IMPACT OF DIETARY NITRATE SUPPLEMENTATION VIA BEETROOT JUICE ON EXERCISING MUSCLE VASCULAR CONTROL IN RATS

by

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Abstract

Introduction: Dietary nitrate (NO_3^-) supplementation, via its reduction to nitrite (NO_2^-) and subsequent conversion to nitric oxide (NO) and other reactive nitrogen intermediates, reduces blood pressure and the O_2 cost of submaximal exercise in humans. Despite these observations, the effects of dietary NO_3^- supplementation on skeletal muscle vascular control during locomotory exercise remain unknown. We tested the hypotheses that dietary NO_3^- supplementation via beetroot juice (BR) would reduce mean arterial pressure (MAP) and increase hindlimb muscle blood flow in the exercising rat. **Methods:** Male Sprague-Dawley rats (3-6 months) were administered either NO_3^- (via beetroot juice; 1 mmol · kg^{-1} · day^{-1}, BR n=8) or untreated (control, n=11) tap water for 5 days. MAP and hindlimb skeletal muscle blood flow and vascular conductance (radiolabeled microsphere infusions) were measured during submaximal treadmill running (20 m · min^{-1}, 5% grade). **Results:** BR resulted in significantly lower exercising MAP (control: 137 ± 3, BR: 127 ± 4 mmHg, P<0.05) and blood [lactate] (control: 2.6 ± 0.3, BR: 1.9 ± 0.2 mM, P<0.05) compared to control. Total exercising hindlimb skeletal muscle blood flow (control: 108 ± 8, BR: 150 ± 11 ml · min^{-1} · 100 g^{-1}, P<0.05) and vascular conductance (control: 0.78 ± 0.05, BR: 1.16 ± 0.10 ml · min^{-1} · 100 g^{-1} · $mmHg^{-1}$, P<0.05) were greater in rats that received beetroot juice compared to control. The relative differences in blood flow and vascular conductance for the 28 individual hindlimb muscles and muscle parts correlated positively with their percent type IIb + d/x muscle fibers (blood flow: r=0.74, vascular conductance: r=0.71, P<0.01 for both). **Conclusion:** These data support the hypothesis that NO_3^- supplementation improves vascular control and elevates skeletal muscle O_2 delivery during exercise predominantly in fast-twitch type II muscles, and provide a potential mechanism by which NO_3^- supplementation improves metabolic control.

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Abbreviations Used

NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO_3^-, nitrate; NO_2^-, nitrite; BR, beetroot juice; HR, heart rate; MAP, mean arterial pressure; VC, vascular conductance; $\dot{V}O_2$, oxygen uptake; BF, blood flow; PO_{2mv}, microvascular partial pressure of oxygen; PCr, phosphocreatine.

Dedication

I would like to dedicate this thesis to my mother and father who have loved and supported me through all of my endeavors. Mom, you have always been there for me and have taught me the importance of family. I have always been able to count on you for inspiration and strength, even through the toughest of days. Dad, thanks for instilling the importance of education in me. You have always set a perfect example for us kids to follow and I intend to always honor the values and lessons you have taught me. I thank God that I have been blessed with a great family and look forward to surrounding my children with the love and fortitude you have shown me. Thanks for all you do!

Love always

Chapter 1 - Introduction

It is now recognized that NO functions as a major contributor to skeletal muscle vascular and metabolic control (reviewed by Joyner & Tschakovsky, 2003). NO is produced endogenously by the reduction of L-arginine to L-citrulline via three distinct NOS isoforms: constitutively expressed eNOS and nNOS, as well as iNOS (reviewed by Stamler & Meissner, 2001). In addition, there is emerging evidence that dietary inorganic NO_3^- delivered, for example, via ingested BR, can be reduced to NO_2^- and, subsequently, NO and other reactive nitrogen intermediates and impact hemodynamic and muscle metabolic function (Larsen *et al.* 2007; Bailey *et al.* 2009). These effects have been divorced from other active BR constituents (i.e., antioxidants; Lansley *et al.* 2011) and, crucially, the reduction of NO_2^- to NO is potentiated by hypoxic and acidic conditions (Cosby *et al.* 2003), which may be present during muscular exercise. In contrast, hypoxic conditions impair NOS function and therefore compromise NO bioavailability from that pathway under the very conditions when NO is requisite to balance O_2 delivery-to-O_2 utilization in skeletal muscle (Ferreira *et al.* 2006ab; Hirai *et al.* 2010).

In humans, acute (2-3 hours) and chronic (3-6 days) dietary NO_3^- ingestion via sodium NO_3^- salt (Larsen *et al.* 2007) or BR (Bailey *et al.* 2009; Vanhatalo *et al.* 2010a; Kenjale *et al.* 2011; Lansley *et al.* 2011) reduces blood pressure, lowers submaximal exercise $\dot{V}O_2$, and has been shown to enhance exercise tolerance. In addition, BR ameliorates the muscle metabolic perturbations found during exercise when breathing a hypoxic inspirate (Vanhatalo *et al.* 2011), improves muscle oxygenation in peripheral artery disease patients (Kenjale *et al.* 2011), and improves human mitochondrial efficiency as measured using the P/O ratio (Larsen *et al.* 2011).

Collectively, these investigations suggest that augmented dietary NO_3^- might serve to maintain or even increase skeletal muscle BF (and hence O_2 delivery) in the presence of reduced O_2 demand, which may be expected to enhance metabolic control via increases in intramyocyte PO_2. However, we are unaware of any measurements of BF and VC within and among skeletal muscles during locomotory exercise. Indeed, within the running rat model it is possible to determine the impact of BR on vascular control across discrete muscle fibre type populations. Such information is essential for resolving the effect of BR on O_2 delivery-to-O_2 utilization matching within and across muscles, which may have important metabolic consequences.

Accordingly, the purpose of the present investigation was to test the hypotheses that ingesting BR for 5 days would, in the face of increased plasma [NO_3^-], [NO_2^-], and lowered MAP: 1) increase BF and VC in locomotory muscles across the spectrum of both high and low oxidative capacities, and 2) and thereby presumably increase the O_2 delivery-to-O_2 utilization ratio thus reducing blood [lactate]. Results from the present investigation may provide mechanistic links between changes in plasma [NO_3^-] and [NO_2^-] and improved muscle oxygenation and metabolic function following NO_3^- supplementation (Kenjale *et al.* 2011; Vanhatalo *et al.* 2011).

Chapter 2 - Literature Review

Less than 10 years after Furchgott and Zawadski's seminal discovery, work done in Salvador Moncada's laboratory revealed that an enzyme known as nitric oxide synthase (NOS) was responsible for NO synthesis via oxidation of the amino acid L-Arginine (Moncada & Higgs, 1993). Since its discovery, NO has been implicated in a multitude of physiological processes including blood flow regulation, cellular respiration, glucose homeostasis, and muscle contraction (reviewed by Joyner & Tschakovsky, 2003). By the mid 1990's a surge of investigations were uncovering the mechanisms by which NO elicits its cell signaling capabilities and the importance of this powerful biological messenger were beginning to come into focus. However, as the literature progressed it became evident that, because of its high reactivity, NO becomes oxidized very quickly. With its short half-life it seemed that local NO production was obligatory to avoid the "scavenging" effects of oxygenated hemoglobin (Hb).

Until recently, NO was thought to be synthesized primarily by the NOS family of enzymes, however Zweier and colleagues (1995) used paramagnetic resonance spectroscopy (EPR) to show an elevated rate of NO synthesis in ischemic rat hearts despite infusion of the comprehensive NOS blocker n-nitro-l-arginine methyl ester (L-NAME). They concluded that high tissue levels of nitrite were responsible and postulated that the acidotic and low intravascular PO_2 levels present during ischemia catalyzed the reduction of NO_2^- into NO_3^-. This was the first investigation to suggest NO_2^- as a potential NO storage pool capable of eliciting a cardioprotective role against tissue ischemia.

NO_2^- and NO

For years, NO_2^- has been used as a meat preservative to prevent botulism (Lundberg & Weitzberg, 1995) and, until recently, was once believed to be an inert byproduct of NO oxidation, offering little physiological significance. Interestingly Furchgott & Bhadrakom (1953) used acidified pharmacological sodium nitrite ($NaNO_2^-$) to induce aortic strip vasodilation leading to the discovery of NO's power as endothelial derived relaxing factor (EDRF) in 1980 . Although the experimental conditions were far outside physiological ranges (i.e. a high dose of NO_2^- was used), the demonstration of the vasoactive properties was evident. By 1990, Classen *et al.* (1990) had shown that NO_2^- reduced blood pressure when given orally to spontaneously

hypertensive rats alluding to a systemic effect of NO_2^- and further suggesting NO forming properties. Classen and his colleagues also questioned the role of NO_3^- in NO_2^- mediated NO production which proved to be very insightful in the years to come. The questions that remained were how NO_2^- was being reduced to NO in vivo, if the reduction was physiologically relevant, and what role NO_3^- played?

Interestingly, NO_2^- reduction to NO is potentiated in environments of reduced pH such that is found in the human stomach. This was eloquently demonstrated by Lundberg et al. (1994) who reported that expelled gastric air from burping contained high levels (800-6000 ppb) of NO gas. Furthermore, expelled NO levels were increased following a meal of lettuce, which is known to contain high levels of NO_3^- adding to the idea that NO_3^- can be reduced to NO_2^- and further to NO *in vivo*. It is noteworthy to mention that the amount of exhaled NO was attenuated 95% after the proton pump inhibitor omeprazole was administered further substantiating that NO_2^- reduction is potentiated in acidic conditions. This was one of the first reports showing the potential for NO_2^- to act as a substrate for enzyme independent NO generation. However, whether this pathway served any role in vascular or metabolic function remained to be answered.

Vasoactive properties of NO_2^-

Modin *et al.* (2001) was perhaps the first to show the vasoactive properties of NO_2^- (at physiologically relevant concentrations) using an isolated vessel preparation. In their experiment, vasoconstriction was induced using phenylephrine while aortic smooth muscle contractions were monitored to assess the speed at which the smooth muscle relaxed as the concentration of NO_2^- was progressively increased. This evidence unveiled the potential of NO_2^- to augment blood flow in areas of reduced pH possible during ischemia and transitions in metabolic demand.

In addition, the reduction of NO_2^- to NO is exacerbated in hypoxic environments, which may be present during muscular exercise. This was demonstrated by Cosby *et al.* (2003) who demonstrated an increase in forearm blood flow in response to infused NO_2^- while simultaneously showing an atrial-venous gradient in which plasma NO_2^- concentration was reduced in the venous circulation. Importantly, the improvements in blood flow were inversely related to Hb O_2 saturation providing evidence that NO_2^- impacts hypoxic vasodilation. Accounting for these data, it is now evident that NO_2^- reduction to NO compliments NOS, and

thus helps maintain NO bioavailability in hypoxic environments where NOS function is compromised.

Xanthine oxidoreductase (XO) has been suggested to facilitate NO_2^- reduction and may protect against ischemia reperfusion injury. Located in the blood, XO is a complex flavoprotein enzyme that has the potential to generate reactive oxygen species such as superoxide and hydrogen peroxide. (McCord, 1985). Moreover, it has also been shown that XO can produce NO by reduction of NO_2^- (Samouilov & Zweier, 2003). This helps to ameliorate post ischemic injury (i.e. infarct size) by providing additional NO to serve as a cardioprotective shield against free radicals and the anoxic environment as demonstrated by Webb *et al.* (2004) in the perfused ischemic rat heart. However, it is important to mention that NO synthesis via XO and NOS is subject to change based on substrate availability and oxygenation status of the surrounding tissue. More specifically, when NO_2^- availability is limited there is a tendency for XO to generate damaging superoxides in lieu of NO. This is similar to NOS in which a reduced availability of L-arginine or O_2 causes an elevated generation of superoxides due to substrate scarcity. Should these conditions arise, there is potential for formation of the peroxynitrite radial which has been shown to be damaging to the endothelium (Beckman *et al. 1990*). Given this information, with an adequate supply of circulating NO_2^-, it is conceivable to render the NO_3^-- NO_2^--NO pathway (regardless of the mode of NO_2^- reduction) as a complimentary backup for anoxic situations in which NO production from NOS is compromised.

Dietary sources of NO_3^- and NO_2^-

With plasma concentration of NO_2^- of paramount importance, many physiologists have now focused their attention towards exogenous sources. The primary source of NO_2^-, aside from the endogenous oxidation of NO described earlier, is from the reduction of the closely related NO_3^- anion. While mammals do not posses the enzymes required for NO_3^- reduction, many bacteria possess powerful NO_3^- reductase enzymes enabling them to convert NO_3^- to NO_2^- in anaerobic environments. These facultative bacteria are located in large numbers within the oral cavity and without them NO_3^- reduction cannot occur (Weitzberg & Lundberg, 1998; Lundberg & Govoni, 2004; Lundberg *et al.* 2004).

For mammals 60-80% of the daily NO_3^- intake is through dietary ingestion of dark green leafy vegetables and their roots (i.e. spinach, carrots, beetroot) (Ysart *et al.* 1999). Upon consumption, NO_3^- is absorbed in the gut quickly elevating plasma levels. The salivary glands then actively take up and secrete NO_3^- in saliva; increasing oral levels nearly 10 fold (Duncan *et al. 1995*). NO_3^- is then reduced to NO_2^- and is swallowed where it is reduced to NO in the digestive tract or re-absorbed into the bloodstream peaking plasma NO_2^- concentrations within 3 hours of ingestion (Dejam *et al.* 2004).

NO_3^- supplementation and muscle metabolic and contractile function

Aside from the vasoactive components, there is data to suggest that the dietary NO_3^- consumption alters muscle metabolic and contractile efficiency (Lundberg *et al.* 2004; Hernandez *et al.* 2012). Elevating NO bioavailability reduces mitochondrial $\dot{V}O_2$ as NO inhibits cytochrome C oxidase activity by competing with O_2 thereby reducing overall mitochondrial respiration (Brown & Cooper, 1994). In addition, recent studies suggests that both NO_2^- and NO serve to increase oxidative phosphorylation efficiency by either reducing proton slippage of the mitochondrial proton pumps or by serving as an alternative terminal electron acceptor, theoretically substituting for O_2 (Clerc *et al.* 2007; Basu *et al.* 2008). Collectively, these improvements in oxidative ATP resynthesis may provide a mechanistic linkage to increased plasma nitrite and reduced whole body exercising $\dot{V}O_2$ (Larsen *et al.* 2007; Bailey *et al.* 2009; Vanhatalo *et al.* 2010a; Kenjale *et al.* 2011; Lansley *et al.* 2011).

NO_3^- and O_2 uptake

Larsen *et al.* (2007) used pharmacological $NaNO_3^-$ as a dietary supplement for 6 days and reported a reduction in submaximal $\dot{V}O_2$ of cycling at 45-80% of $\dot{V}O_2$max. This was accomplished without an increase in glycolytic energy production as confirmed by unchanged blood lactate levels. Bailey *et al.* (2009) took this concept yet another step by supplementing NO_3^--rich beetroot juice (BR) in place of the sodium NO_3^- salt to see if the metabolic effects could be elicited via dietary supplementation. Three days of supplementation reduced submaximal $\dot{V}O_2$ by 19% while simultaneously increasing time to exhaustion. Interestingly, the

$\dot{V}O_2$ slow component was also reduced suggesting that NO_3^- ingestion may increase exercise tolerance, which carries many implications for various diseases states where exercise capacity is reduced (i.e. heart failure, reviewed by Poole *et al.* 2010). In addition to the remarkable metabolic findings, systemic blood pressure was reduced significantly in those in the BR group with others reporting similar results (Gladwin *et al.* 2000; Larsen *et al.* 2007; Kenjale *et al.* 2011; Lansley *et al.* 2011a,b; Vanhatalo *et al.* 2011). Given that blood flow is mediated in part by vasodilatory mechanisms, it is possible that NO_3^- supplementation may also elevate exercising vascular conductance manifested by a reduced driving pressure but maintained (or slightly elevated) blood flow.

Of late, a barrage of studies on the effects of dietary NO_3^- supplementation has taken shape investigating the possibility of augmented exercise performance, amelioration of certain chronic disease symptoms, and elevated tolerance to hypoxic environments. While the exact mechanisms of the NO_3^--NO_2^--NO pathway are not yet fully understood, it is quite clear that the relatively stable anions NO_3^- and NO_2^- are far from inert as once thought. The studies discussed in this review have helped further illuminate the role and potential benefits of NO_3^- and NO_2^- in NO bioavailability and cellular function and have paved the road for future investigations.

Chapter 3 - Methods

Ethical approval

A total of 19 young adult male Sprague-Dawley rats (3-4 months old; body mass=416 ± 12 g) were used in the present investigation. Rats were maintained on a 12:12 hr light-dark cycle with food and water available *ad libitum*. All experimental procedures were conducted under the guidelines established by *The Journal of Physiology* (Drummond, 2009) and approved by the Institutional Animal Care and Use Committee of Kansas State University. All rats were familiarized with running on a custom-built motor-driven treadmill for 5 min · day^{-1} at a speed of 20 m · min^{-1} up a 5% grade for ~5 days.

BR Supplementation

Rats were assigned randomly to receive either tap water (control; *n*=11) or 5 days of BR supplementation (BR; *n*=8) (dose; 1 mmol · kg^{-1} · day^{-1} diluted in 100 ml of tap water; Beet it™, James White Drinks, Ipswich, UK) with consumption monitored daily. Preliminary studies in our laboratory demonstrated this dose elevated plasma [NO_3^-] and [NO_2^-] to levels approximating those seen in humans following NO_3^- supplementation (Lundberg *et al.* 2004; Bailey *et al.* 2009; Kenjale *et al.* 2011). Moreover, this dose compares closely to NO_3^- doses administered to humans after accounting for the ~7x greater resting metabolic rate in rats compared to humans (Musch *et al.* 1988).

Instrumentation and regional BF measurements

Rats were first anesthetized using a 5% isoflurane-O_2 mixture. Subsequently, while maintained on a 2-3% isoflurane-O_2 mixture, a catheter (PE-10 connected to PE-50; Clay Adams Brand, Sparks, MD, USA) was placed in the ascending aorta via the right carotid artery. A second catheter (PE-10 connected to PE-50) was placed surgically in the caudal (tail) artery as described previously (Musch *et al.* 1992). Both catheters were tunneled subcutaneously to the dorsal aspect of the cervical region and exteriorized through a puncture wound in the skin. Following incision closure, anesthesia was terminated and the animal was given 1-2 hours to recover before initiation of the final experimental protocol (Flaim *et al.* 1984).

After recovery, the rat was 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 then connected to a pressure transducer (Gould Statham P23ID, Valley View, OH, USA) maintained at the same height as the animal and exercise was initiated. Treadmill speed was increased progressively over a ~30 s period to a speed of 20 m · min^{-1} (5% grade, ~60% $\dot{V}O_2$ 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 the 3 min mark the pump connected to the caudal artery catheter was activated and withdrawal was initiated at a rate of 0.25 ml · 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 injected into the aortic arch for determination of regional BF. Following the microsphere injection ~0.2 ml of blood was sampled from the carotid artery catheter for the determination of [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 injection 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).

Determination of regional BF and VC

Following the second microsphere infusion, rats were euthanized with a sodium pentobarbital overdose (≥50 mg · kg^{-1}, infused into the carotid artery catheter). The thorax 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 ·

$min^{-1} \cdot 100\ g^{-1}$. Adequate mixing of the microspheres was verified for each rat, 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^{-1} · 100 g^{-1} · · $mmHg^{-1}$.

Blood sampling and measurement of Plasma NO_3^- and NO_2^-

A blood sample was collected from control and BR group rats to assess differences in plasma [NO_3^-] and [NO_2^-]. Following instrumentation and before regional BF measurements ~0.8 ml of blood was drawn from the caudal artery catheter and centrifuged at 5000 g at 4°C for 6 minutes. Plasma was subsequently extracted and immediately frozen at -80 °C for later analysis of [NO_3^-] and [NO_2^-].

All measurements of plasma NO_3^- and NO_2^- were performed within 30 min 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].

Statistical analysis

Plasma [NO_3^-] and [NO_2^-] were compared using unpaired Student's t-tests. All other data were compared within (rest vs. exercise) and among (control vs. BR) groups using mixed 2-way ANOVAs and Student-Newman-Keuls *post hoc* tests where appropriate. Pearson product-moment correlations and linear regressions were used to determine relationships between variables. Muscle fibre type composition was based on the percentage of type I, type IIa, type IIb, and type IId/x fibres in the individual muscles and muscle parts of the rat hindlimb as

reported by Delp & Duan (1996). Significance was set at $P<0.05$ and values are expressed as mean ± SEM.

Chapter 4 - Results

There was no between group differences in the total hindlimb muscle/body mass ratio (control: 8.8 ± 0.2, BR: 8.3 ± 0.2 %, $P>0.05$) despite modest differences in total body mass (control: 442 ± 14, BR: 384 ± 8 g, $P<0.05$).

Effects of BR on plasma [NO_3^-] and [NO_2^-]

Plasma and [NO_2^-] were significantly greater in rats receiving BR when compared to control (Figure 1).

Effects of BR on HR, MAP, and blood [lactate] at rest and during exercise

HR, MAP, and blood [lactate] values are presented in Table 1. Rats receiving BR had significantly lower exercising but not resting MAP ($P=0.48$) compared to control. There were no differences in resting blood [lactate]. Exercising blood [lactate] was lower in the BR group compared to control.

Effects of BR on skeletal muscle BF and VC at rest and during exercise

There were no differences in total resting hindlimb BF (control: 16 ± 2, BR: 20 ± 4 ml · min^{-1} · 100 g^{-1}, $P=0.30$) or VC (control: 0.12 ± 0.01, BR: 0.15 ± 0.02 ml · min^{-1} · 100 g^{-1} · $mmHg^{-1}$, $P=0.20$). There were no differences in resting BF or VC in any of the 28 individual hindlimb muscles or muscle parts (Table 2). Total exercising hindlimb muscle BF and VC was higher in BR supplemented rats compared to control (Figure 2). Specifically, BR resulted in greater BF in 17, and VC in 21, of the 28 individual hindlimb muscles or muscle parts compared to control (Table 3). All individual muscles and muscle parts demonstrating greater BF are comprised of ≥66% type IIb + d/x muscle fibers whereas VC was higher in muscles and muscle parts ranging from 14-100% type IIb + d/x muscle fibers. Relative differences in BF and VC with BR (i.e. % Δ BF and VC; respectively) were significantly positively correlated with the percentage of type IIb + d/x muscle fibres in the individual hindlimb muscles and muscle parts

(Figure 3). Figure 4 illustrates the marked differences in % Δ BF and VC for the extremes of muscle fiber type composition (i.e., all muscles composed of 100% and ≤20% type IIb + d/x muscle fibers) of the individual muscles and muscle parts of the hindlimb.

Effects of BR on renal and splanchnic BF and VC at rest and during exercise

Renal and splanchnic BF and VC values are presented in Table 4. Renal VC was significantly higher in rats receiving BR compared to control at rest ($P<0.05$). Liver VC was greater during exercise in BR supplemented rats compared to control ($P<0.05$).

Chapter 5 - Discussion

The principal novel finding of this investigation was that 5 days of BR supplementation in healthy rats elevated markedly plasma [NO_3^-] and [NO_2^-] and augmented total hindlimb muscle BF and VC during submaximal locomotory exercise with targeted increases in the type IIb + d/x muscles and muscle parts. That the changes in exercising muscle BF were evident despite a reduction in exercising MAP demonstrates, for the first time, that dietary NO_3^- serves as a powerful controller of muscle O_2 perfusion presumably following its reduction to NO_2^- and NO *in vivo.* These results are important from several perspectives, in particular, because elevations in BF, and therefore O_2 delivery, have the potential to raise PO_{2mv} and hence the O_2 driving pressure across the capillary-myocyte interface (per Fick's Law). This ultimately enhances oxidative function, thereby reducing glycolytic metabolism dependence, as supported by reduced exercising blood [lactate] (Table 2).

Effects of BR on plasma [NO_3^-], [NO_2^-] and MAP

Crucially, both plasma [NO_3^-] and [NO_2^-] (Figure 1) rose to levels approximating what has been shown previously in humans following NO_3^- supplementation (Bailey *et al.* 2009; Vanhatalo *et al.* 2010a; Kenjale *et al*. 2011; Masschelein *et al.* 2012). While there were no differences in resting MAP between groups there was a ~10 mmHg (Table 1) lower MAP during exercise in rats receiving BR compared to control. The exercising MAP data presented herein are particularly interesting given that the effects of NO_3^- supplementation have been primarily studied in humans at rest. Interestingly, rats given BR had significantly higher resting renal VC (Table 3) suggesting that dietary NO_3^- reduces basal vasomotor tone and may play a cardioprotective role in renal vascular diseases as proposed previously (Lundberg *et al*. 2008; Tsuchiya *et al*. 2010; Carlström *et al*. 2011).

Effects of BR on exercising inter- and intra-muscular hindlimb BF and VC

The most striking result of the present investigation was the higher exercising BF and VC in BR rats compared to control. Recent studies performed in humans have shown an apparent increase in skeletal muscle blood volume estimated using near-infrared spectroscopy following

NO_3^- or NO_2^- supplementation (Cosby *et al.* 2003; Bailey *et al.* 2009; Kenjale *et al.* 2011; Masschelein *et al.* 2012). However, muscle blood volume is not a measurement of BF per se and, therefore, to our knowledge, this is the first study investigating the effects of NO_3^- supplementation on inter- and intra-muscular BF and VC at rest and during exercise.

The augmented BF and VC in the present investigation was observed predominantly in fast-twitch type IIb + d/x muscles illustrating a fibre type selective effect of dietary NO_3^- supplementation on vascular control (Figures 3 and 4). This could be due, in part, to the lower PO_{2mv} observed during contractions in muscles composed of primarily type II vs. type I fibres (Behnke *et al.* 2003; McDonough *et al.* 2005; Ferreira *et al.* 2006c). Cosby *et al.* (2003) demonstrated that NO_2^- reduction to NO is potentiated in low O_2 environments via deoxyhemoglobin, deoxymyoglobin, and/or xanthine oxidoreductase. As a result, the reduction of NO_2^- to NO within the microvasculature of predominantly glycolytic type II muscles is likely amplified following NO_3^- supplementation, thereby increasing NO-mediated vasodilation in those muscles. Additionally, sympathetic adrenergic vasoconstriction occurs to a greater extent within more glycolytic type II compared to more oxidative type I muscles (Behnke *et al.* 2011) and the attenuation of skeletal muscle sympathetic vasoconstriction (i.e. functional sympatholysis) within glycolytic muscles during contractions (Thomas *et al.* 1994) is mediated, at least in part, by NO (Thomas & Victor, 1998; Dinenno & Joyner, 2004). This likely contributes to the observed muscle fibre type selective increases in BF and VC seen presently with BR during exercise but not at rest (Table 3).

The lack of BF differences within the highly oxidative muscles could potentially account for the disparities among NO_3^--induced improvements in short-term high intensity exercise (Bailey *et al.* 2009, Lansley *et al.* 2011) but not long duration exercise performance of highly trained endurance athletes (Cermak *et al.* 2012; Wilkerson *et al.* 2012). Any potential improvements in exercise performance following NO_3^- supplementation may be limited to exercise testing protocols that recruit fast-twitch type II muscle fibres. There may also be a BF independent effect as supported by the faster rate and greater magnitude of muscle force development in mouse fast-twitch but not slow-twitch muscle following NO_3^- supplementation reported recently by Hernandez *et al.* (2012).

BR resulted in substantially higher hindlimb skeletal muscle BF and VC (Figure 2) despite no reductions in BF or VC to renal or splanchnic organs during exercise compared to control (Table 3), which may indicate a central effect, where NO_3^- elevates cardiac output (and hence skeletal muscle BF) via increases in stroke volume. Dietary NO_3^- has previously been shown to attenuate ventricular dysfunction via improved cardiac contractility in Doxorubicin-induced cardiomyopathy (Zhu *et al.* 2011). However, it seems more reasonable to suggest that the increases in BF seen herein result from a combination of peripheral and central components in which the increases in peripheral VC alleviate afterload, affording improvements in cardiac output and thus BF via an increase in stroke volume rather than a redistribution effect via vasoconstriction of the renal and splanchnic vascular beds. Therefore, the present data stand in stark contrast to the higher BF in the type IIb + d/x fibres of aged rats observed by Musch et al. (2004) given that the higher BFs in that report occurred concomitant with lower BF in slow twitch muscles and splanchnic organs.

The elevated skeletal muscle BF with BR supplementation documented presently becomes particularly important when considering that elevating local O_2 delivery ($\dot{Q}O_2$) relative to demand ($\dot{V}O_2$) improves the $\dot{Q}O_2/\dot{V}O_2$ relationship thereby increasing the O_2 pressure head (PO_{2mv}) for blood-myocyte O_2 flux as dictated by Fick's law of diffusion. Even if $\dot{V}O_2$ remains unchanged (and it is likely that it decreases via improvement in mitochondrial or muscle contractile efficiency, Larsen *et al.* 2007; Bailey *et al.* 2009; Vanhatalo *et al.* 2010a), the ~38% increase in total hindlimb BF (Figure 2) would be expected to increase mean PO_{2mv} substantially. Accordingly, the reduced PCr breakdown and improved exercise tolerance following BR reported by Jones and colleagues (Bailey *et al.* 2010; Vanhatalo *et al.* 2011) may have been mediated, in part, by elevated O_2 driving pressures in the microvasculature which reduce PCr breakdown (Haseler *et al.* 1998; Vanhatalo *et al.* 2010b) and speed PCr recovery kinetics during hypoxia (Haseler *et al.* 1999). This mechanism is consistent with the lower blood [lactate] found herein with the BR group during exercise but remains to be tested specifically (Table 1).

Experimental considerations and future directions

A major strength of the present investigation lies in the techniques used to measure inter- and intra-muscular BF and VC that, due to technical and ethical limitations, are unavailable in humans. In this regard, the measurements of BF and VC heterogeneity across the spectrum of varying muscle fibre type composition presented herein provide a unique perspective as regards the effects of dietary NO_3^- on skeletal muscle vascular control. This, in combination with the ability to measure both whole-body exercise performance (Copp *et al.* 2010a) and skeletal muscle microvascular function (e.g., PO_{2mv}, Behnke *et al.* 2003), identifies the rat as a valuable research tool for future studies examining the mechanistic bases of the beneficial effects of dietary NO_3^- supplementation in humans. These data have significant clinical implications for a host of disease conditions associated with reduced NO bioavailability and concomitant vascular and metabolic dysfunction, which culminates typically in compromised exercise tolerance (e.g., chronic heart failure; reviewed by Poole *et al.* 2012). A prime example illustrating the potential clinical benefits of BR has already been demonstrated by Kenjale *et al.* (2011) who showed an ~18% increase in peak walk time and time to claudication in peripheral artery disease patients following a single dose of BR.

The differences in total body mass between groups cannot account for the greater exercising blood flows in BR rats given: 1) the hindlimb mass/body mass ratios were not different between groups and blood flows were normalized to muscle mass, 2) data from other laboratories (Armstrong *et al.* 1985) as well as a comparison between the present control data and previous data from our laboratory (Copp *et al.* 2010b) indicate that varying body masses elicit similar BF values at matched treadmill speeds, 3) subsets of body mass-matched control (n = 5, 405 ± 8 g) and BR (n = 5, 398 ± 8, P=0.52) rats from the present investigation confirm that BR results in significantly higher muscle BF versus control (control: 94 ± 13, BR: 155 ± 13 ml · min^{-1} · $100g^{-1}$, P=0.01).

Conclusions

This study is the first to investigate the effects of dietary NO_3^- supplementation on total, inter-, and intra-muscular hindlimb BF and VC both at rest and during submaximal locomotory

exercise. In healthy rats BR supplementation for 5 days elicited marked elevations of plasma [NO_3^-] and [NO_2^-] and lower exercising MAP compared to control. Moreover, BR resulted in a higher total hindlimb muscle BF and VC with targeted increases in the muscles and muscle parts comprised of principally type II + d/x muscle fibres. These data provide compelling that dietary NO_3^- increases muscle O_2 delivery in a fibre-type dependent manner following its reduction to NO_2^- and NO *in vivo*. This investigation offers novel insight into the role of NO_3^- in vascular control and provides a mechanistic linkage between elevated plasma [NO_3^-] and augmented metabolic control found in humans during exercise (Bailey *et al.* 2009; Bailey *et al.* 2010; Larsen *et al.* 2010; Kenjale *et al.* 2011; Vanhatalo *et al.* 2011).

Table 1. Effects of 5 days of BR supplementation on HR, MAP, and blood [lactate] at rest and during exercise.

	HR (bpm)		MAP (mmHg)		Blood [lactate] (mM)	
	Control	**BR**	**Control**	**BR**	**Control**	**BR**
Rest	405 ± 8	409 ± 13	138 ± 3	132 ± 7	0.9 ± 0.1	0.7 ± 0.1
Exercise	525 ± 9 †	521 ± 6 †	137 ± 3	127 ± 4*	2.6 ± 0.3†	1.9 ± 0.2*†

Data are mean ± SEM. *P<0.05 vs. control, †P<0.01 vs. rest.

Table 2. Effects of BR supplementation on resting hindlimb muscle BF (ml · min-1 · 100 g-1) and VC (ml · min-1 · 100 g-1 · mmHg-1).

	BF		VC	
	Control	**BR**	**Control**	**BR**
Ankle extensors				
Soleus (9%)	84 ± 15	102 ± 25	0.62 ± 0.11	0.75 ± 0.18
Plantaris (80%)	15 ± 2	10 ± 1	0.11 ± 0.01	0.08 ± 0.01
Gastrocnemius, red (14%)	42 ± 6	50 ± 15	0.31 ± 0.05	0.37 ± 0.10
Gastrocnemius, white (100%)	14 ± 2	10 ± 2	0.10 ± 0.02	0.08 ± 0.01
Gastrocnemius, mixed (91%)	14 ± 2	15 ± 3	0.10 ± 0.02	0.11 ± 0.02
Tibialis posterior (73%)	17 ± 2	15 ± 4	0.12 ± 0.01	0.11 ± 0.02
Flexor digitorum longus (68%)	21 ± 3	10 ± 2	0.15 ± 0.02	0.07 ± 0.01
Flexor halicus longus (71%)	13 ± 2	10 ± 1	0.09 ± 0.01	0.07 ± 0.01
Ankle flexors				
Tibialis anterior, red (63%)	19 ± 3	19 ± 8	0.14 ± 0.02	0.13 ± 0.05
Tibialis anterior, white (80%)	19 ± 2	16 ± 3	0.14 ± 0.02	0.12 ± 0.02
Extensor digitorum longus (76%)	16 ± 2	14 ± 3	0.12 ± 0.01	0.10 ± 0.02
Peroneals (67%)	17 ± 3	18 ± 3	0.12 ± 0.02	0.13 ± 0.02
Knee extensors				
Vastus intermedius (4%)	43 ± 8	87 ± 18	0.32 ± 0.06	0.64 ± 0.26
Vastus medialis (82%)	14 ± 2	22 ± 7	0.10 ± 0.01	0.16 ± 0.05
Vastus lateralis, red (35%)	39 ± 6	78 ± 23	0.28 ± 0.04	0.57 ± 0.16
Vastus lateralis, white (100%)	15 ± 2	13 ± 2	0.11 ± 0.01	0.10 ± 0.01
Vastus lateralis, mixed (89%)	16 ± 1	26 ± 7	0.12 ± 0.01	0.19 ± 0.05
Rectus femoris, red (66%)	22 ± 4	27 ± 11	0.16 ± 0.03	0.19 ± 0.07
Rectus femoris, white (100%)	15 ± 2	15 ± 4	0.11 ± 0.01	0.11 ± 0.02
Knee flexors				
Biceps femoris anterior (100%)	10 ± 1	10 ± 1	0.07 ± 0.01	0.08 ± 0.01
Biceps femoris posterior (92%)	11 ± 1	13 ± 3	0.08 ± 0.01	0.10 ± 0.02
Semitendinosus (83%)	12 ± 2	16 ± 4	0.08 ± 0.01	0.12 ± 0.03
Semimembranosus, red (72%)	15 ± 2	24 ± 7	0.11 ± 0.02	0.18 ± 0.05
Semimembranosus, white (100%)	13 ± 2	11 ± 2	0.09 ± 0.01	0.08 ± 0.01
Thigh adductors				
Adductor longus (5%)	115 ± 7	136 ± 12	0.84 ± 0.06	1.06 ± 0.12
Adductor magnus & brevis (89%)	15 ± 3	21 ± 5	0.12 ± 0.02	0.15 ± 0.04
Gracilis (77%)	16 ± 2	19 ± 3	0.11 ± 0.02	0.14 ± 0.02
Pectineus (69%)	17 ± 2	24 ± 6	0.12 ± 0.01	0.18 ± 0.04

Data are mean ± SEM. Values in parentheses indicate % type IIb + d/x according to Delp & Duan (1996). Control; n=11, BR; n=8. *P<0.05 vs. control.

Table 3. Effects of BR supplementation on exercising hindlimb muscle BF (ml · min^{-1} · 100 g^{-1}) and VC (ml · min^{-1} · 100 g^{-1} · mmHg^{-1}).

	BF		VC	
	Control	**BR**	**Control**	**BR**
Ankle extensors				
Soleus (9%)	296 ± 42	312 ± 33	2.14 ± 0.30	2.43 ± 0.23
Plantaris (80%)	207 ± 15	247 ± 15*	1.50 ± 0.10	1.94 ± 0.10*
Gastrocnemius, red (14%)	452 ± 44	500 ± 39	3.27 ± 0.98	3.93 ± 0.29*
Gastrocnemius, white (100%)	42 ± 7	66 ± 11*	0.30 ± 0.05	0.51 ± 0.08*
Gastrocnemius, mixed (91%)	149 ± 12	209 ± 17*	1.08 ± 0.08	1.64 ± 0.11*
Tibialis posterior (73%)	118 ± 17	133 ± 17	0.85 ± 0.12	1.05 ± 0.14
Flexor digitorum longus (68%)	99 ± 14	103 ± 15	0.71 ± 0.09	0.81 ± 0.11
Flexor halicus longus (71%)	75 ± 10	86 ± 9	0.54 ± 0.06	0.67 ± 0.06
Ankle flexors				
Tibialis anterior, red (63%)	343 ± 35	368 ± 31	2.47 ± 0.23	2.88 ± 0.20
Tibialis anterior, white (80%)	119 ± 14	161 ± 19*	0.85 ± 0.09	1.26 ± 0.13*
Extensor digitorum longus (76%)	55 ± 7	80 ± 10*	0.39 ± 0.05	0.62 ± 0.07*
Peroneals (67%)	128 ± 11	166 ± 7*	0.93 ± 0.08	1.31 ± 0.06*
Knee extensors				
Vastus intermedius (4%)	359 ± 39	348 ± 40	2.60 ± 0.27	2.75 ± 0.31
Vastus medialis (82%)	114 ± 18	163 ± 30	0.82 ± 0.12	1.28 ± 0.25*
Vastus lateralis, red (35%)	388 ± 43	449 ± 43	2.81 ± 0.28	3.56 ± 0.37*
Vastus lateralis, white (100%)	33 ± 5	45 ± 8	0.24 ± 0.03	0.35 ± 0.06*
Vastus lateralis, mixed (89%)	168 ± 21	227 ± 16*	1.22 ± 0.14	1.77 ± 0.14*
Rectus femoris, red (66%)	224 ± 33	310 ± 30*	1.62 ± 0.23	2.45 ± 0.26*
Rectus femoris, white (100%)	101 ± 13	178 ± 31*	0.72 ± 0.08	1.39 ± 0.23*
Knee flexors				
Biceps femoris anterior (100%)	50 ± 8	77 ± 14*	0.36 ± 0.05	0.61 ± 0.11*
Biceps femoris posterior (92%)	79 ± 8	130 ± 10*	0.58 ± 0.06	1.03 ± 0.08*
Semitendinosus (83%)	56 ± 6	75 ± 12*	0.40 ± 0.04	0.58 ± 0.09*
Semimembranosus, red (72%)	119 ± 14	174 ± 15*	0.86 ± 0.10	1.37 ± 0.11*
Semimembranosus, white (100%)	33 ± 6	61 ± 11*	0.24 ± 0.04	0.48 ± 0.09*
Thigh adductors				
Adductor longus (5%)	316 ± 38	329 ± 45	2.28 ± 0.27	2.58 ± 0.34
Adductor magnus & brevis (89%)	83 ± 8	108 ± 15*	0.60 ± 0.05	0.85 ± 0.12*
Gracilis (77%)	42 ± 15	57 ± 9*	0.30 ± 0.03	0.45 ± 0.07*
Pectineus (69%)	54 ± 8	81 ± 13*	0.39 ± 0.06	0.64 ± 0.10*

(Table 3 caption)

Data are mean ± SEM. Values in parentheses indicate % type IIb + IId/x muscle fibres according to Delp & Duan (1996). Control; n=11, BR; n=8. *P<0.05 vs. control. All 28 muscles and muscle parts of the hindlimb demonstrated elevated exercising BF and VC compared to rest within control and BR groups (P<0.05 for all).

Table 4. Effects of BR supplementation on BF (ml · min^{-1} · 100 g^{-1}) and VC (ml · min^{-1} · 100 g^{-1} · mmHg^{-1}) to the kidneys and organs of the splanchnic region measured at rest and during exercise.

	At rest				During exercise			
	BF		VC		BF		VC	
	Control	BR	Control	BR	Control	BR	Control	BR
Kidney	434 ± 33	566 ± 44	3.22 ± 0.30	4.30 ± 0.25*	421 ± 42	460 ± 51	3.04 ± 0.28	3.62 ± 0.39
Stomach	84 ± 7	91 ± 18	0.61 ± 0.06	0.66 ± 0.11	67 ± 13	59 ± 12†	0.49 ± 0.10	0.45 ± 0.08†
Adrenals	577 ± 85	664 ± 67	4.25 ± 0.68	5.22 ± 0.69	400 ± 63	540 ± 142	2.87 ± 0.44	4.30 ± 1.19
Spleen	339 ± 49	447 ± 104	2.47 ± 0.36	3.26 ± 0.69	62 ± 14†	108 ± 27†	0.44 ± 0.10†	0.85 ± 0.22†
Pancreas	118 ± 10	179 ± 66	0.86 ± 0.07	1.26 ± 0.43	110 ± 15	172 ± 74	0.80 ± 0.11	1.31 ± 0.53
Sm. intestine	313 ± 20	297 ± 36	2.30 ± 0.18	2.22 ± 0.22	240 ± 26†	255 ± 40	1.73 ± 0.18	2.00 ± 0.32
Lg. intestine	124 ± 13	147 ± 15	0.91 ± 0.10	1.11 ± 0.08	127 ± 16	155 ± 22	0.92 ± 0.10	1.20 ± 0.15
Liver **	37 ± 14	32 ± 4	0.27 ± 0.10	0.25 ± 0.04	17 ± 3	34 ± 9	0.12 ± 0.02	0.26 ± 0.07*

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

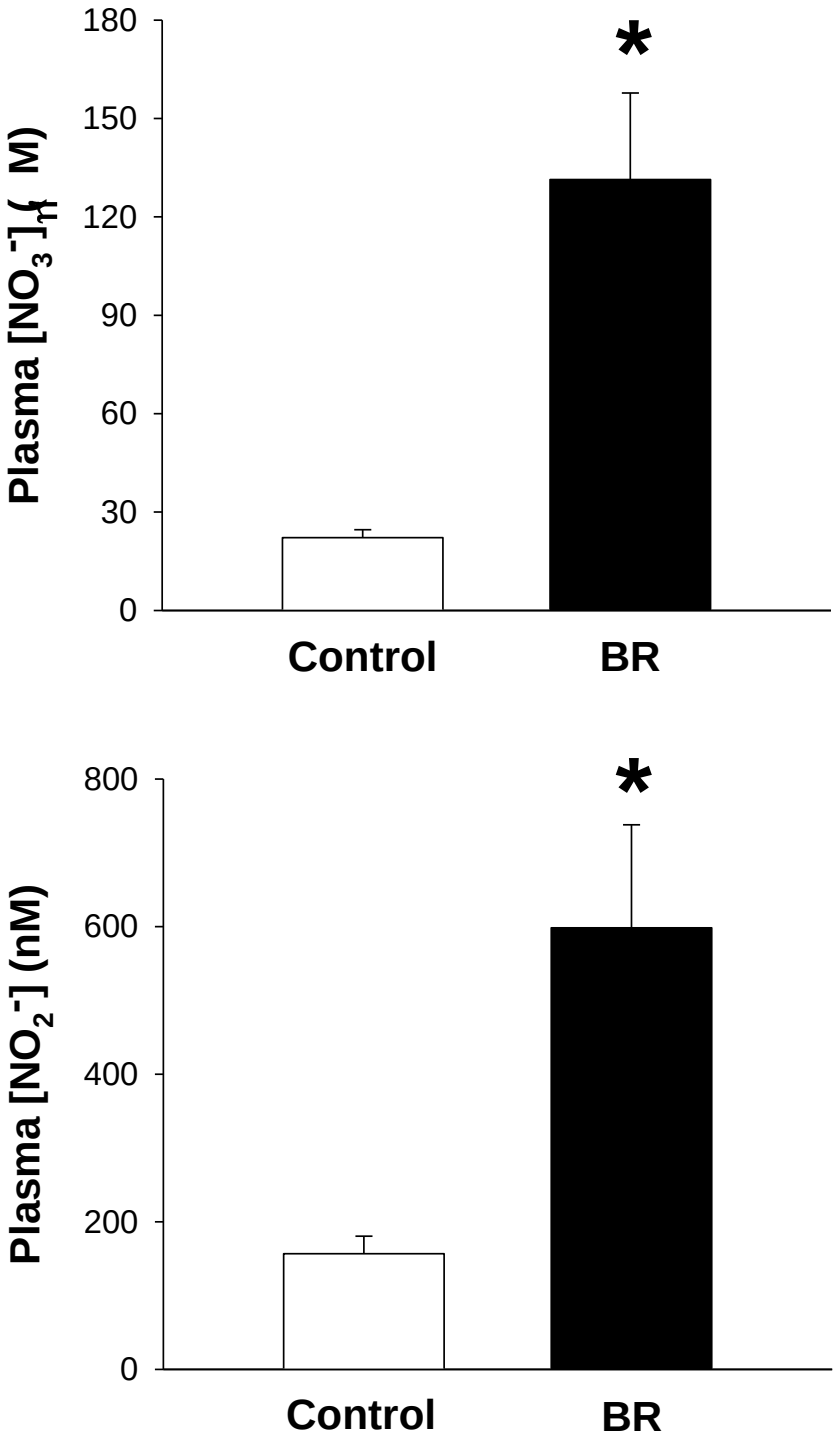

Figure 1. Effects of dietary NO_3^- supplementation with BR on plasma [NO_3^-] and [NO_2^-]. * *P*<0.05 vs. control.

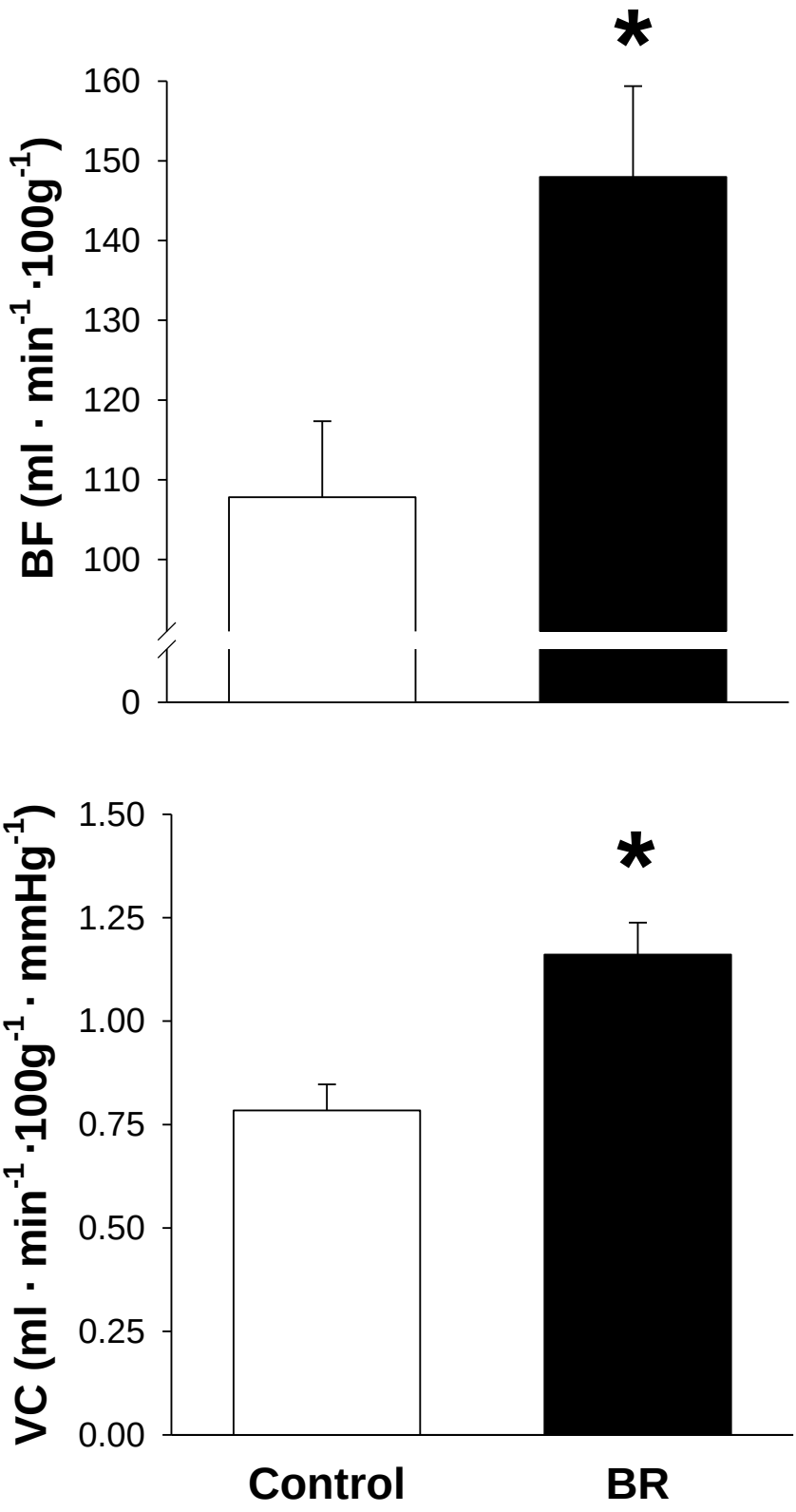

Figure 1. Effects of dietary NO_3^- supplementation with BR on total hindlimb muscle BF and VC during submaximal locomotory exercise. * *P*<0.05 vs. control.

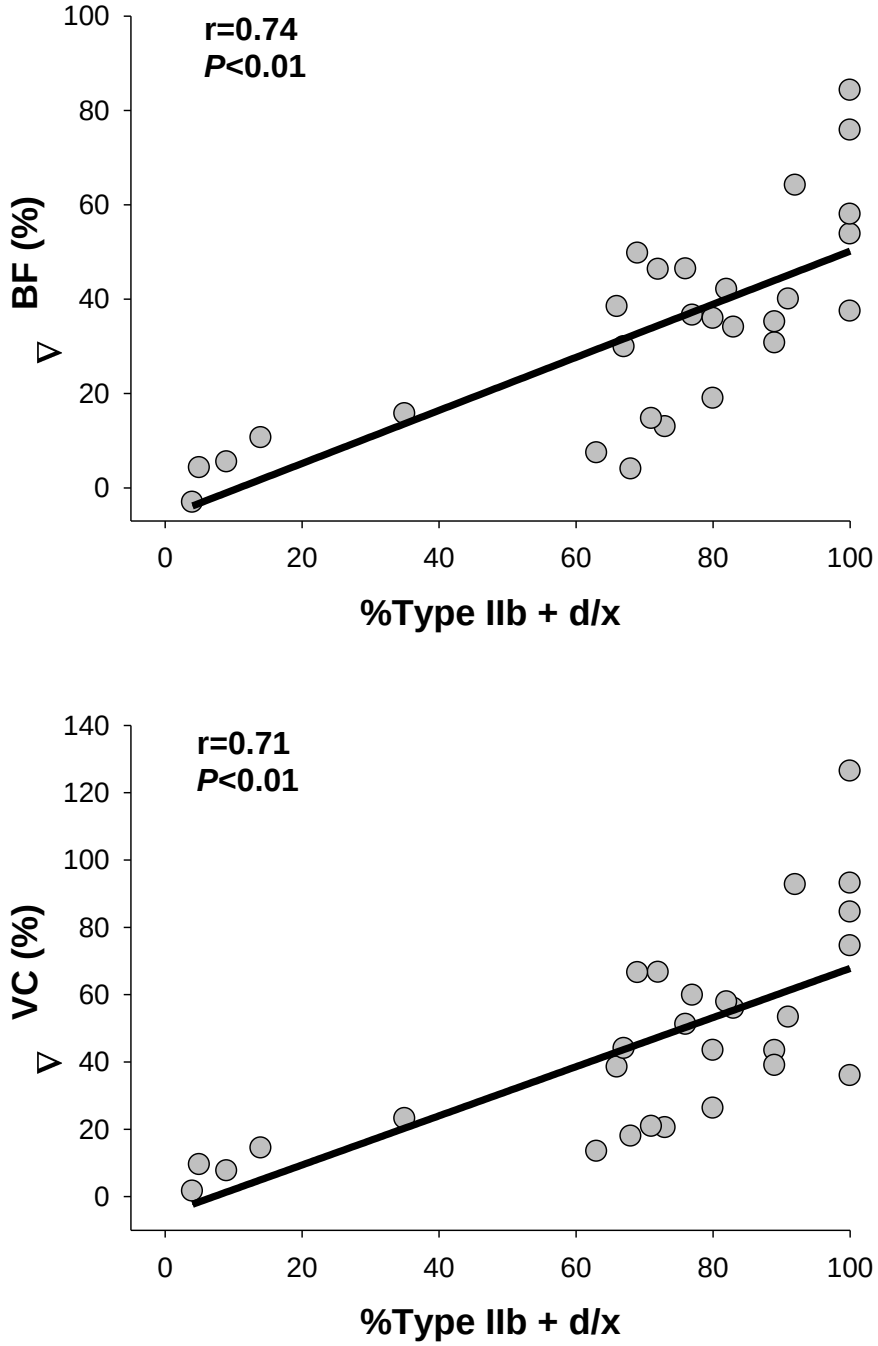

Figure 2. Relationship between the relative changes in total hindlimb muscle BF and VC (% Δ BF and VC, respectively) with dietary NO_3^- supplementation with BR during submaximal locomotory exercise and the percentage of type IIb + d/x fibres found in the individual muscles and muscle parts of the rat hindlimb according to Delp & Duan (1996).

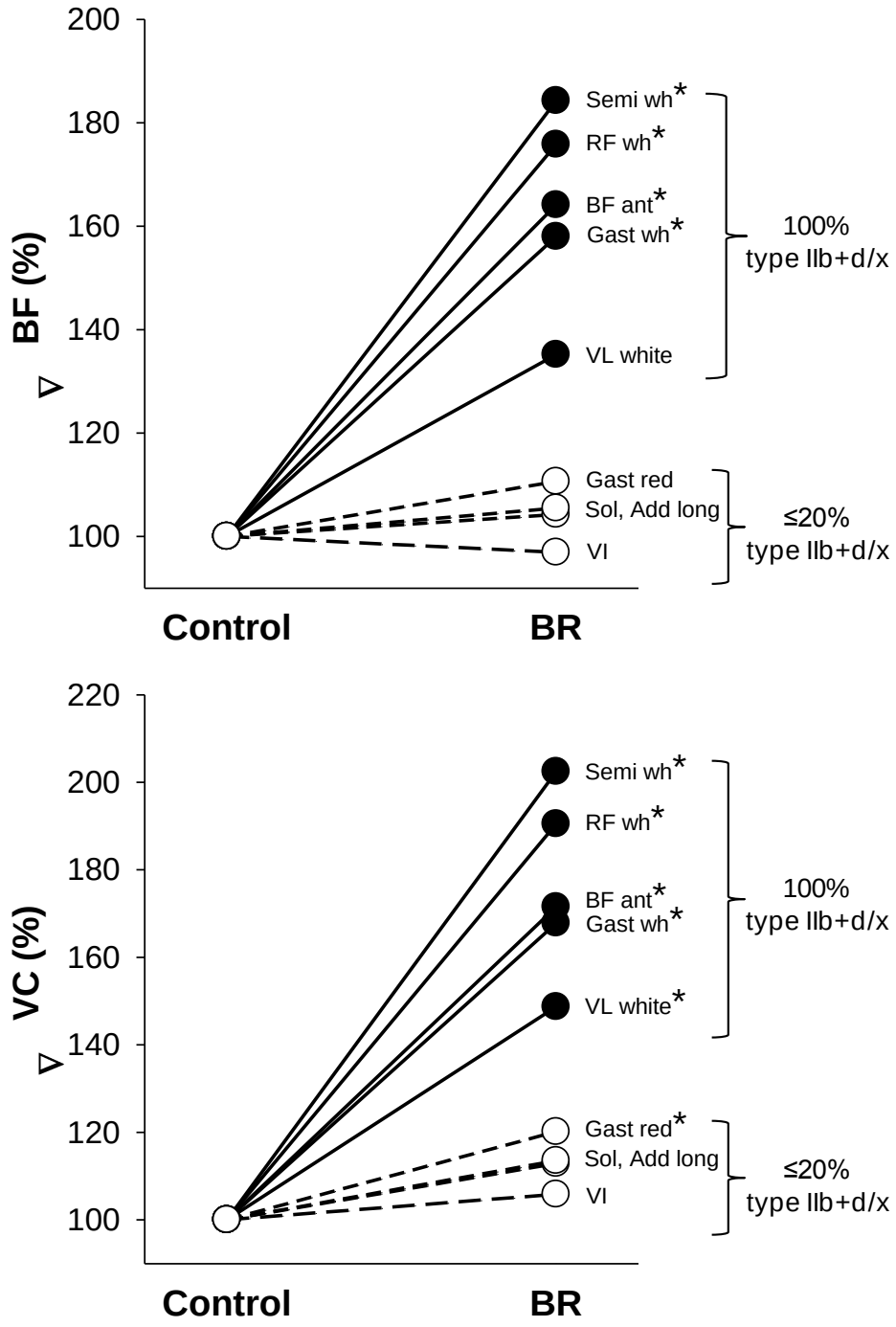

Figure 3. Relative changes in BF and VC (% Δ BF and VC, respectively) for NO_3^- supplemented rats compared to control during submaximal locomotory exercise for all hindlimb muscles and muscle parts comprised of 100% type IIb + d/x fibres (solid lines and symbols) and ≤20% type IIb + d/x fibres (dashed lines and open symbols) according to Delp & Duan (1996). * *P*<0.05 vs. control.

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