derived from glycolysis (ATP_{Gly}) are shown. The reductions in ATP_{Total}, ATP_{Ox}, and ATP_{PCr} were statistically significant. See text for further details. *Significantly different from placebo ($P < 0.05$).

reductions in the mean systolic BP (−5 mmHg) and diastolic BP (−2 mmHg) as well as mean arterial pressure (−2 mmHg) over *days 4–6*, supporting the notion that both systolic and diastolic BP can be reduced following NO_3^- administration (52, 74). These data, along with evidence for a beneficial role of NO_2^- in hypoxic vasodilation (27) and in the protection of tissues from ischemia-reperfusion injury (28, 73), suggest that a diet rich in vegetables containing a high nitrate content might confer benefits to cardiovascular health (26, 36).

Effects of Dietary Nitrate on Pulmonary Gas Exchange

During low-intensity exercise, the pulmonary $\dot{V}O_2$ amplitude was reduced by 25% during prone knee extension exercise following NO_3^- supplementation, consistent with our previous study during upright cycling (3). In our previous study, this reduction in the pulmonary $\dot{V}O_2$ amplitude was accompanied by an increased blood volume in the thigh (consistent with vasodilatation and increased muscle O_2 availability) and a reduction in muscle fractional O_2 extraction, as estimated using near-infrared spectroscopy (3). A reduction in the O_2 cost of low-intensity submaximal exercise has also been observed following pharmacological NO_3^- administration (52). Conversely, inhibition of nitric oxide synthase (NOS) has been reported to increase $\dot{V}O_2$ in dogs at rest (62). Neither of the two previous human studies assessed the influence of dietary NO_3^- on resting $\dot{V}O_2$, and thus it was unclear whether a possible reduction in resting $\dot{V}O_2$ contributed to the reduced $\dot{V}O_2$ during submaximal exercise. An important finding in the present study was that $\dot{V}O_2$ and the calculated ATP_{Ox} at rest were unaffected by NO_3^- ingestion; thus the reduced O_2 cost appears to be manifest only during skeletal muscle contraction. If NO_3^- supplementation had improved mitochondrial respiratory efficiency, a reduction in resting $\dot{V}O_2$ might have been expected given that there is considerable mitochondrial activity both in skeletal and nonskeletal muscle tissues during resting conditions.

Consistent with our previous results (3), neither $\dot{V}_E$ nor HR was significantly altered by dietary NO_3^- supplementation. This suggests that the reduction in $\dot{V}O_2$ is not a consequence of a reduction of the energy cost of cardiorespiratory processes but is specific to the contracting skeletal muscles. Another intriguing observation from the present study was that the 5–6% reduction in "steady-state" $\dot{V}CO_2$ following nitrate supplementation was not statistically significant and was less than the reduction in steady-state $\dot{V}O_2$, leading to a small but nonsignificant increase in RER. A slight shift in substrate utilization toward a relatively greater use of carbohydrate, perhaps as a consequence of a NO-mediated increase in myocyte glucose uptake (64), might provide a partial explanation for this effect. Additional muscle metabolic studies are required to resolve this issue.

During high-intensity knee-extension exercise, there was a nonsignificant tendency for the primary $\dot{V}O_2$ amplitude to be reduced following NO_3^- supplementation (15%); in contrast, we previously observed an increased primary $\dot{V}O_2$ amplitude during severe-intensity cycling following BR ingestion (3). The reason for this disparity is unclear. However, it may be related to the intensity at which the subjects were exercising. Larsen et al. (52) previously reported that NO_3^- administration was only effective in reducing $\dot{V}O_2$ at intensities up to 80% of the $\dot{V}O_{2\,max}$. Therefore, it is possible that NO_3^- supplementation is effective in reducing the primary $\dot{V}O_2$ amplitude only during exercise that is below the so-called critical power (38). In contrast to our previous study, in which we observed a slower phase II $\dot{V}O_2$ time constant (τ) following NO_3^- supplementation (3), the $\dot{V}O_2$ τ was unchanged in the present study. Differences in exercise modality and body position may explain the differences in the effects on $\dot{V}O_2$ kinetics between the studies.

Following dietary NO_3^- supplementation, the amplitude of the $\dot{V}O_2$ slow component was significantly reduced (by 52%) but the $\dot{V}O_2$ attained at the termination of exercise was not different, results that are consistent with our previous study (3). Interestingly, inhibition of NOS has been shown to have the opposite effect, i.e., to increase the amplitude of the $\dot{V}O_2$ slow component during high-intensity exercise in humans (41). In animal models, NOS inhibition has been reported to reduce mechanical efficiency and impair exercise performance in rats (49) but to reduce the fatigue index during high-intensity contractions in an isolated canine muscle model (30). Although the results are inconsistent and controversial (23), it is clear that NO plays a potentially important role in regulating muscle force production and the dynamic $\dot{V}O_2$ response to muscular exercise (23, 59).

Effects of Dietary Nitrate on Muscle Energetics

During low-intensity exercise, we observed a reduction in the steady-state amplitude of muscle [PCr] degradation and [P_i] and [ADP] accumulation following NO_3^- supplementation, while muscle pH was not significantly altered. It was also calculated that muscle ATP_{total} was significantly reduced with NO_3^- supplementation as a consequence of a reduced ATP_{Ox} and ATP_{PCr}. It should be noted that these estimates of muscle ATP turnover rates involve numerous assumptions and may be subject to considerable error. However, the ^{31}P-MRS data, in conjunction with a reduction in the $\dot{V}O_2$ steady state, suggest that the liberation of energy from the CK reaction and oxida-

tive metabolism was reduced, with the rate of anaerobic glycolysis, which is not expected to make a significant energetic contribution during low-intensity exercise, being essentially unchanged. Collectively, these data suggest that the reduced O_2 cost of exercise following NO_3^- supplementation is consequent to an improved coupling between ATP hydrolysis and skeletal muscle force production rather than an increased mitochondrial P/O ratio. This is highlighted in Fig. 7, which indicates that the slope of the relationship between "steady-state" $\dot{V}O_2$ and muscle [PCr] is similar in the placebo and NO_3^--supplemented conditions, i.e., the changes in $\dot{V}O_2$ and [PCr] are broadly proportional following NO_3^- supplementation. If NO_3^- supplementation had reduced the O_2 cost of exercise exclusively through a specific reduction in the mitochondrial O_2 cost of ATP synthesis, muscle [PCr] degradation and ADP accumulation would not have been expected to change. It appears, instead, that NO_3^- supplementation reduced ATP hydrolysis for the same work rate, which, in turn, reduced PCr degradation and ADP and P_i accumulation. Based on established models of respiratory control, these changes would reduce the stimulus/stimuli for oxidative phosphorylation (12, 13, 17, 57), consistent with the reduced steady-state $\dot{V}O_2$ we have observed (Fig. 3).

During high-intensity exercise, the effect of NO_3^- supplementation on the [PCr] response was analogous to the changes observed in $\dot{V}O_2$. Specifically, there was a clear trend for a reduction in the primary [PCr] amplitude (16%) with no change in the kinetic parameters, and an appreciable reduction in the [PCr] slow component amplitude (59%). Similar to low-intensity exercise, NO_3^- supplementation did not affect pH dynamics during high-intensity exercise. The calculated muscle ATP_{total} was significantly reduced with NO_3^- supplementation as a consequence of a reduced ATP_{Ox} and ATP_{PCr}. Despite there being a clear trend for NO_3^- supplementation to reduce [ADP] and [P_i] accumulation during high-intensity exercise, these changes did not attain statistical significance.

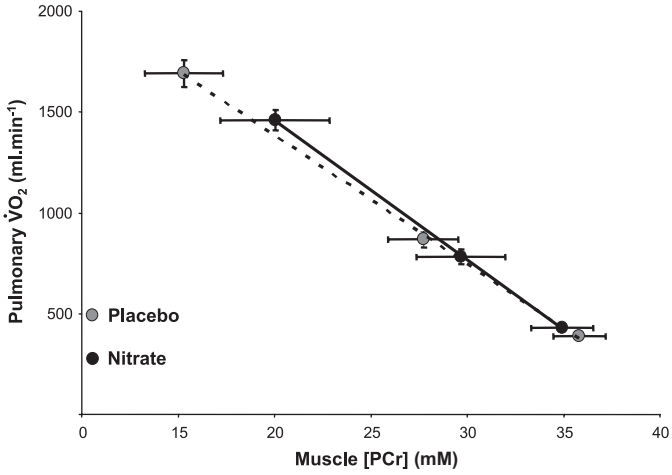

Fig. 7. Group mean ± SE muscle [PCr] plotted against pulmonary $\dot{V}O_2$ at rest, after 4 min of low-intensity exercise and after 6 min of high-intensity exercise following dietary nitrate and placebo supplementation. Note that the lines of best fit for these responses are similar, suggesting reciprocal changes in [PCr] and $\dot{V}O_2$ following dietary nitrate supplementation. This suggests that the improved exercise efficiency following nitrate supplementation is a consequence of a reduced ATP cost of muscle contraction rather than to changes in the mitochondrial P/O ratio.

Overall, the changes in the pulmonary $\dot{V}O_2$ and muscle metabolic responses with NO_3^- supplementation were similar during low-intensity and high-intensity exercise, which suggests that a reduction in the total ATP cost of force production was primarily responsible for the observed effects at both intensities (Fig. 7). During intense constant-work-rate exercise, the rate of ATP turnover within the recruited myocytes is not constant but increases as exercise proceeds such that there is a progressive loss of efficiency (5, 61). NO_3^- supplementation appears to have ameliorated this effect, as evidenced by the commensurate reductions in the $\dot{V}O_2$ and [PCr] slow component amplitudes.

Effects of Dietary Nitrate on Exercise Tolerance

Dietary NO_3^- supplementation resulted in a 25% improvement in the time to task failure during high-intensity exercise. This confirms our previous report in which NO_3^- supplementation resulted in a 17% increase in the time to task failure during high-intensity cycle exercise (3). In the present study, the time to task failure tended to be correlated with the mean plasma [NO_2^-] measured over *days 4–6* of NO_3^- supplementation ($r = 0.73$; $P = 0.06$). The reduced [PCr] slow component amplitude resulted in a sparing of [PCr] over the first 360 s of exercise, and this also tended to be related to the time to task failure ($r = 0.71$; $P = 0.07$). Therefore, dietary NO_3^- supplementation, by reducing the ATP cost of force production, facilitated a sparing of the finite PCr stores and a reduction in the O_2 cost of exercise (particularly in the slow phase), culminating in an improved tolerance of intense exercise. It has been shown that at the termination of high-intensity constant-work-rate exercise, intramuscular [PCr] and pH reach consistently low values, and [P_i] reaches a consistently high value, that may limit continued muscle function (70). Interventions that reduce the $\dot{V}O_2$ or [PCr] slow component amplitude have previously been associated with improved exercise performance (2, 4, 78; see 37 for review). While the causes of skeletal muscle fatigue during intense exercise are debated (75), the reduced muscle metabolic perturbation (i.e., reduced fall in [PCr] and reduced accumulation of [ADP] and [P_i]) following NO_3^- supplementation appears to have enabled high-intensity exercise to be sustained for longer before these metabolites reached "critical" values.

Possible Mechanisms for the Reduced ATP Cost of Force Production with Dietary Nitrate

The results of this study suggest that the improvements in exercise efficiency and exercise tolerance associated with NO_3^- supplementation are a result of a reduced rate of muscle ATP turnover for a given work rate. The pertinent question is therefore: by which mechanism(s) does NO_3^- supplementation reduce the ATP cost of muscle force production? The ATP turnover rate in the contracting skeletal myocytes is predominantly determined by two ATPase pathways: actomyosin-ATPase, which facilitates the interaction between actin and myosin, and Ca^{2+}-ATPase, which is responsible for sarcoplasmic reticulum (SR) Ca^{2+} pumping; membrane depolarization (Na^+-K^+-ATPase) is responsible for a further small (~7%) fraction of the total ATP turnover (6). While the relative energetic requirements of actomyosin-ATPase and Ca^{2+}-ATPase during contraction is equivocal (6, 72, 80), it is clear

that the O_2 cost of contraction is reduced by interventions that inhibit both the actomyosin-ATPase (46, 72) and the Ca^{2+}-ATPase (65, 66). There is evidence that NO reduces Ca^{2+} cycling (33, 71) and slows cross-bridge cycling kinetics (25, 34), indicating that NO may modulate the ATP cost of force production. Our observation of a reduced O_2 cost of exercise following dietary NO_3^- supplementation might therefore be related to a reduction in the ATP cost of cross-bridge cycling and/or Ca^{2+} handling. However, whereas NO reduces muscle force development in in vitro preparations (25, 34), in the present study it enabled the same submaximal power output to be sustained for longer.

We are not able to exclude the possibility that NO_3^- supplementation also increased the mitochondrial P/O ratio during exercise and/or that some other factor associated with BR ingestion caused a reduced PCr breakdown. For example, greater homogeneity of perfusion relative to metabolic rate, perhaps as a consequence of an extension of the tissue O_2 gradient due to NO-mediated inhibition of mitochondria closest to the capillary (14, 68), might reduce muscle metabolic perturbation. NO-mediated mitochondrial biogenesis (58) might also reduce muscle metabolic perturbation for the same work rate (35, 39). However, these changes would not be expected to reduce the steady-state $\dot{V}O_2$ for a given submaximal work rate. CK, the enzyme which catalyzes [PCr] splitting, is a dimer containing several sulfhydryl groups (24). Administration of NO donors results in reversible inhibition of CK via *S*-nitrosylation (1), which might also explain our observed reductions in [PCr] degradation. However, the proportionally similar reductions in $\dot{V}O_2$ and [PCr] suggests that the likely effect of NO_3^- supplementation is a reduced ATP cost of force production during skeletal muscle contraction.

The results of this study have a number of potentially important implications. For example, it is possible that the protective effect of NO_2^- on infarct size that has been reported in experimental models of myocardial ischemia (20, 63, 73) might be due to an NO-mediated reduction in the energy (and O_2) cost of contraction rather than, or perhaps in addition to, enhanced perfusion of ischemic areas. Moreover, it has recently been reported that Tibetans residing at 4,200-m altitude have >10-fold-higher circulating concentrations of bioactive NO products including NO_2^- compared with low-altitude dwellers (24). While enhanced NO bioavailability might facilitate blood flow and tissue O_2 delivery in hypobaric hypoxia through effects on peripheral vasodilation (24), it is also possible that the physiological adaptation to high altitude involves an NO-mediated reduction in the energy (and O_2) cost of physical activity. This would be consistent with reports that altitude training can improve submaximal cycling efficiency (29).

We have attributed the various physiological changes observed in the present study to increased dietary nitrate consumption consequent to beetroot juice consumption. This interpretation is based on the similarity of our results (increased plasma [nitrite] and improved exercise efficiency) to those observed when the diet is supplemented with sodium nitrate (52). However, we wish to stress that beetroot juice is also rich in antioxidants and phenols (48), and it is possible that these compounds contributed either independently, or synergistically with nitrate, to the results obtained. Additional studies are required to identify the extent to which other metabolically active compounds in beetroot juice influence BP and the physiological responses to exercise.

Conclusions

Dietary nitrate supplementation effectively doubled plasma [nitrite] and reduced both systolic and diastolic BP, effects that are consistent with an elevated NO bioavailability. The $\dot{V}O_2$ required for the same work rate was reduced during both low-intensity and high-intensity knee-extension exercise following NO_3^- supplementation. On average, during low-intensity exercise, the amplitude of the increase in $\dot{V}O_2$ above the resting baseline was reduced by 25% and the absolute $\dot{V}O_2$ (including the resting baseline) was reduced by 11%. Similarly, following 6 min of high-intensity exercise, the $\dot{V}O_2$ amplitude was reduced by 21% and the absolute $\dot{V}O_2$ was reduced by 14%, although the $\dot{V}O_2$ at the termination of exercise was not different between conditions. At both exercise intensities, the reduction in $\dot{V}O_2$ was accompanied by a reduction in muscle [PCr] of similar magnitude, while pH was unchanged. These in vivo data suggest that the reduction in $\dot{V}O_2$ following NO_3^- supplementation is principally a result of a reduced rate of ATP turnover in the contracting myocytes, i.e., a blunting of the changes in high-energy phosphates would reduce the stimuli to oxidative phosphorylation. The reduction in muscle metabolic perturbation with NO_3^- supplementation, including a sparing of the rate of depletion of the finite PCr reserve, was associated with an improved tolerance of high-intensity exercise.

DISCLOSURES

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

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