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Effects of nitrate supplementation via beetroot juice on contracting rat skeletal muscle microvascular oxygen pressure dynamics

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Abstract

NO₃⁻ supplementation via beetroot juice (BR) augments exercising skeletal muscle blood flow subsequent to its reduction to NO₂⁻ then NO. We tested the hypothesis that enhanced vascular control following BR would elevate the skeletal muscle O₂ delivery/O₂ utilization ratio (microvascular PO₂, *PmvO*₂) and raise the *PmvO*₂ during the rest-contractions transition. Rats were administered BR (~0.8 mmol/kg/day, *n*=10) or water (control, *n*=10) for 5 days. *PmvO*₂ was measured during 180 s of electrically-induced (1 Hz) twitch spinotrapezius muscle contractions. There were no changes in resting or contracting steady-state *PmvO*₂. However, BR slowed the *PmvO*₂ fall following contractions onset such that time to reach 63% of the initial *PmvO*₂ fall increased (MRT₁; control: 16.8±1.9, BR: 24.4±2.7 s, *p*<0.05) and there was a slower relative rate of *PmvO*₂ fall ($\Delta_1 PmvO_2/\tau_1$; control: 1.9±0.3, BR: 1.2±0.2 mmHg/s, *p*<0.05). Despite no significant changes in contracting steady state *PmvO*₂, BR supplementation elevated the O₂ driving pressure during the crucial rest-contractions transients thereby providing a potential mechanism by which BR supplementation may improve metabolic control.

Keywords

exercise; blood flow; nitrite; nitric oxide

1. Introduction

At exercise onset, skeletal muscle blood flow (*Qm*) is elevated via a complex array of mechanical (muscle pump) as well as vasodilatory controllers (Joyner & Wilkins, 2007) in an effort to balance O₂ delivery (*QO*₂)-to-utilization (*VO*₂). Across species, for small and

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large muscle mass exercise QO_2 increases between 5 and 6 L per L $\dot{V}O_2$ irrespective of muscle fiber-type composition (Whipp & Ward, 1982; Ferreira *et al.* 2006; Jones *et al.* 2012; reviewed by Poole & Jones, 2012) serving to meet the rising metabolic demands during the rest-contraction transient. However, owing to a lower intercept of the QO_2 -to- $\dot{V}O_2$ relation across the spectrum of muscle fiber-types, muscles or muscle portions comprised of fast-twitch (type II) fibers have a significantly lower QO_2 than their slow-twitch (type I) counterparts such that their fractional O_2 extraction at rest is higher and thus their microvascular PO_2 ($Pm\dot{V}O_2$) lower (Behnke *et al.* 2003; McDonough *et al.* 2005; Ferreira *et al.* 2006). Consequently, during contractions when $\dot{V}O_2$ rises there is less room for further increases in fractional O_2 extraction giving rise to extremely low $Pm\dot{V}O_2$ values. This is particularly important given that low $Pm\dot{V}O_2$ values lead to reduced intramyocyte $Pm\dot{V}O_2$ (Hogan *et al.* 1992; Richardson *et al.* 1999; Kindig *et al.* 2003; Haseler *et al.* 2004; reviewed by McDonough *et al.* 2005) making it probable that the metabolic behavior of type II fibers (i.e. slowed $\dot{V}O_2$ kinetics, elevated rate of glycolysis and lactic acid production) results, at least in part, from this phenomenon.

The ubiquitous signaling molecule nitric oxide (NO) plays a fundamental role in exercise induced vasodilation and thus QO_2 (Hirai *et al.* 2004; reviewed by Joyner & Tschakovsky, 2003) and also increases muscle mitochondrial oxidative (Larsen *et al.* 2012) and contractile (Andrade *et al.* 1998) efficiency. Emerging evidence suggests dietary nitrate (NO_3^-), ingested for example via sodium NO_3^- salt or beetroot juice (BR), may impact skeletal muscle hemodynamic, metabolic and contractile function following its non-enzymatic reduction to nitrite (NO_2^-) and NO *in vivo* (Larsen *et al.* 2007, Bailey *et al.* 2009, Bailey *et al.* 2010, Hernandez *et al.* 2012, Ferguson *et al.* 2013). In humans, acute NO_3^- supplementation via BR has been linked to improvements in muscle tissue oxygenation during exercise in a hypoxic environment (Vanhatalo *et al.* 2011, Masschelein *et al.* 2012) and has been demonstrated to enhance local tissue oxygenation in peripheral artery disease patients in whom reduced local O_2 delivery is a defining characteristic responsible for exercise intolerance (Kenjale *et al.* 2011).

Recently, our laboratory demonstrated that BR supplementation in rats (NO_3^- dose 1 mmol/kg/day for 5 days) elevates QO_2 preferentially in muscles comprised of fast-twitch fibers during treadmill running (Ferguson *et al.* 2013). The resultant Qm increase would presumably elevate the $QO_2/\dot{V}O_2$ ratio and thus $Pm\dot{V}O_2$, thereby improving metabolic control, which may help explain mechanistically the lowered arterial [lactate] seen in the running rat following BR supplementation (Ferguson *et al.* 2013).

To our knowledge there have been no reports on the effects of NO_3^- supplementation on the $Pm\dot{V}O_2$ profile of contracting skeletal muscle. We hypothesized that 5 days of BR supplementation (NO_3^- dose: ~0.8 mmol/kg/day) would increase $Pm\dot{V}O_2$ across the rest/contraction transient (i.e. slow $Pm\dot{V}O_2$ on kinetics) and raise the subsequent steady-state $Pm\dot{V}O_2$.

2. Methods

2.1 Animal selection and care

Twenty young adult male Sprague-Dawley rats (average body mass = 410 ± 14 g, Charles River Laboratories, Wilmington, MA) were used in this investigation. Rats were maintained in accredited animal facilities at Kansas State University on a 12/12 hr light-dark cycle with food and water provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and conducted according to National Institute of Health guidelines.

2.2 Supplementation protocol

Rats received 5 days of BR supplementation (BR; n=10) with a NO_3^- dose of 1 mmol/kg/day (Beet it™, James White Drinks, Ipswich UK, diluted with 100 ml of tap water) or untreated tap water (control; n=10) with consumption monitored daily (average NO_3^- consumption for BR rats = 0.79 ± 0.04 (range = 0.66–0.90) mmol/kg/day). This dose is similar to the sodium NO_3^- dose administered to rats by Carlström et al. (2010) and accounts for the resting metabolic rate of the Sprague Dawley rat (~7× faster than humans, Musch *et al.* 1988). Moreover, we have reported recently that this identical BR dose and administration period elevates plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ to levels similar to those seen in humans and improves skeletal muscle O_2 delivery in exercising rats (Ferguson *et al.* 2013).

2.3 Surgical preparation

Rats were anaesthetized with a 5% isoflurane- O_2 mixture and maintained subsequently on 3% isoflurane- O_2 . The carotid artery was cannulated and a catheter (PE-10 connected to PE-50, Intra-Medic polyethylene tubing, Clay Adams Brand, Becton, Dickinson and Company, Sparks, MD) inserted into carotid artery catheter for measurement of MAP and HR, infusion of the phosphorescent probe (see below), and arterial blood sampling. A second catheter was placed in the caudal artery. The incisions were then closed and rats were transitioned progressively to pentobarbital sodium anesthesia (administered into the caudal artery catheter to effect) with the level monitored continuously via the toe-pinch and blink reflexes and anesthesia supplemented as necessary. Rats were then placed on a heating pad to maintain core temperature at ~38 °C (measured via rectal probe). Overlying skin and fascia were reflected carefully from the mid-dorsal caudal region of each rat and the right spinotrapezius muscle was carefully exposed in a manner which ensured the integrity of the neural and vascular supply to the muscle (Bailey *et al.* 2000). Silver wire electrodes were sutured (6–0 silk) to the rostral (cathode) and caudal (anode) regions of the muscle. The exposed spinotrapezius muscle was continuously superfused with a warmed (38°C) Krebs–Henseleit bicarbonate buffered solution equilibrated with 5% CO_2 –95% N_2 and surrounding exposed tissue was covered with Saran wrap (Dow Brands, Indianapolis, IN). The spinotrapezius muscle was selected specifically based on its mixed muscle fiber-type composition and citrate synthase activity close to that found in human quadriceps muscle (Delp & Duan 1996; Leek *et al.* 2001).

2.4 Experimental protocol

The phosphorescent probe palladium meso-tetra (4 carboxyphenyl)porphyrin dendrimer (R2: 15–20 mg·kg⁻¹ dissolved in 0.4 ml saline) was infused via the carotid artery catheter. After a brief stabilization period (~10 min), the common end of the light guide of a frequency domain phosphorimeter (PMOD 5000, Oxygen Enterprises, Philadelphia, PA) was positioned ~2–4 mm superficial to the dorsal surface of the exposed right spinotrapezius muscle over a randomly selected muscle field absent of large vessels thus ensuring that the region contained principally capillary blood. PmvO_2 was measured via phosphorescence quenching (see below) and reported at 2 s intervals throughout the duration of the 180 s contraction protocol (1 Hz, ~6 V, 2 ms pulse duration) elicited via a Grass stimulator (model S88, Quincy, MA). Following the contraction period it was ensured that PmvO_2 returned to baseline values (indicative of preserved vasomotor function). Rats were euthanized via pentobarbital sodium overdose (50 mg/kg administered into the carotid artery catheter).

2.5 PmvO_2 measurement and curve-fitting

The Stern-Volmer relationship allows the calculation of PmvO_2 through the direct measurement of a phosphorescence lifetime via the following equation (Rumsey *et al.*, 1988):

$$PmvO_2 = [\tau^\circ / \tau - 1] / (k_Q \times \tau^\circ)$$

Where k_Q is the quenching constant and τ° and τ are the phosphorescence lifetimes in the absence of O_2 and the ambient O_2 concentration, respectively. For R2, k_Q is 409 $\text{mmHg}^{-1} \cdot \text{s}^{-1}$ and τ° is 601 μs (Lo *et al.*, 1997) and these characteristics do not change over the physiological range of pH and temperature in the rat *in vivo* and, therefore, the phosphorescence lifetime is determined directly by the O_2 pressure (Rumsey *et al.*, 1988; Lo *et al.*, 1997).

The R2 phosphorescent probe binds to albumin, and consequently, is uniformly distributed throughout the plasma. A previous study from our laboratory investigated systematically the compartmentalization of R2 and confirmed that it remains within the microvasculature of exposed muscle over the duration considered in the present experiments, thereby ensuring a valid $PmvO_2$ measurement (Poole *et al.*, 2004).

Curve-fitting of the measured $PmvO_2$ responses was performed with commercially available software (SigmaPlot 11.01, Systat Software, San Jose, CA) and the data were fit with either a one- or two-component model as described below:

$$\text{One component: } PmvO_2(t) = PmvO_2(BL) - \Delta PmvO_2(1 - e^{-(t - TD)/\tau})$$

$$\text{Two component: } PmvO_2(t) = PmvO_2(BL) - \Delta_1 PmvO_2(1 - e^{-(t - TD_1)/\tau_1}) + \Delta_2 PmvO_2(1 - e^{-(t - TD_2)/\tau_2})$$

where $PmvO_2(t)$ represents the $PmvO_2$ at any given time t , $PmvO_2(BL)$ corresponds to the pre-contracting resting baseline $PmvO_2$, Δ_1 and Δ_2 are the amplitudes for the first and second component, respectively, TD_1 and TD_2 are the time delays for each component, and τ_1 and τ_2 are the time constants (i.e., time to 63% of the final response value) for each component. Goodness of fit was determined using the following criteria: 1) the coefficient of determination, 2) sum of the squared residuals, and 3) visual inspection and analysis of the model fits to the data and the residuals. The MRT of the kinetics response was calculated for the first component in order to provide an index of the overall principal kinetics response according to the following equation:

$$MRT_1 = TD_1 + \tau_1$$

where TD_1 and τ_1 are as described above. The delta of the initial $PmvO_2$ fall following contractions onset was normalized to τ_1 ($\Delta_1 PmvO_2 / \tau_1$) to provide an index of the relative rate of fall. Additionally, the time taken to reach 63% of the initial $PmvO_2$ fall was determined independently from the modeling procedures (T_{63}) to ensure appropriateness of the model fits. Specifically, the raw $PmvO_2$ data were interpolated, and the time coinciding with 63% of the total amplitude ($\Delta_{\text{total}} PmvO_2$) was determined.

2.6 Blood sampling and measurement of plasma $[NO_3^-]$ and $[NO_2^-]$

A pre-supplementation period blood sample was taken from all rats to assess plasma $[NO_3^-]$. In accordance with IACUC guidelines these pre-supplementation blood samples (i.e., when the rats were not instrumented with catheters) were taken from the sub-orbital plexus using a glass capillary pipette. Approximately ~0.8 ml of blood was sampled and centrifuged in heparinized tubes at 6000 g at 4°C for 6 minutes, plasma was extracted and frozen immediately at -80°C for later analysis. This required sampling strategy precluded

accurate determination of pre-supplementation plasma $[\text{NO}_2^-]$ because of hemolysis in some samples.

Post-supplementation blood samples were collected following surgical instrumentation via the caudal artery catheter to assess 1) plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ and 2) pH, PO_2 , and % O_2 saturation. For plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$, ~0.8 ml of blood was drawn into heparinized tubes, rapidly centrifuged and frozen as described above. A second ~0.3 ml blood sample was drawn and analyzed for pH, PO_2 , and % O_2 saturation (Nova Stat Profile M, Nova Biomedical, Waltham, MA, USA).

All measurements of plasma NO_3^- and NO_2^- were performed within 30 minutes of thawing via chemiluminescence with an Ionic/Sievers NO analyzer (NOA 280i, Sievers Instruments, Boulder, CO, USA). In order to obtain plasma NO_2^- levels and to avoid potential reduction of NO_3^- , potassium iodide in acetic acid was used as a reductant. This reductant possesses the ability to reduce NO_2^- to NO but is incapable of reducing higher oxides of nitrogen (i.e. NO_3^-) thus increasing the specificity for NO_2^- . Plasma NO_3^- concentrations were then obtained using the same apparatus with the stronger reductant vanadium chloride in hydrochloric acid at a temperature of 95°C. This stronger reductant reduces the sum of all nitrogen oxides with an oxidation state of +2 or higher (predominantly NO_3^- [μM]) but also includes NO_2^- and nitrosothiols [nM].

2.7 Statistical analysis

Data are presented as mean $\pm$ SEM. Results were compared with mixed 2-way ANOVAs (plasma $[\text{NO}_3^-]$, MAP, and HR) with Student-Newman-Keuls *post hoc* tests where appropriate or unpaired Student's t-tests ($[\text{NO}_2^-]$, blood gases, PmvO_2 kinetics parameters). Significance was accepted at $p < 0.05$.

3. Results

3.1 Plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$

Rats receiving BR had significantly higher plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ when compared to control (Figure 1).

3.2 MAP, HR, and arterial blood gasses

There were no differences in MAP or HR between control and BR rats prior to contractions or during the contracting steady-state (Table 1). Arterial blood pH (control: 7.50 ± 0.01 , BR: 7.51 ± 0.01 , $p > 0.05$), PO_2 (control: 101 ± 3 , BR: 94 ± 3 mmHg, $p > 0.05$), and O_2 saturation (control: 98 ± 1 , BR: $98 \pm 1\%$ mmHg, $p > 0.05$) were not different between control and BR rats.

3.3 PmvO_2 parameters

Representative raw PmvO_2 profiles, their model fits, and residuals for control and BR rats are presented in Figure 2. The responses were adequately fit by a one-component model in 2 of 10 control rats and 5 of 10 BR rats. The more complex two-component model was indicated in 8 of 10 control and 5 of 10 BR rats. The r^2 (control: 0.99 ± 0.01 , BR: 0.98 ± 0.01) and sum of squared residuals (control: 20.2 ± 3.1 , BR: 19.2 ± 3.7 mmHg) for both groups suggested the appropriateness of the model fits. Table 2 presents the average PmvO_2 kinetics parameters. There were no differences in the $\text{PmvO}_{2(\text{BL})}$. However, following the onset of contractions BR resulted in a longer TD_1 , smaller first-component and overall (i.e., PmvO_2 baseline minus steady-state, $\Delta_{\text{total}}\text{PmvO}_2$) amplitudes, and slower PmvO_2 kinetics (i.e., longer MRT_1 and lower $\Delta\text{PmvO}_2/\tau$) following the onset of contractions. There were no significant differences in steady-state PmvO_2 during contractions. Importantly, within the control and BR groups the model-dependent MRT_1 and model-independent T_{63} were not

different (Table 2) increasing confidence in the model parameters. This conclusion was also supported by the very small and non-systematic pattern of the residuals of the model fits (Figure 2, middle panel).

4. Discussion

The primary novel finding of the present investigation was that, relative to control, 5 days of BR supplementation elevated plasma $[\text{NO}_2^-]$ and $[\text{NO}_3^-]$ and slowed significantly the PmvO_2 fall during the crucial rest-contraction transient despite no significant steady-state effects in the mixed fiber-type rat spinotrapezius muscle. The slowed PmvO_2 response (i.e. longer time delay, MRT, and slower relative rate of PmvO_2 fall) following the onset of contractions reflects an elevated QO_2/VO_2 ratio within the skeletal muscle microvasculature which serves to increase the pressure head vital for capillary-myocyte O_2 flux and potentially enhance oxidative function. Improvements in metabolic control at the onset of contractions would be expected to reduce glycolytic metabolism dependence, ultimately attenuating the accumulation of fatigue-associated metabolites. Therefore, these results suggest that acute BR supplementation may provide advantageous effects within skeletal muscle with important implications for exercise tolerance in health and disease.

4.1 Effects of BR on the PmvO_2 kinetics profile

The principal novel finding of this investigation was the slower PmvO_2 kinetics in BR supplemented rats. Behnke *et al.* (2003) found fiber type differences in the PmvO_2 response to electrical stimulation that included a longer time delay, MRT, and reduced rate of PmvO_2 fall at the onset of contractions in soleus (slow) versus peroneal (fast) muscles. Considering that the spinotrapezius muscle consists of predominantly fast-twitch type II muscles (59% type II; Delp & Duan, 1996) it appears that BR supplementation shifts the PmvO_2 profile, at least during the transient, to resemble the characteristic response seen in muscles composed of type I fibers. A recent study by Hirai *et al.* (2012) reported a similar slowing of the PmvO_2 kinetics in rats that completed an 8-week exercise training program compared to sedentary rats. The slower PmvO_2 fall in that report following exercise training was mediated by elevations in NO bioavailability (Hirai *et al.* 2012). In this regard it quite remarkable that a relatively short term (5 days) dose of a non-pharmacological aid mimics the effects of 8-weeks of exercise training with regards to effects on the microvascular oxygenation profile during muscle contractions.

Given our recent report that BR supplementation raises exercising muscle steady-state QO_2 substantially (Ferguson *et al.* 2013), the higher QO_2/VO_2 ratio seen herein may be due to a faster rate of QO_2 increase following the onset of contractions. In addition, BR supplementation may reduce VO_2 due to improvements in mitochondrial and/or muscle contractile efficiency (Larsen *et al.* 2007; Larsen *et al.* 2012; Bailey *et al.* 2009; Bailey *et al.* 2010; Vanhatalo *et al.* 2010; Hernandez *et al.* 2012). These mechanisms may contribute to the slower PmvO_2 kinetics found in the present investigation and could explain, at least in part, the reduced PCr breakdown and improved exercise tolerance following BR supplementation shown by Jones and colleagues (Bailey *et al.* 2010; Lansley *et al.* 2011; Vanhatalo *et al.* 2011). Moreover, elevations in O_2 pressures within the microvasculature reduce PCr breakdown (Haseler *et al.* 1998, Vanhatalo *et al.* 2011) and speed PCr recovery in hypoxic conditions (Haseler *et al.* 1999), which may also help explain the improvements in exercise tolerance seen in peripheral artery disease patients following acute BR supplementation (Kenjale *et al.* 2011). In this regard, BR supplementation may have vital implications for other diseases hallmarked by reduced QO_2 and elevated metabolic perturbation during exercise (e.g., chronic heart failure, reviewed by Poole *et al.* 2012) and could emerge as a non-pharmacological therapeutic modality used to increase adherence to, and efficacy of, cardiac rehabilitation programs in which exercise is a primary component.

In the present investigation we hypothesized initially that BR supplementation would elevate $PmvO_2$ not only across the rest-contraction transient but also during the contracting steady-state. However, no such steady-state $PmvO_2$ elevation was evident. This may be due, in part, to the apparent fiber-type specific effects of NO_3^- on skeletal muscle vascular control (Ferguson *et al.* 2013) and contractile function (Hernandez *et al.* 2012). Specifically, it has been demonstrated that BR supplementation elevates blood flow explicitly to exercising muscles and muscle parts composed predominantly of fast-twitch type IIB+d/x fibers (Ferguson *et al.* 2013). Moreover, a fiber-type selective effect also exists whereby NO_3^- supplementation augments Ca^{2+} handling and rate of force development in isolated mouse fast-twitch but not slow-twitch muscle (Hernandez *et al.* 2012). Considering that the spinotrapezius muscle utilized presently is composed of approximately 52% type IIB+d/x fibers (Delp & Duan, 1996) and that the elevations in blood flow demonstrated by Ferguson *et al.* (2013) were present only in muscles and muscle parts comprised of 66% type IIB+d/x muscle fibers it is possible that the spinotrapezius lacks the fiber-type composition necessary to illustrate the effects of BR supplementation on contracting steady-state $PmvO_2$. However, it is important not to understate the improved $PmvO_2$ kinetics seen herein given that humans and animals are rarely at a metabolic steady-state, but instead transition frequently among differing metabolic rates (reviewed by Jones *et al.* 2011; Poole & Jones, 2012). Therefore, the slowed $PmvO_2$ kinetics elicited by BR may underlie mechanistically the faster pulmonary $\dot{V}O_2$ kinetics evident following BR consumption in certain circumstances (i.e. advanced age, Kelly *et al.* 2012).

4.2 Experimental considerations and future directions

The current experimental model differs from voluntary dynamic exercise in several respects, the most pertinent of these relates to the different skeletal muscle recruitment patterns evoked by electrical stimulation versus voluntary muscle contraction (Gollnick *et al.* 1974). This is particularly important when considering the fiber-type disparities discussed above. Namely, by using electrical stimulation it is presumed that all motor units are activated within a given stimulation field thereby contracting muscle fibers across the spectrum of both slow- and fast-twitch types. Therefore, the current experimental preparation maximized the opportunity to reveal any potential fiber-type specific effects of BR supplementation within the mixed fiber-type spinotrapezius muscle. In this regard, investigation into the effects of BR supplementation on the $PmvO_2$ profile in a muscle composed of primarily type IIB+d/x fibers are warranted and may unveil additional and/or greater effects both during the contracting steady state and across the rest-contraction transient. Moreover, blood flow measurements would allow calculation of muscle oxygen consumption using the Fick relationship (McDonough *et al.* 2001) thereby providing further insights into the potential metabolic effects of NO_3^- supplementation.

4.3 Conclusions

BR supplementation for 5 days slowed substantially (~45%) the $PmvO_2$ fall across the crucial rest-contraction transient with BR rats eliciting a longer time delay, MRT and blunted rate of $PmvO_2$ fall. However, in this mixed fiber-type muscle, steady-state $PmvO_2$ was not elevated significantly. This may be due to the fiber-type composition of the spinotrapezius muscle in that an effect may potentially be evident in muscles comprised of a greater proportion of fast-twitch fibers. The slowed kinetics seen following BR supplementation reflects an improved ability to increase O_2 delivery relative to metabolic demand (i.e. raise $QO_2/\dot{V}O_2$ ratio) following the onset of muscle contractions. Muscle $QO_2/\dot{V}O_2$ matching is compromised in multiple disease states (e.g. chronic heart failure, PAD) exacerbating metabolic perturbations and sowing the seeds for exercise intolerance. Consequently, these results provide compelling evidence that BR supplementation provides advantageous effects on skeletal muscle vascular and metabolic function during exercise.

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References

- Andrade FH, Reid MB, Allen DG, Westerblad H. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J. Physiol. (Lond.)*. 1998; 509:577–586. [PubMed: 9575305]
- Bailey JK, Kindig CA, Behnke BJ, Musch TI, Schmid Schoenbein GW, Poole DC. Spinotrapezius muscle microcirculatory function: effects of surgical exteriorization. *Am J Physiol Heart Circ Physiol*. 2000; 279:H3131–H3137. [PubMed: 11087272]
- Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O₂ cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J. Appl. Physiol*. 2009; 107:1144. [PubMed: 19661447]
- Bailey SJ, Fulford J, Vanhatalo A, Winyard PJ, 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*. 2010; 109:135–148. [PubMed: 20466802]
- Behnke BJ, McDonough P, Padilla DJ, Musch TI, Poole DC. Oxygen exchange profile in rat muscles of contrasting fibre types. *J. Physiol. (Lond.)*. 2003; 549:597–605. [PubMed: 12692174]
- Carlström M, Persson AEG, Larsson E, Hezel M, Scheffer P, Teerlink T, Weitzberg E, Lundberg J. Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. *Cardiovasc. Res*. 2011; 89:574–585. [PubMed: 21097806]
- Delp MD, Duan C. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J. Appl. Physiol*. 1996; 80:261. [PubMed: 8847313]
- Federspiel WJ, Popel AS. A theoretical analysis of the effect of the particulate nature of blood on oxygen release in capillaries. *Microvasc. Res*. 1986; 32:164–189. [PubMed: 3762425]
- Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, Poole DC. Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *J. Physiol. (Lond.)*. 2013; 591:547–557. [PubMed: 23070702]
- Ferreira LF, McDonough P, Behnke BJ, Musch TI, Poole DC. Blood flow and O₂ extraction as a function of O₂ uptake in muscles composed of different fiber types. *Res Phys Neuro Biol*. 2006; 153:237–249.
- Gollnick PD, Piehl K, Saltin B. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J. Physiol. (Lond.)*. 1974; 241:45–57. [PubMed: 4278539]
- Groebe K, Thews G. Calculated intra- and extracellular PO₂ gradients in heavily working red muscle. *Am. J. Physiol*. 1990; 259:H84–H92. [PubMed: 2375415]
- Haseler LJ, Hogan MC, Richardson RS. Skeletal muscle phosphocreatine recovery in exercise-trained humans is dependent on O₂ availability. *J. Appl. Physiol*. 1999; 86:2013–2018. [PubMed: 10368368]
- Haseler LJ, Richardson RS, Videen JS, Hogan MC. Phosphocreatine hydrolysis during submaximal exercise: the effect of F_IO₂. *J. Appl. Physiol*. 1998; 85:1457–1463. [PubMed: 9760341]
- Haseler L, Kindig C, Richardson R, Hogan M. The role of oxygen in determining phosphocreatine onset kinetics in exercising humans. *J. Physiol. (Lond.)*. 2004; 558:985–992. [PubMed: 15169844]
- Hernández A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E, Westerblad H. Dietary nitrate increases tetanic [Ca²⁺] and contractile force in mouse fast-twitch muscle. *J. Physiol. (Lond.)*. 2012; 590:3575–3583. [PubMed: 22687611]
- Hirai DM, Copp SW, Ferguson SK, Holdsworth CT, McCullough D, Behnke BJ, Musch TI, Poole DC. Exercise training and muscle microvascular oxygenation: functional role of nitric oxide. *J. Appl. Physiol*. 2012; 113:557–565. [PubMed: 22678970]

- Hogan MC, Arthur PG, Bebout DE, Hochachka PW, Wagner PD. Role of O₂ in regulating tissue respiration in dog muscle working in situ. *J. Appl. Physiol.* 1992; 73:728–736. [PubMed: 1400003]
- Jones AM, Grassi B, Christensen PM, Krstrup P, Bangsbo J, Poole DC. Slow component of VO₂ kinetics: mechanistic bases and practical applications. *Med. Sci. Sports Exerc.* 2011; 43:2046–2062. [PubMed: 21552162]
- Jones AM, Krstrup P, Wilkerson DP, Berger NJ, Calbet JA, Bangsbo J. Influence of exercise intensity on skeletal muscle blood flow, O₂ extraction and O₂ uptake on-kinetics. *J. Physiol. (Lond.)*. 2012; 590:4363–4376. [PubMed: 22711961]
- Joyner MJ, Tschakovsky ME. Nitric oxide and physiologic vasodilation in human limbs: where do we go from here? *Can J Appl Physiol.* 2003; 28:475–490. [PubMed: 12955873]
- Joyner MJ, Wilkins BW. Exercise hyperaemia: is anything obligatory but the hyperaemia? *J. Physiol. (Lond.)*. 2007; 583:855–860.
- Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, Jones AM. Effects of short-term dietary nitrate supplementation on blood pressure, O₂ uptake kinetics, and muscle and cognitive function in older adults. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2012 DOI: 10.1152/ajpregu.00406.2012.
- Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson J, Vanbruggen M, Privette G, Yim E, Kraus WE, Allen JD. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *J. Appl. Physiol.* 2011; 110:1582–1591. [PubMed: 21454745]
- Kindig CA, Howlett RA, Hogan MC. Effect of extracellular PO₂ on the fall in intracellular PO₂ in contracting single myocytes. *J. Appl. Physiol.* 2003; 94:1964–1970. [PubMed: 12533498]
- Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, Gilchrist M, Benjamin N, Jones AM. Acute dietary nitrate supplementation improves cycling time trial performance. *Med. Sci. Sports Exerc.* 2011; 43:1125–1131. [PubMed: 21471821]
- Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, Dimenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O₂ cost of walking and running: a placebo-controlled study. *J. Appl. Physiol.* 2011; 110:591–600. [PubMed: 21071588]
- Larsen FJ, Weitzberg E, Lundberg JO, Eklom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiologica.* 2007; 191:59. [PubMed: 17635415]
- Larsen FJ, Schiffer TA, Weitzberg E, Lundberg JO. Regulation of mitochondrial function and energetics by reactive nitrogen oxides. *Free. Rad. Biol. Med.* 2012; 53:1919–1928. [PubMed: 22989554]
- Leek BT, Mudaliar SR, Henry R, Mathieu Costello O, Richardson RS. Effect of acute exercise on citrate synthase activity in untrained and trained human skeletal muscle. *Am. J. Physiol-Reg. I.* 2001; 280:R441.
- Lo LW, Vinogradov SA, Koch CJ, Wilson DF. A new, water soluble, phosphor for oxygen measurements in vivo. *Adv. Exp. Med. Biol.* 1997; 428:651–656. [PubMed: 9500111]
- Masschelein E, Van Thienen R, Wang X, Van Schepdael A, Thomis M, Hespel P. Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. *J. Appl. Physiol.* 2012; 113:736–745. [PubMed: 22773768]
- McDonough P, Behnke BJ, Kindig CA, Poole DC. Rat muscle microvascular PO₂ kinetics during the exercise off-transient. *Exp. Physiol.* 2001; 86:349–356. [PubMed: 11429652]
- McDonough P, Behnke BJ, Padilla DJ, Musch TI, Poole DC. Control of microvascular oxygen pressures in rat muscles comprised of different fibre types. *J. Physiol. (Lond.)*. 2005; 563:903–913. [PubMed: 15637098]
- Musch TI, Bruno A, Bradford GE, Vayonis A, Moore RL. Measurements of metabolic rate in rats: a comparison of techniques. *J. Appl. Physiol.* 1988; 65:964–970. [PubMed: 3139623]
- Poole DC, Behnke BJ, McDonough P, McAllister RM, Wilson DF. Measurement of muscle microvascular oxygen pressures: compartmentalization of phosphorescent probe. *Microcirculation.* 2004; 11:317–326. [PubMed: 15280071]
- Poole DC, Hirai DM, Copp SW, Musch TI. Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. *Am. J. Physiol. Heart-C.* 2012; 302:H1050–H1063.

- Poole DC, Jones AM. Oxygen uptake kinetics. *Compr. Physiol.* 2012; 2:933–996. [PubMed: 23798293]
- Richardson RS, Leigh JS, Wagner PD, Noyszewski EA. Cellular PO₂ as a determinant of maximal mitochondrial O₂ consumption in trained human skeletal muscle. *J. Appl. Physiol.* 1999; 87:325–331. [PubMed: 10409591]
- Rumsey WL, Vanderkooi JM, Wilson DF. Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue. *Science.* 1988; 241:1649–1651. [PubMed: 3420417]
- Tsuchiya K, Tomita S, Ishizawa K, Abe S, Ikeda Y, Kihira Y, Tamaki T. Dietary nitrite ameliorates renal injury in L-NAME-induced hypertensive rats. *Nitric Oxide.* 2010; 22:98–103. [PubMed: 20005970]
- Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey T, Wilkerson DP, Benjamin N, Winyard PG, Jones AM. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am. J. Physiol-Reg. I.* 2010; 299:R1121–R1131.
- Vanhatalo A, Fulford J, Bailey SJ, Blackwell JR, Winyard PJ, Jones AM. Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia. *J. Physiol. (Lond.).* 2011; 589:5517–5528. [PubMed: 21911616]
- Whipp BJ, Ward SA. Cardiopulmonary coupling during exercise. *J. Exp. Biol.* 1982; 100:175–193. [PubMed: 6816892]

Highlights

- Dietary NO_3^- (via beetroot juice, BR) augments exercising muscle blood flow
- We examined the effects of BR on contracting rat muscle microvascular O_2 pressure (PmvO_2) dynamics
- BR slowed the PmvO_2 fall across the crucial rest-contractions transition
- There was no effect of BR on contracting steady-state PmvO_2
- This elevated O_2 driving pressure may improve metabolic control during exercise

Figure 1.

Top panel: Pre- and post-supplementation plasma $[\text{NO}_3^-]$ for control and BR rats. Bottom panel: Post-supplementation plasma $[\text{NO}_2^-]$ for control and BR rats. * $p < 0.05$ versus control.

Figure 2.

Top panel: Representative P_{mvO_2} profiles (black lines) and their model fits (gray lines) for one control and one BR rat. Middle panel: P_{mvO_2} residuals demonstrate excellent model fits. Bottom panel: Absolute P_{mvO_2} difference (BR-control) for responses shown in top panel. Time "0" represents the onset of contractions. Note greatest effect of BR across the initial transient (i.e. 0-60 s).

Table 1
MAP and HR prior to and during the steady-state of electrically-induced muscle contractions for control and BR rats

	Control	BR
<u>Rest (pre-contractions)</u>		
MAP (mmHg)	123±8	110±9
HR (bpm)	360±11	342±22
<u>Contracting steady-state</u>		
MAP (mmHg)	123±8	111±7
HR (bpm)	360±11	349±17

Values are mean±SEM. There were no differences within or between groups ($p>0.05$).

Table 2
Microvascular partial pressure of O₂ (PmvO₂) kinetics parameters during contractions
for control and BR rats

	Control	BR
PmvO₂(BL), mmHg	30.2±2.0	28.6±2.3
Δ₁PmvO₂, mmHg	16.8±1.4	12.0±1.5 *
Δ₂PmvO₂, mmHg	4.1±0.7	5.6±1.9
Δ_{total}PmvO₂, mmHg	13.5±1.4	9.2±1.3 *
P_{mv}O₂(steady-state), mmHg	17.4±1.6	18.5±1.0
TD₁, s	6.9±1.4	11.8±1.8 *
TD₂, s	46.7±10.2	26.0±3.5
τ₁, s	9.9±1.1	12.6±1.9
τ₂, s	88.0±16.2	76.6±12.9
MRT₁, s	16.8±1.9	24.4±2.7 *
T₆₃, s	16.2±1.6	23.8±3.2 *
Δ₁PmvO₂/τ₁, mmHg/s	1.9±0.3	1.2±0.2 *

Values are mean±SEM. Where second component model averages are shown the value reflects only those rats where a two-component model was applied to describe the PmvO₂ data (control: n=8 of 10; BR: n=5 of 10). PmvO₂(BL), pre-contracting PmvO₂; Δ₁PmvO₂, amplitude of the first component; Δ₂PmvO₂, amplitude of the second component; Δ_{total}PmvO₂; overall amplitude regardless of one- or two-component model fit; PmvO₂(steady-state), contracting steady-state PmvO₂; TD₁, time delay for the first component; TD₂, time delay for the second component; τ₁, time constant for the first component; τ₂, time constant for the second component; MRT, mean response time describing the overall kinetics response; T₆₃, time to reach 63% of the overall response determined independent of modeling procedures; Δ₁PmvO₂/τ₁, parameter describing the relative rate of PmvO₂ fall.

*
p<0.05 versus control.