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Dose dependent effects of nitrate supplementation on cardiovascular control and microvascular oxygenation dynamics in healthy rats

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Running title: Dose dependent vascular effects of dietary nitrate supplementation in rats

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Abstract

High dose nitrate (NO_3^-) supplementation via beetroot juice (BR, 1 mmol/kg/day) lowers mean arterial blood pressure (MAP) and improves skeletal muscle blood flow and O_2 delivery/utilization matching thereby raising microvascular O_2 pressure (PO_2mv). We tested the hypothesis that a low dose of NO_3^- supplementation, consistent with a diet containing NO_3^- rich vegetables (BRLD, 0.3 mmol/kg/day), would be sufficient to cause these effects. Male Sprague-Dawley rats were administered a low dose of NO_3^- (0.3 mmol/kg/day; *n*=12), a high dose (1 mmol/kg/day; BRHD, n=6) or tap water (control, *n*=10) for 5 days. MAP, heart rate (HR), blood flow (radiolabeled microspheres) and vascular conductance (VC) were measured during submaximal treadmill exercise (20 m/min, 5% grade, equivalent to ~60% of maximal O_2 uptake). Subsequently, PO_2mv (phosphorescence quenching) was measured at rest and during 180 s of electrically-induced twitch contractions (1 Hz, ~6 volts) of the surgically-exposed spinotrapezius muscle. BRLD and BRHD lowered resting (control: 139±4, BRLD: 124±5, BRHD: 128±9 mmHg, $P<0.05$) and exercising (control: 138±3, BRLD: 126±4, BRHD: 125±5 mmHg, $P<0.05$) MAP to a similar extent. For BRLD this effect occurred in the absence of altered exercising hindlimb muscle(s) blood flow or spinotrapezius PO_2mv (rest and across the transient response at the onset of contractions, all $P>0.05$), each of which increased significantly for the BRHD condition (all $P<0.05$). Whereas BRHD slowed the PO_2mv kinetics significantly (i.e., >mean response time, MRT; control: 16.6±2.1, BRHD: 23.3±4.7 s) following the onset of contractions compared to control, in the BRLD group this effect did not reach statistical significance (BRLD: 20.9±1.9 s, $P=0.14$). These data demonstrate that while low dose NO_3^- supplementation lowers MAP it does so in the absence of augmented muscle blood flow, VC and PO_2mv; all of which are elevated at a higher dose. Thus, in healthy animals, a high dose of NO_3^- supplementation seems necessary to elicit significant changes in exercising skeletal muscle O_2 delivery/utilization.

Key words: nitric oxide; exercise; dietary nitrate; nitrite; mean arterial pressure; blood flow

1. Introduction

A fundamental tenet of exercise physiology is that blood flow (BF) increases following exercise onset to meet the rising skeletal muscle energetic demands. This hyperemic response is mediated by a host of vasodilatory controllers (Joyner and Wilkins, 2007) and it is now widely accepted that nitric oxide (NO) plays a deterministic role in regulating not only O_2 delivery (QO_2) (Hirai *et al.* 1994; reviewed by Joyner and Tschakovsky, 2004), but also O_2 utilization ($\dot{V}O_2$) within the skeletal muscle (Andrade *et al.* 1998; Larsen *et al* 2012). A growing body of evidence suggests that ingestion of inorganic nitrate (NO_3^-), for example via beetroot juice (BR), can, following a step-wise reduction, elevate NO bioavailability and thus impact skeletal muscle hemodynamic and metabolic function during exercise (Larsen *et al.* 2007; Bailey *et al.* 2009; Vanhatalo *et al.* 2010; Kenjale *et al.* 2011; Lansley *et al.* 2011a,b; Ferguson *et al.* 2013a,b).

In humans, NO_3^- supplementation via BR reduces blood pressure and enhances exercise tolerance in both healthy (Bailey *et al.* 2009; Vanhatalo *et al.* 2010; Lansley *et al.* 2011a,b; Cermak *et al.* 2012; Wylie *et al.* 2013b) and patient populations (i.e., peripheral arterial disease, Kenjale *et al.* 2011). These effects appear to have a dose-dependent response with no additional improvement in exercise tolerance after ingesting BR containing 16.8 compared to 8.4 mmol NO_3^- (Wylie *et al*. 2013a). The precise mechanisms for these improvements are not yet fully understood. However, recent investigations using murine models implicate enhanced exercising muscle BF (i.e., $\uparrow QO_2$, Ferguson *et al.* 2013a) and $QO_2/\dot{V}O_2$ matching (e.g. microvascular PO_2; PO_2mv, Ferguson *et al.* 2013b) combined with greater contractile efficiency (e.g. $\downarrow \dot{V}O_2$; Hernandez *et al.* 2012).

Many disease states impair exercise tolerance and its restoration is a primary therapeutic goal. What is not known is whether lower doses of NO_3^- alter cardiovascular control and muscle oxygenation (i.e. P*mv*O_2, which sets the pressure head for capillary-myocyte O_2 flux) during exercise. Specifically, one question of paramount ecological importance is whether NO_3^- dosing consistent with an individual eating a diet rich in leafy greens and other NO_3^- sources can achieve the cardiovascular and muscular benefits without the necessity for supplementation *per se*. Thus, we tested the hypotheses that a low dose of NO_3^- supplementation (i.e. consistent with a diet containing NO_3^- rich vegetables, 0.3 mmol/kg/day) would be sufficient to 1) raise plasma [NO_3^-] and [NO_2^-], 2) lower mean arterial pressure (MAP) at rest and during exercise, 3) elevate BF and vascular conductance (VC) in locomotory muscles of the hindlimb and 4) raise the PO_2*mv* of the mixed fiber-type spinotrapezius muscle during the crucial rest-contractions transient.

Methods

Animal selection and care

Thirty-one young adult male Sprague-Dawley rats (~3-4 months of age, Charles River Laboratories, Wilmington, MA,USA) 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 Institutes of Health guidelines. All rats were familiarized with running on a custom-built motor-driven treadmill for 5 min/day at a speed of 20 m/min up a 5% grade for ~5 days.

Supplementation protocol

Rats were randomly assigned to receive 5 days of BR supplementation with either a low NO_3^- dose of 0.3 mmol/kg/day (BRLD; n=14), a higher NO_3^- dose of 1 mmol/kg/day (BRHD; n=6, Beet it™, James White Drinks, Ipswich UK) or untreated tap water (control; n=11) with consumption monitored. For both BRLD and BRHD rats, two days' worth of BR was diluted in 100 ml of tap water (average daily fluid consumption ~50-60 ml/day). This lower NO_3^- dose (0.3 mmol/kg/day) represents a dose found in a diet containing NO_3^- rich vegetables, while the higher NO_3^- dose (1 mmol/kg/day) represents a dietary supplement with a NO_3^- concentration similar to that used by Jones and colleagues (Bailey *et al.* 2009; Vanhatalo *et al.* 2010; Lansley *et al.* 2011a,b) after accounting for the resting metabolic rate of rats (~7x that of humans, Henson *et al.* 1987; Musch *et al.* 1988). In an effort to minimize the unnecessary utilization of additional animals, both control and BRHD data presented herein represent a randomly selected subset of animals published recently. The BRHD data represent a NO_3^- dose of 1 mmol/kg/day and

demonstrate a significant vascular effect of supplementation (Ferguson *et al.* 2013ab). Data from the BRLD group were obtained within the same time-frame as control and BRHD groups presented in Ferguson *et al.* (2013ab, e.g. within 16 weeks). In this way any potential seasonal differences or variations in rat-chow content were avoided.

Surgical instrumentation

Rats were anaesthetized with a 5% isoflurane-O_2 mixture and maintained subsequently on 3% isoflurane-O_2. The carotid artery was isolated and cannulated with a catheter (PE-10 connected to PE-50, Intra-Medic polyethylene tubing, Clay Adams Brand, Becton, Dickinson and Company, Sparks, MD) for the 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 given >1 hr to recover (Flaim *et al.* 1984).

Protocol 1: Measurement of hindlimb skeletal muscle blood flow

After recovery, rats were placed on the treadmill and the caudal artery catheter was connected to a 1 ml syringe chambered in a Harvard infusion/withdrawal pump (model 907, Cambridge, MA, USA). The carotid artery catheter was connected to a pressure transducer (Gould Statham P23ID, Valley View, OH, USA) maintained at the same height as the animal. Exercise was initiated and treadmill speed was increased progressively over a ~30 s period to a speed of 20 m/min (5% grade, ~60% $\dot{V}O_2$ O_2 max; Musch *et al.* 1988). The rat continued to exercise for another 2.5 min until a total time of 3 min was reached. At 3 min the Harvard pump was activated and withdrawal was initiated at a rate of 0.25 ml $\cdot$ min^{-1}. Simultaneously, HR and MAP were measured and recorded using the carotid artery catheter. The carotid artery catheter

was then disconnected from the pressure transducer and 0.5-0.6 × 10^6 15 μm diameter radiolabeled microspheres (^{57}Co or ^{85}Sr in random order; Perkin Elmer, Waltham, MA, USA) were infused into the aortic arch for determination of regional BF. Following the microsphere infusion ~0.2 ml of blood was sampled from the carotid artery catheter for the determination of blood lactate concentration ([lactate]) (Nova Stat Profile M, Nova Biomedical, Waltham, MA, USA) after which exercise was terminated.

Following a minimum 1 hr recovery period, a second microsphere infusion (differently radio-labeled than the first) was performed while the rat sat quietly on the treadmill for the determination of resting BF, HR and MAP. This experimental strategy (i.e. exercise before rest) mitigates potential influences of the pre-exercise anticipatory response on resting skeletal muscle BF measurements (Armstrong *et al.* 1989).

Protocol 2: Measurement of spinotrapezius muscle PO_2mv

Following the second (resting) microsphere infusion, rats were anesthetized progressively using diluted pentobarbital sodium anesthesia (administered into the caudal artery catheter to effect) with the level of anesthesia monitored continuously via the toe-pinch and blink reflexes. 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 that 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).

The phosphorescent probe palladium meso-tetra (4 carboxyphenyl)porphyrin dendrimer (R2: 15–20 $mg \cdot kg^{-1}$ 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 phosphorometer (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. PO_2mv 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).

PO_2mv measurement and curve-fitting

The Stern-Volmer relationship allows the calculation of PO_2mv through the direct measurement of a phosphorescence lifetime via the following equation (Rumsey *et al.*, 1988):

$$PO_{2mv} = [(\tau^\circ / \tau)-1]/(k_Q \times \tau^\circ)$$

where k_Q is the quenching constant and $\tau°$ 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 $mmHg^{-1} \cdot s^{-1}$ and $\tau°$ 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 PO_2mv measurement (Poole *et al.*, 2004).

Curve-fitting of the measured PO_2mv 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:

One component: $PO_2mv_{(t)} = PO_2mv_{(BL)} - \Delta\ PO_2mv\ (1 - e^{-(t - TD)/\tau})$

Two component: $PO_2mv_{(t)} = PO_2mv_{(BL)} - \Delta_1\ PO_2mv\ (1 - e^{-(t - TD_1)/\tau_1}) + \Delta_2\ PO_2mv\ (1 - e^{-(t - TD_2)/\tau_2})$

where $PO_2mv_{(t)}$ represents the PO_2mv at any given time t, $PO_2mv_{(BL)}$ corresponds to the pre-contracting resting baseline PO_2mv, Δ_1 and Δ_2 are the amplitudes for the first and second components, 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 mean response time (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 PO_2mv fall following contractions onset was normalized to τ_1 ($\Delta_1 PO_2mv / \tau_1$) to provide an index of the relative rate of fall. Additionally, the time taken to reach 63% of the initial PO_2mv fall was determined independently from the modeling procedures (T_{63}) to ensure appropriateness of the model fits. Specifically, the raw PO_2mv data were interpolated, and the time coinciding with 63% of the total amplitude (Δtotal PO_2mv) was determined.

Determination of BF and VC

Following euthanasia, the thorax of each rat was opened and placement of the carotid artery catheter was confirmed before the internal organs and individual muscles and muscle parts of the hindlimb were identified and excised. Upon removal, tissues were weighed and placed promptly into counting vials. Radioactivity of each tissue was determined with a gamma scintillation counter (Packard Auto Gamma Spectrometer, model 5230, Downers Grove, IL, USA). Tissue BF was then calculated using the reference sample method (Musch & Terrell, 1992) and expressed as ml/min/100 g. Adequate mixing of the microspheres was verified for each microsphere infusion as demonstrated by a <15% difference in BF to the right and left

kidneys and to the right and left hindlimb musculature. VC was calculated by normalizing BF to MAP and expressed as ml/min/100 g · ·mmHg^{-1}.

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

Post-supplementation blood samples were collected following surgical instrumentation via the caudal artery catheter to assess 1) plasma [NO_3^-] and [NO_2^-] and 2) pH, PO_2, and $\%O_2$ saturation. For plasma [NO_3^-] and [NO_2^-], ~0.8 ml of blood was drawn into heparinized tubes and rapidly centrifuged at 6000 g at 4°C for 6 minutes. Plasma was then extracted and frozen immediately at -80°C for later analysis. 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]. Consequently, the signals obtained using potassium iodide were subtracted from those with vanadium chloride to provide a clearer representation of the NO_3^- concentrations.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Results were compared within (rest vs. exercise) and between (control vs. BRLD vs. BRHD) using mixed 2-way analysis of variance (ANOVA) with Student-Newman-Keuls *post hoc* tests where appropriate. Significance was accepted at $P<0.05$.

Results

There were no between-group differences in the total hind-limb muscle/body mass ratio (control: 4.4 ± 0.1, BRLD: 4.3 ± 0.1, BRHD: 4.2 ± 0.2%, $P>0.05$). BRHD, but not BRLD, rats had significantly higher plasma [NO_3^-] and [NO_2^-] when compared to control (Figure 1).

Protocol 1: BF and VC at rest and during exercise

MAP and HR. MAP at rest was reduced in BRLD rats compared to control. However, despite >10 mmHg lower average, resting MAP was not significantly different in BRHD rats when compared to control ($P=0.20$, Figure 2). During treadmill exercise MAP was reduced for both BRLD and BRHD groups when compared to control (Figure 2). Neither HR at rest (control: 408 ± 8, BRLD: 408 ± 17, BRHD: 407 ± 18 beats/min, $P>0.05$) or during exercise (control: 528 ± 10, BRLD: 508 ± 14, BRHD: 525 ± 7 beats/min, $P>0.05$) was altered for BRLD or BRHD.

Blood gases, blood [lactate], hematocrit. There were no between-group differences in arterial PO_2, PCO_2, or hematocrit at rest or during exercise (data not shown, $P>0.05$ for all). Resting and exercising arterial blood [lactate] were not different among groups (Table 1, $P>0.05$ for all) but tended ($P=0.12$) to be lower for BRHD during exercise.

BF and VC. There were no differences in resting total hindlimb skeletal muscle BF (control: 16 ± 2, BRLD: 19 ± 2, BRHD: 21 ± 5 ml/min/100g, $P>0.05$) or VC (control: 0.12 ± 0.01, BRLD: 0.15 ± 0.02, BRHD: 0.16 ± 0.03 ml/min/100g/mmHg, $P>0.05$) between groups.

Similarly, there were no differences in resting BF or VC in any of the 28 individual hindlimb muscles or muscle parts (Table 1).

Total exercising hindlimb muscle BF and VC were greater in BRHD rats when compared to BRLD and control rats (Figure 3). Specifically, BRHD supplemented rats had greater BF in 15, and VC in 20, of the 28 individual hindlimb muscles and muscle parts (Table 2) when compared to control and BRLD rats. There were no between-group differences in BF or VC at rest or during exercise to organs of the splanchnic region (Table 3).

Protocol 2: PO_2mv parameters

Representative raw PO_2mv profiles of control, BRLD and BRHD rats are presented in Figure 4. The responses were fit by a one-component model in 2 of 11 control, 6 of 12 BRLD and 2 of 5 BRHD rats while the more complex two-component model was utilized for the remainder. The r^2 (control: 0.99 ± 0.01, BRLD: 0.98 ± 0.01, BRHD: 0.98 ± 0.01) and low sum of squared residuals (control: 20.2 ± 3.1, BRLD: 18.3 ± 2.7, BRHD: 21.1 ± 7 mmHg) for both groups supported that the model fits were suitable. Table 4 presents the average PO_2mv baselines and kinetics parameters. There were no differences in the $PO_2mv_{(BL)}$ between groups. However, following the onset of contractions BRHD, but not BRLD, demonstrated a longer TD_1, smaller first-component amplitude and slower PO_2mv kinetics (i.e., longer MRT_1). Overall (i.e., $PO_2mv_{(BL)}$ minus $PO_2mv_{(steady\text{-}state)}$, $\Delta_{total}PO_2mv$) amplitudes tended to be less in BRHD (P=0.06) but failed to reach statistical significance. There were no differences in $PO_2mv_{(steady\text{-}state)}$ during contractions between groups.

4. Discussion

The principal original finding of this investigation is that low dose NO_3^- supplementation via beetroot juice (0.3 mmol/kg/day, BRLD), at levels consistent with a diet rich in leafy green vegetables consumed by humans, lowers resting and exercising MAP. Moreover, this occurred in the absence of increased exercising skeletal muscle BF and VC which are both elevated after higher supplementation doses (1 mmol/kg/day, BRHD). That BRLD impacts cardiovascular control at levels that do not detectably increase circulating NO_3^- or NO_2^- suggests that central mechanisms of cardiovascular control, possibly within the rostral ventrolateral medulla (RVLM) region of the brain, are extremely sensitive to altered dietary NO_3^- intake. Given that, in humans, reductions in systolic blood pressure of ~10 mmHg (as seen with BRLD herein) have been estimated to decrease the risk of stroke by ~35% and ischemic heart disease by ~25% (MacMahon *et al.* 1990; Lewington *et al.* 2002; Law *et al.* 2003; Lawes *et al.* 2003; Larsen *et al.* 2007,2010), these findings have strong potential clinical significance. It is also pertinent that employing low dose inorganic NO_3^- supplementation may avoid or reduce the opportunity for development of NO_3^- tolerance and endothelial dysfunction (Vanhatalo *et al.* 2010; Omar *et al.* 2012) which occur especially after chronic administration of organic NO_3^- (see Wylie *et al.* 2013a for discussion).

Relationship to existing literature

The randomly selected subset of data from results initially reported from our laboratory (Ferguson *et al.* 2013a) demonstrate clearly the effects of BRHD as regards elevated skeletal muscle(s) BF and VC and reduced MAP during treadmill running. Also, as demonstrated in Figure 4, PO_2mv kinetics were slowed significantly and PO_2mv decreased less during contractions of the spinotrapezius muscle, effectively raising the microvascular O_2 driving

pressure especially across the transition period when mitochondrial O_2 uptake would be rising most rapidly (Ferguson *et al.* 2013b). These effects can be explained by the greater O_2 delivery combined with a reduced mitochondrial O_2 utilization at a given workload as demonstrated in intact humans (Larsen *et al.* 2007; Bailey *et al.* 2009; Larsen *et al.* 2010; Wylie *et al.* 2013a) as well as in isolated mitochondria (Larsen *et al.* 2012). With respect to the BRLD and based upon the dose-response relationship reported by Wylie *et al.* (2013a), where a graded fall in MAP was observed from 4.2 to 8.4 mmol NO_3^- (supplemented via BR with no additional decrease at 16.8 mmol), we were surprised that, in rats, MAP fell to the same extent (i.e., ~10 mmHg) with both doses. Paramount to setting the NO_3^- supplement doses herein was the consideration of the faster metabolic rate found in the resting rat (~27 ml/kg/min, Musch *et al.* 1988) when compared to humans (3.5ml/kg/min, i.e. ~7-8 fold higher per unit mass, Henson *et al.* 1987). Taking this into account, the doses employed (i.e., BRLD, 0.3; BRHD, 1 mmol/kg/day) correspond broadly to 3.2 and 7.4 mmol/day, respectively in humans. Thus, with respect to species differences, the NO_3^- derived hypotensive response becomes saturated at lower doses in rats than in their human counterparts. This effect is very different from the skeletal muscle vascular response and that of PO_2mv which are manifested only at the higher dose.

Mechanisms for reduction of mean arterial pressure

The increased VC observed in the skeletal muscle(s) of BRHD rats explain, at least in part, the reduction in MAP found at the higher dose. However, in marked contrast, skeletal muscle VC and BF were unchanged for BRLD (which was not unexpected given that circulating NO_3^-/NO_2^- levels were not different from control values) and thus, the reduced MAP for these animals must have a different explanation. There is evidence that NO bioavailability (from both endothelial and neuronal nitric oxide synthase, eNOS and nNOS) in higher cardiovascular neural

control centers, for example the RVLM, impacts sympathetic outflow and controls, or at least influences, MAP regulation and can lead to a reduction and/or redistribution of cardiac output (e.g. Mayorov, 2005; Gao *et al.* 2008). Although not measured in this investigation, it is possible that BRLD may have altered NO bioavailability in the RVLM. Indeed, elevated NO bioavailability via overexpression of eNOS in the RVLM induces significant hypotension by elevating gamma-aminobutyric acid (GABA) production (Kishi *et al.* 2001). Thus, one putative mechanism for the hypotensive response invoked by BRLD relates to increased NO bioavailability in the RVLM from non-nNOS/eNOS dependent sources which may ultimately reduce sympathetic outflow. A greater NO_3^- dose may intensify this effect which could be responsible, at least in part, for the elevated renal BF and VC seen in BRHD rats herein. Furthermore, the presence of antioxidants in the BR supplement may contribute to this effect (Gao *et al.* 2007) and thus play a synergistic role with NO_3^-. This novel aspect of BR and NO_3^- mediated cardiovascular control carries clinical implications for those suffering from renal vascular diseases (Lundberg *et al.* 2008; Carlstöm *et al.* 2011) and warrants future investigation.

Alternative mechanisms of BR induced reductions in MAP include impacts on other vascular beds, apart from the skeletal muscle vasculature. For example, BRHD rats demonstrated 26% greater renal BF at rest (Table 3) suggesting that the renal circulation may be prone to NO_2^- induced vascular effects. Given that resting renal BF was not significantly elevated in BRLD rats, this particular effect may require greater local concentrations of NO_2^- to elicit significant vascular effects within the renal circulation. While not statistically significant, a mild impact of the renal circulation on the resting MAP of BRLD rats is certainly plausible and should be investigated further. What's more, the lower PO_2 environment of the venous circulation may enhance the reduction of circulating NO_2^- to NO and other reactive nitrogen species, which

would likely cause venodilation and a reduction in MAP. This effect would likely be exacerbated during exercise due to the robust reductions in venous blood PO_2 (to values <30 mmHg, Gonzalez *et al.* 1994) offering a partial explanation for the lower exercising MAP values presented in figure 2.

Experimental considerations

The rat is a widely used and valuable experimental model to study cardiovascular and muscle metabolic control particularly during exercise. Moreover, dietary, pharmaceutical and exercise conditions can be well controlled and standardized within this population. However, determination of "equivalent" NO_3^- doses is challenging and basing such on resting metabolic rate differences found between rats and humans is only a first approximation. Importantly, in the BRHD group plasma [NO_3^-] and [NO_2^-] were raised to levels approximating those seen in humans following consumption of a dietary supplement high in NO_3^- (Lundberg & Govoni, 2004; Kenjale *et al.* 2011) providing additional confidence in the doses used herein. What is particularly exciting with respect to the present results is that BRLD represented a dose that evinced clear cardiovascular responses in the absence of a significant increase in plasma NO_3^- or NO_2^- and the vascular and metabolic consequences of such. This observation unveils a novel, potentially exciting, and heretofore unappreciated consequence of BR-derived NO_3^- supplementation.

Conclusions

This investigation was the first to examine the effects of a low (0.3 mmol/kg/day) and high (1 mmol/kg/day) dose of NO_3^- supplementation on vascular responses at rest and during submaximal locomotory exercise in the rat. Despite the absence of elevations of plasma [NO_3^-]

or [NO_2^-], BRLD evoked reductions in MAP at rest and during exercise. These cardiovascular improvements were further amplified in BRHD rats which demonstrated augmented skeletal muscle BF and VC, which likely resulted in the slowed PO_2*mv* fall during the crucial rest-contraction transition presented herein. These results carry tremendous clinical implications particularly for those at risk for stroke, renal, and ischemic heart diseases (MacMahon *et al.* 1990; Lewington *et al.* 2002; Law *et al.* 2003; Lawes *et al.* 2003; Larsen *et al.* 2007, 2010; Tsuchiya *et al.* 2010; Carlström *et al.* 2011). Given the sensitivity of the RVLM to NO bioavailability (Mayorov, 2005; Gao *et al.* 2008), it seems logical to postulate an interaction between NO_3^-, NO_2^- and central cardiovascular control centers located in the brain (i.e. RVLM). Understanding these interactions will further provide a mechanistic linkage between elevated NO_3^- ingestion and the improved cardiovascular and metabolic function seen in humans (Bailey *et al.* 2009, 2010; Larsen *et al.* 2010; Kenjale *et al.* 2011; Vanhatalo *et al.* 2011; Masschelein *et al.*2012; Wylie *et al.* 2013b) and animals (Ferguson *et al.* 2013a,b; Hernandez *et al.* 2013) and thus should be the focus of future investigations.

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Table 1. Effects of low dose and high dose BR supplementation on resting hindlimb muscle BF (ml/min/100g) and VC (ml/min/100g/mmHg).

	BF			VC		
	Control	**BRLD**	**BRHD**	**Control**	**BRLD**	**BRHD**
Ankle extensors						
Soleus (9%)	87 ± 16	119 ± 17	107 ± 33	0.62 ± 0.11	0.96 ± 0.12	0.79 ± 0.24
Plantaris (80%)	15 ± 2	16 ± 2	9 ± 2	0.11 ± 0.01	0.13 ± 0.01	0.07 ± 0.02
Gastrocnemius, red (14%)	43 ± 7	56 ± 9	58 ± 19	3.07 ± 0.05	0.44 ± 0.07	0.43 ± 0.13
Gastrocnemius, white (100%)	14 ± 2	12 ± 2	10 ± 3	0.10 ± 0.05	0.10 ± 0.02	0.08 ± 0.02
Gastrocnemius, mixed (91%)	14 ± 2	16 ± 2	16 ± 4	0.10 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
Tibialis posterior (73%)	17 ± 2	18 ± 3	16 ± 5	0.12 ± 0.01	0.14 ± 0.02	0.12 ± 0.03
Flexor digitorum longus (68%)	19 ± 2	24 ± 2	10 ± 2	0.15 ± 0.02	0.19 ± 0.02	0.08 ± 0.01
Flexor halicus longus (71%)	13 ± 2	12 ± 2	9 ± 2	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01
Ankle flexors						
Tibialis anterior, red (63%)	19 ± 3	27 ± 7	21 ± 10	0.14 ± 0.02	0.21 ± 0.06	0.15 ± 0.07
Tibialis anterior, white (80%)	20 ± 2	18 ± 3	16 ± 4	0.14 ± 0.02	0.15 ± 0.02	0.12 ± 0.03
Extensor digitorum longus (76%)	16 ± 2	15 ± 2	15 ± 4	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.03
Peroneals (67%)	17 ± 3	15 ± 2	19 ± 4	0.12 ± 0.02	0.12 ± 0.02	0.14 ± 0.03
Knee extensors						
Vastus intermedius (4%)	46 ± 9	84 ± 18	93 ± 48	0.32 ± 0.06	0.66 ± 0.13	0.69 ± 0.35
Vastus medialis (82%)	15 ± 2	17 ± 3	25 ± 9	0.10 ± 0.01	0.13 ± 0.02	0.19 ± 0.06
Vastus lateralis, red (35%)	40 ± 6	66 ± 16	83 ± 31	0.28 ± 0.04	0.19 ± 0.04	0.61 ± 0.22
Vastus lateralis, white (100%)	16 ± 2	14 ± 2	13 ± 3	0.11 ± 0.01	0.11 ± 0.02	0.10 ± 0.02
Vastus lateralis, mixed (89%)	16 ± 1	21 ± 4	29 ± 9	0.12 ± 0.01	0.17 ± 0.04	0.21 ± 0.06
Rectus femoris, red (66%)	23 ± 4	24 ± 4	29 ± 14	0.16 ± 0.03	0.19 ± 0.04	0.20 ± 0.10
Rectus femoris, white (100%)	15 ± 2	16 ± 3	17 ± 5	0.11 ± 0.01	0.13 ± 0.02	0.12 ± 0.03
Knee flexors						
Biceps femoris anterior (100%)	11 ± 1	9 ± 1	10 ± 2	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
Biceps femoris posterior (92%)	11 ± 2	12 ± 2	15 ± 3	0.08 ± 0.01	0.10 ± 0.01	0.11 ± 0.02
Semitendinosus (83%)	12 ± 2	14 ± 2	18 ± 5	0.08 ± 0.01	0.11 ± 0.02	0.14 ± 0.03
Semimembranosus, red (72%)	15 ± 2	19 ± 3	28 ± 9	0.11 ± 0.02	0.15 ± 0.02	0.21 ± 0.06
Semimembranosus, white (100%)	13 ± 2	11 ± 1	12 ± 2	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Thigh adductors						
Adductor longus (5%)	115 ± 7	142 ± 12	148 ± 12	0.84 ± 0.06	1.12 ± 0.10	1.19 ± 0.12
Adductor magnus & brevis (89%)	15 ± 3	16 ± 3	23 ± 7	0.11 ± 0.02	0.13 ± 0.02	0.17 ± 0.05
Gracilis (77%)	16 ± 3	17 ± 2	20 ± 4	0.11 ± 0.02	0.13 ± 0.01	0.15 ± 0.03
Pectineus (69%)	17 ± 2	23 ± 4	28 ± 7	0.12 ± 0.01	0.19 ± 0.03	0.21 ± 0.04

Data are mean ± SEM. Values in parentheses indicate % type IIb + d/x according to Delp & Duan (1996). Control; *n*=10, BRLD; *n*=14, BRHD; *n*=6.

Table 2. Effects of low dose and high dose BR supplementation on exercising hindlimb muscle BF (ml/min/100g) and VC (ml/min/100g/mmHg).

	BF			VC		
	Control	**BRLD**	**BRHD**	**Control**	**BRLD**	**BRHD**
Ankle extensors						
Soleus (9%)	297 ± 46	251 ± 19	328 ± 39	2.15 ± 0.33	2.04 ± 0.20	2.60 ± 0.26
Plantaris (80%)	208 ± 17	194 ± 18	249 ± 10†	1.51 ± 0.11	1.56 ± 0.17	2.00 ± 0.06*†
Gastrocnemius, red (14%)	444 ± 48	391 ± 40	507 ± 42	3.21 ± 0.32	3.13 ± 0.28	4.07 ± 0.31*†
Gastrocnemius, white (100%)	44 ± 7	42 ± 8	71 ± 14*†	0.31 ± 0.05	0.32 ± 0.05	0.56 ± 0.10*†
Gastrocnemius, mixed (91%)	153 ± 13	135 ± 12	214 ± 19*†	1.11 ± 0.08	1.08 ± 0.10	1.71 ± 0.11*†
Tibialis posterior (73%)	121 ± 18	111 ± 11	137 ± 22	0.88 ± 0.13	0.89 ± 0.09	1.10 ± 0.18
Flexor digitorum longus (68%)	103 ± 15	113 ± 19	106 ± 19	0.74 ± 0.10	0.92 ± 0.17	0.84 ± 0.15
Flexor halicus longus (71%)	74 ± 11	69 ± 11	88 ± 11	0.53 ± 0.07	0.55 ± 0.08	0.70 ± 0.07
Ankle flexors						
Tibialis anterior, red (63%)	347 ± 38	303 ± 22	388 ± 35†	2.50 ± 0.26	2.38 ± 0.16	3.08 ± 0.19†
Tibialis anterior, white (80%)	120 ± 15	109 ± 11	170 ± 24*†	0.86 ± 0.10	0.86 ± 0.08	1.34 ± 0.16*†
Extensor digitorum longus (76%)	57 ± 8	60 ± 6	83 ± 13*†	0.46 ± 0.05	0.47 ± 0.04	0.66 ± 0.09*†
Peroneals (67%)	129 ± 13	123 ± 10	172 ± 8*†	0.93 ± 0.09	0.98 ± 0.08	1.38 ± 0.05*†
Knee extensors						
Vastus intermedius (4%)	371 ± 41	320 ± 28	358 ± 54	2.68 ± 0.29	2.54 ± 0.23	2.88 ± 0.41
Vastus medialis (82%)	122 ± 18	111 ± 13	185 ± 36	0.88 ± 0.12	0.87 ± 0.10	1.48 ± 0.28*†
Vastus lateralis, red (35%)	393 ± 47	327 ± 29	486 ± 48†	2.84 ± 0.32	2.60 ± 0.23	3.91 ± 0.39*†
Vastus lateralis, white (100%)	32 ± 5	33 ± 5	51 ± 10*†	0.23 ± 0.03	0.26 ± 0.04	0.40 ± 0.07*†
Vastus lateralis, mixed (89%)	169 ± 23	151 ± 14	243 ± 17*†	1.23 ± 0.16	1.20 ± 0.11	1.95 ± 0.13*†
Rectus femoris, red (66%)	233 ± 35	245 ± 15	33 ± 37*†	1.69 ± 0.24	1.95 ± 0.13	2.64 ± 0.30*†
Rectus femoris, white (100%)	103 ± 14	94 ± 8	197 ± 38*†	0.74 ± 0.09	0.75 ± 0.06	1.55 ± 0.27*†
Knee flexors						
Biceps femoris anterior (100%)	49 ± 9	50 ± 7	77 ± 16*†	0.36 ± 0.06	0.40 ± 0.05	0.63 ± 0.13*†
Biceps femoris posterior (92%)	79 ± 9	81 ± 9	135 ± 13	0.57 ± 0.07	0.65 ± 0.07	1.09 ± 0.10*†
Semitendinosus (83%)	57 ± 7	51 ± 5	80 ± 13*†	0.41 ± 0.05	0.40 ± 0.04	0.63 ± 0.11*†
Semimembranosus, red (72%)	120 ± 16	116 ± 10	172 ± 17*†	0.87 ± 0.11	0.93 ± 0.10	1.38 ± 0.13*†
Semimembranosus, white (100%)	34 ± 6	32 ± 4	67 ± 14*†	0.25 ± 0.04	0.25 ± 0.03	0.54 ± 0.11*†
Thigh adductors						
Adductor longus (5%)	321 ± 42	268 ± 20	333 ± 51	2.32 ± 0.29	2.17 ± 0.20	2.66 ± 0.40
Adductor magnus & brevis (89%)	82 ± 9	71 ± 7	116 ± 19*†	0.59 ± 0.06	0.60 ± 0.06	0.93 ± 0.14*†
Gracilis (77%)	42 ± 5	41 ± 6	60 ± 11	0.30 ± 0.03	0.33 ± 0.06	0.48 ± 0.08*
Pectineus (69%)	49 ± 7	59 ± 8	84 ± 17*	0.35 ± 0.05	0.46 ± 0.06	0.68 ± 0.14*†

Data are mean ± SEM. Values in parentheses indicate % type IIb + d/x according to Delp & Duan (1996). Control; n=10, BRLD; n=14 BRHD; n=6. *P<0.05 vs. control. †P<0.05 vs. BRLD.

Table 3. Effects of low dose and high dose BR supplementation on kidney and splanchnic region organ BF (ml/min/100g) and VC (ml/min/100g/mmHg) at rest and during exercise.

	Rest			Exercise		
	Control	**BRLD**	**BRHD**	**Control**	**BRLD**	**BRHD**
BF						
Kidney	414 ± 29	447 ± 32	521 ± 32*	421 ± 47	372 ± 35	436 ± 52†
Stomach	82 ± 8	94 ± 13	71 ± 16	68 ± 15	51 ± 6†	49 ± 9
Adrenals	557 ± 91	601 ± 57	667 ± 67	349 ± 79	321 ± 49	594 ± 188
Spleen	345 ± 54	332 ± 37	501 ± 134	63 ± 16†	81 ± 14†	99 ± 33†
Pancreas	117 ± 11	112 ± 14	86 ± 15	115 ± 15	103 ± 10	116 ± 28
Sm. Intestine	304 ± 17	361 ± 38	269 ± 38	247 ± 30	238 ± 21†	255 ± 53
Lg. Intestine	131 ± 13	153 ± 18	139 ± 18	135 ± 15	126 ± 13	142 ± 21
Liver**	37 ± 13	32 ± 6	40 ± 8	17 ± 4	18 ± 2	41 ± 11
VC						
Kidney	3.04 ± 0.27	3.65 ± 0.32	4.15 ± 0.27*	3.04 ± 0.31	3.13 ± 0.43	3.51 ± 0.44
Stomach	0.59 ± 0.06	0.75 ± 0.10	0.54 ± 0.09	0.50 ± 0.11	0.41 ± 0.05†	0.39 ± 0.07
Adrenals	4.06 ± 0.72	4.71 ± 0.37	5.42 ± 0.80	2.86 ± 0.49	2.51 ± 0.37†	4.79 ± 1.57
Spleen	2.50 ± 0.40	2.63 ± 0.27	3.71 ± 0.85	0.45 ± 0.11†	0.64 ± 0.28†	0.79 ± 0.28
Pancreas	0.85 ± 0.08	0.88 ± 0.11	0.65 ± 0.09	0.83 ± 0.11	0.83 ± 0.08	0.92 ± 0.23†
Sm. Intestine	2.18 ± 0.14	2.87 ± 0.28	2.06 ± 0.25	1.72 ± 0.20	1.89 ± 0.17†	2.00 ± 0.43
Lg. Intestine	0.95 ± 0.10	1.21 ± 0.14	1.09 ± 0.11	0.98 ± 0.10	1.00 ± 0.10	1.13 ± 0.16
Liver**	0.26 ± 0.12	0.21 ± 0.04	0.26 ± 0.05	0.12 ± 0.03	0.15 ± 0.02	0.32 ± 0.08

Data are mean ± SEM. *, $P < 0.05$ vs. Control. †, $P < 0.05$ vs. rest. **Indicates arterial, not portal, BF and VC.

Table 4. Microvascular partial pressure of O_2 (PO_2mv) kinetics parameters at rest and during contractions for control, BRLD and BRHD rats.

	Control	BRLD	BRHD
$PO_2mv_{(BL)}$ (mmHg)	31.1 ± 2.0	30.1 ± 1.9	26.4 ± 2.8
Δ_1PO_2mv (mmHg)	16.7 ± 1.5	12.9 ± 0.7*	11.8 ± 0.9*
Δ_2PO_2mv (mmHg)	3.9 ± 0.8	3.6 ± .3	3.8 ± 0.4
$\Delta_{total}PO_2mv$ (mmHg)	13.6 ± 1.6	11.1 ± 1	9.6 ± 1.5
$PO_2mv_{(steady\text{-}state)}$(mmHg)	18 ± 1.7	19 ± 1.4	17.7 ± 2.0
TD_1 (s)	6.7 ± 1.5	8.6 ± 0.9	12.9 ± 2.8*
TD_2 (s)	49.8 ± 11.2	54 ± 15.6	27 ± 5.5
τ_1 (s)	9.9 ± 1.2	8.6 ± 0.9	10.4 ± 2.2
τ_2 (s)	89.4 ± 18.7	93.9 ± 21.2	86.1 ± 10.9
MRT_1 (s)	16.6 ± 2.1	20.9 ± 1.9	23.3 ± 4.7*
$\Delta_1PO_2mv/\ \tau_1$ (mmHg/s)	1.9 ± 0.3	1.3 ± 0.2	1.4 ± 0.3

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 PO_2mv data (control: n=9, BRLD: n=6, BRHD: n=3). $PmvO_{2(BL)}$, pre-contraction PO_2mv; Δ_1PO_2mv, amplitude of the first component; Δ_2PO_2mv, amplitude of the second component; $\Delta_{total}PO_2mv$, overall amplitude regardless of one- or two-component model fit; $PO_2mv_{(steady\text{-}state)}$, PO_2mv during contracting steady-state; TD_1, time delay for the first component; TD_2, time delay for the second component; τ_1, time constant for the first component; τ_2, time constant for the second component; MRT_1, mean response time describing the overall kinetics response; $\Delta_1PO_2mv/\ \tau_1$, parameter describing the relative rate of PO_2mv fall.

*$P<0.05$ vs. control.

Figure captions

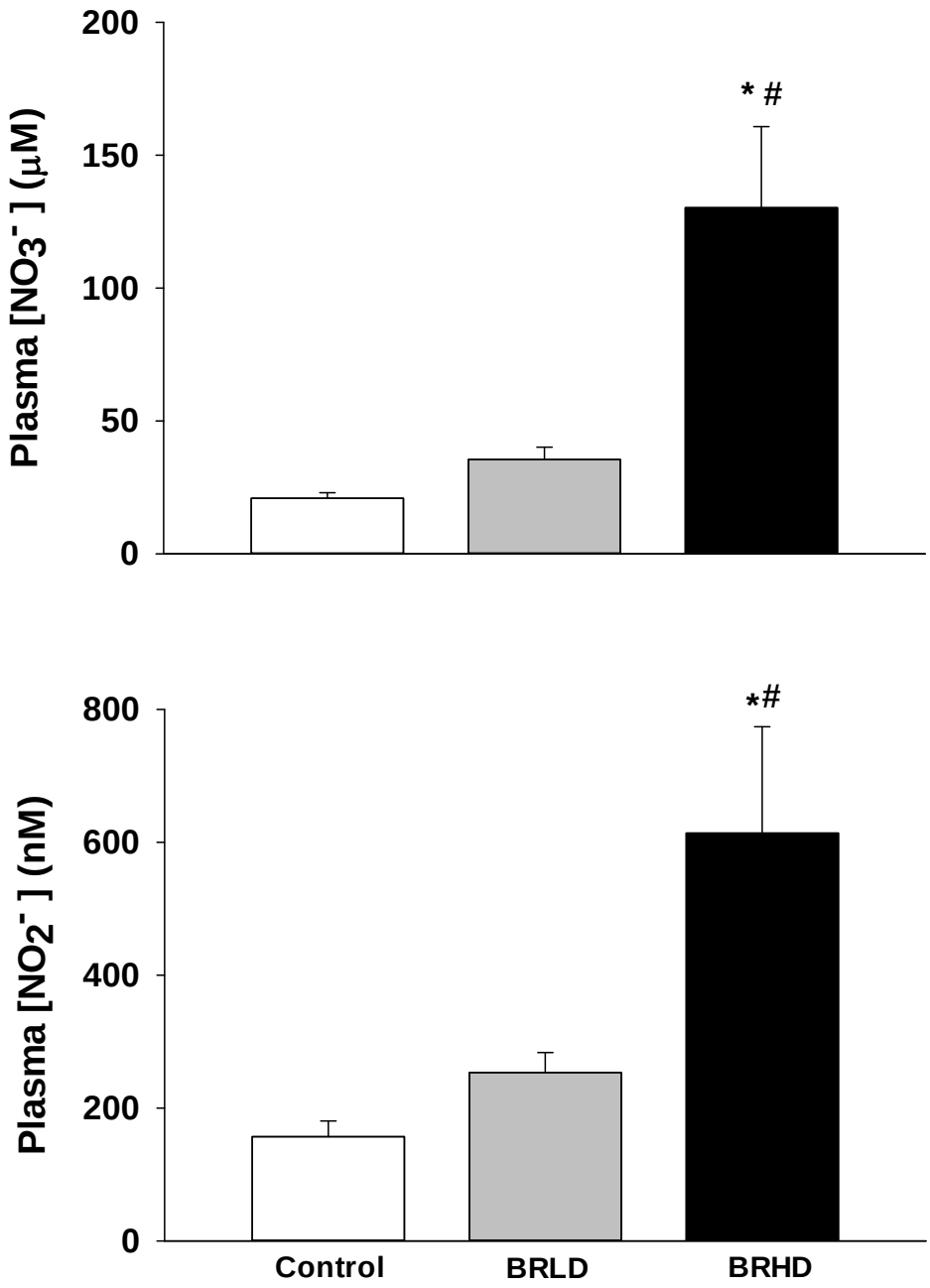
Figure 1. Top panel: Post-supplementation plasma [NO_3^-] for control, BRLD and BRHD rats. Bottom panel: Post-supplementation plasma [NO_2^-] for control, BRLD and BR rats. *P<0.05 versus control #P<0.05 versus BRLD.

Figure 2. Resting and exercising mean arterial pressures for control, BRLD and BRHD rats. *P<0.05 versus control.

Figure 3. Total hindlimb BF and VC for control, BRLD and BRHD rats during submaximal locomotory exercise. *P<0.05 versus control.

Figure 4. Raw representative PO_2*mv* profiles for control, BRLD and BRHD rats during 180 s of 1-Hz twitch contractions.

Figure 1.


200
150
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Plasma [NO3-] (μM)
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Plasma [NO2-] (nM)
*#
Control
BRLD
BRHD

Figure 2.

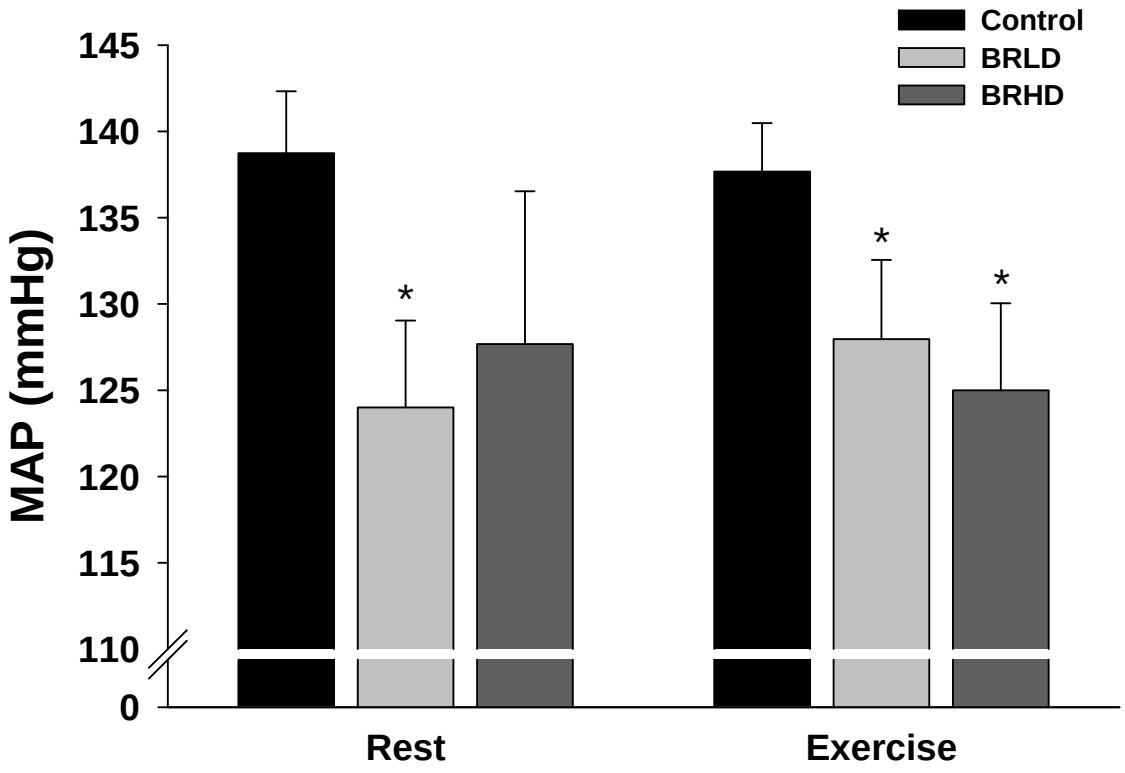

Control
BRLD
BRHD
MAP (mmHg)
145
140
135
130
125
120
115
110
0
*
*
*
Rest
Exercise

Figure 3.

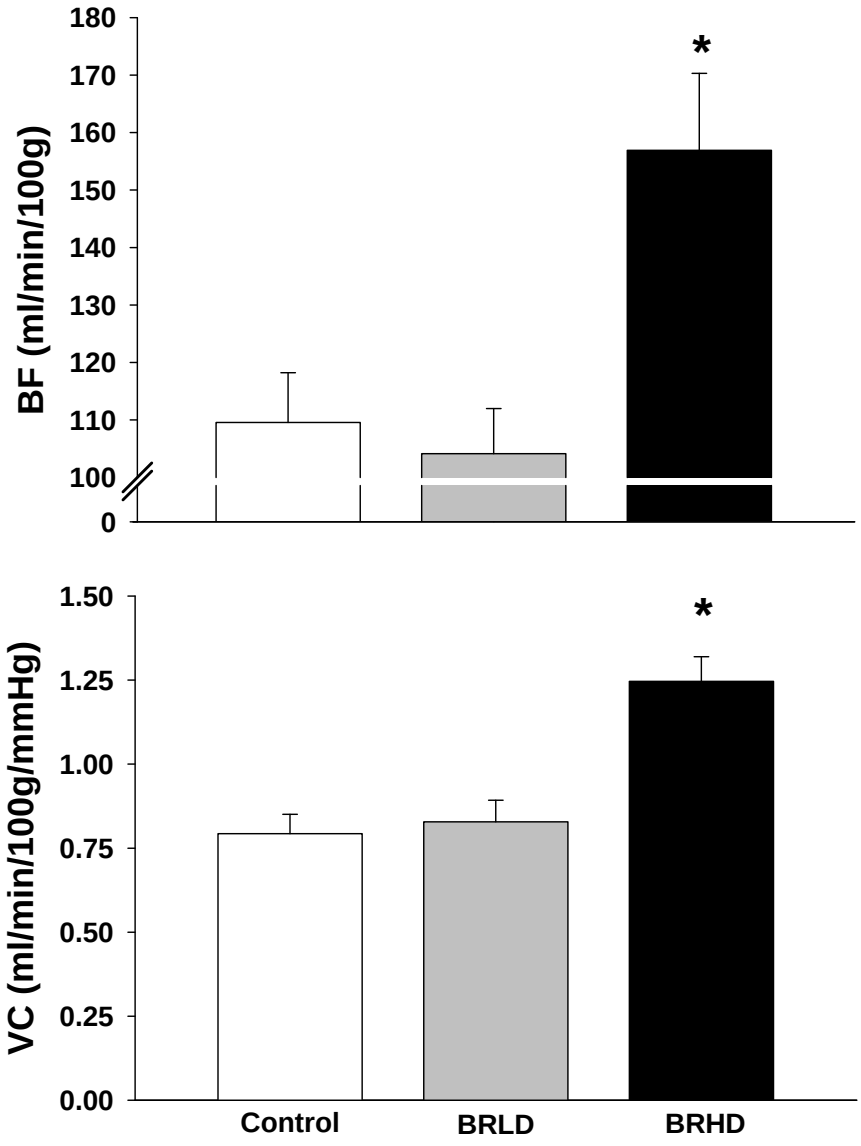
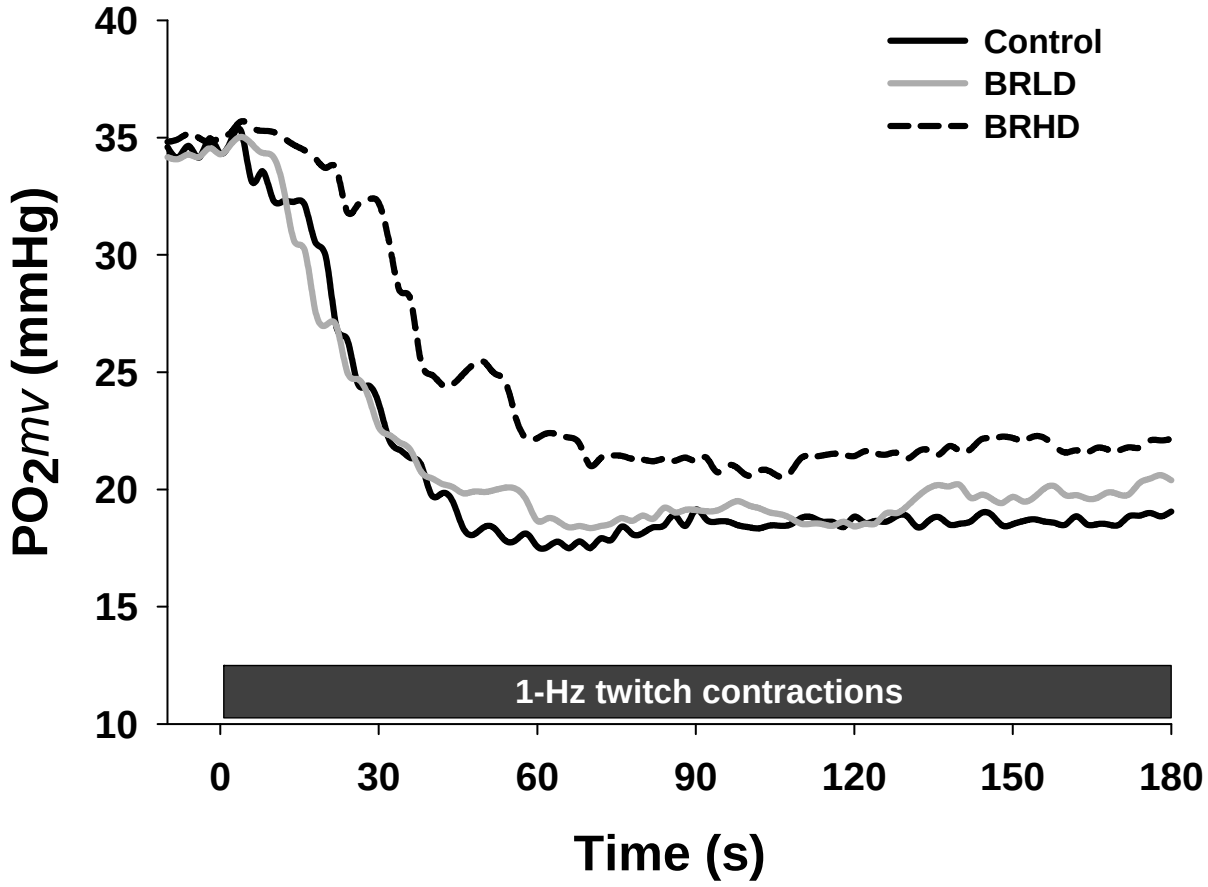

BF (ml/min/100g)
180
170
160
150
140
130
120
110
100
0
*
VC (ml/min/100g/mmHg)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
Control
BRLD
BRHD

Figure 4.


Control
BRLD
BRHD
PO_{2mv} (mmHg)
40
35
30
25
20
15
10
1-Hz twitch contractions
0
30
60
90
120
150
180
Time (s)