SKELETAL MUSCLE VASCULAR AND METABOLIC CONTROL: IMPACTS OF
EXOGENOUS VS. ENDOGENOUS NITRIC OXIDE SYNTHESIS

by

SCOTT KOHMAN FERGUSON

B.S., Kansas State University, 2010
M.S., Kansas State University, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

The purpose of this dissertation is to expand our knowledge on the physiological effects of the ubiquitous signaling molecule nitric oxide (NO). Focus is given to the impacts of the nitrate (NO_3^-) nitrite (NO_2^-) NO pathway on skeletal muscle vascular and metabolic function during exercise. The NO_3^--NO_2^--NO pathway has garnered tremendous research interest due to its ability to upregulate NO bioavailability independently of NO synthase (NOS) function and thus impact the metabolic responses to exercise. Chapter 2 demonstrates that NO_3^- supplementation via beetroot juice (BR) augments the skeletal muscle vascular responses to exercise. Five days of BR supplementation resulted in a significantly higher skeletal muscle blood flow (BF) and vascular conductance (VC) during exercise when compared to control. The increases in BF and VC were preferentially directed to muscles and muscle portions comprised predominantly of fast twitch fibers. Furthermore, exercising blood [lactate] was reduced, suggesting improved metabolic control. In chapter 3, BR resulted in a slower fall in the microvascular PO_2 (PO_2mv, the soul driving force for blood myocyte O_2 flux) during the crucial rest-contraction transition thereby preserving the pressure head needed to move O_2 from the capillary into the myocyte. Chapter 4 examines the effects of BR on fast vs. slow twitch muscles in which BR raised the PO_2mv during the steady state of muscle contractions in fast but not slow twitch muscles, likely due to the lower PO_2mv at rest and throughout muscle contractions within these tissues. Chapter 5 investigates the effects of direct arterial NO_2^- infusion on skeletal muscle BF and VC during exercise in rats with NOS blockade via N^G-nitro-L arginine methyl ester. NO_2^- infusion restored MAP and VC to levels observed in healthy control animals (with intact NOS function) highlighting the potential for a NO_2^- based therapy to positively impact vascular function in those with compromised NOS function such that is evident in many prevalent

diseases. These results provide crucial mechanistic insight into the improved exercise tolerance observed in humans following NO_3^- supplementation whilst also challenging our current understanding of NO's role in physiology and pathophysiology.

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Major Professor
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Dedication

To my parents, Karen and Ron Ferguson. Thank you for all of your love and for teaching me the importance of higher education. Both of you have been, and continue to be, excellent examples to follow.

Love always, your son,

Scott

Chapter 1 - Introduction

Daily living requires frequent transitions from rest to exercise in which skeletal muscle contraction initiates an immediate rise in O_2 demand ($\dot{V}O_2$). Capillary-myocyte flux is dictated by the passive process of diffusion in which the PO_2 within the microvasculature (PO_2mv) serves as the sole driving force required to deliver O_2 to the myocyte, and ultimately the mitochondria. At exercise onset, the fall in PO_2mv is constrained by an immediate and rapid increase in skeletal muscle O_2 delivery (BF, $\dot{Q}O_2$) which can exceed 5-6 L/min per L increase in $\dot{V}O_2$(39) and is accomplished by a vast array of neurohumoral (↑ cardiac output and redistribution of BF to the active tissues), peripheral mechanical activation (i.e. muscle pump and stimulation of group III afferent nerve responses), as well as local metabolic and humoral vasodilators (60, 79).

Of the local vasodilators, nitric oxide (NO) has received considerable attention since it was first identified as endothelial derived relaxation factor, produced by innermost layer of blood vessels (53, 95). Until recently, it was believed that NO was synthesized solely by the 5-electron reduction of L-Arginine, an O_2 dependent reaction carried out by the NO synthase (NOS) family of enzymes (89). Nitrate (NO_3^-) and nitrite (NO_2^-) were understood to be relatively inert end products of NO metabolism and thus were used as a systemic marker of NO bioavailability. However, over the past decade a flurry of investigations now suggests that NO_3^- and NO_2^- can be recycled to yield NO in a series of reactions that are NOS independent (11, 78). Briefly, when NO_3^- is consumed it is rapidly absorbed within the small intestine and enters the circulation. It is then actively taken up and secreted into the oral cavity via the salivary glands, thus increasing oral NO_3^- concentrations ~10 fold. NO_3^- is then reduced to NO_2^- via facultative anaerobic bacteria that are concentrated on the surface of the tongue (30), is swallowed and absorbed into

the blood stream, elevating plasma [NO_2^-]. The remaining one-electron reduction of NO_2^- to NO

is known to be facilitated in low PO_2/pH environments (26, 83, 84) such that is present to

varying degrees in skeletal muscle during exercise (14, 88).

The effects of this pathway on the physiological responses to exercise were first

described by Larsen et al. (78) who in 2007 reported that an acute oral dose of sodium NO_3^-

($NaNO_3^-$) reduced the O_2 cost of submaximal treadmill exercise. This was later expanded upon

by Bailey et al. (11) who employed NO_3^- rich beetroot juice (BR) to increase plasma [NO_3^-] and

[NO_2^-] which ultimately reduced pulmonary $\dot{V}O_2$ during submaximal exercise ($\uparrow$ efficiency) and

increased the time to exhaustion during high intensity exercise. Further investigations have

demonstrated reductions in the metabolic perturbations of exercise at simulated altitude (113),

elevated muscle contractile efficiency (10) as well as improvements in time to fatigue during

intense intermittent exercise (110, 121) (reviewed by 56). In addition, work performed by

Weitzberg and Lundberg et al. (19, 48, 75, 78, 82, 84) has suggested that dietary NO_3^- may

improve muscle mitochondrial efficiency which, when combined with the improved contractile

efficiency reported by Bailey et al. (10), could explain the lower $\dot{V}O_2$ observed during

submaximal exercise.

Collectively, these results are in stark contrast to the well-established work rate/$\dot{V}O_2$

relationship for a given submaximal exercise. More specifically, at the onset of moderate-

intensity exercise (i.e below the gas exchange threshold) pulmonary $\dot{V}O_2$, which closely reflects

gas exchange at the myocyte, rises in an exponential fashion until it reaches a steady state (~2-3

minutes in a healthy individual (117)). A linear increase in $\dot{V}O_2$ becomes apparent when plotted

against external work rate, with a slope approximating 10ml O_2/Watt/min; a relationship that was

thought to hold true irrespective of age, training status, or diet of the individual (58). In this

regard, the above highlighted effects of dietary NO_3^- are challenging fundamental tenants of exercise physiology.

The mechanisms by which dietary NO_3^- supplementation improves exercise tolerance remain to be fully elucidated, however investigations from our laboratory have provided crucial insight into the impacts of this intervention on skeletal muscle vascular and metabolic function during exercise. The investigation discussed in chapter 2 answers a crucial question surrounding the effects of dietary NO_3^- supplementation on skeletal muscle $\dot{Q}O_2$. Prior to this investigation, it had been speculated that elevations in plasma $[NO_2^-]$ would increase NO bioavailability (a potent vasodilator) which could improve the matching of $\dot{Q}O_2$ to $\dot{V}O_2$ within the active tissue bed ($\uparrow\dot{Q}O_2$). Utilizing the running rat model and the radiolabeled microsphere technique we were able to measure inter- and intra-muscular blood flow during whole body dynamic exercise in rats supplemented with NO_3^- rich BR. This technique provides accurate measurement of BF within and among discrete muscles and muscle portions and thus affords a more comprehensive interpretation of the diverse heterogeneity of skeletal muscle BF during exercise than can be obtained using measurements of tissue oxygenation (i.e. near infrared spectroscopy) in humans.

The microcirculation serves as the final frontier for the cardiovascular system as it is the site of blood-myocyte gas exchange. As previously mentioned, capillary-myocyte O_2 flux is determined by the PO_2mv and thus, alterations in this pressure ultimately impact metabolic control. The elevations in $\dot{Q}O_2$ reported in chapter two prompted us to investigate the effects of NO_3^- on the skeletal muscle PO_2mv profile at rest and during muscle contractions. This question is addressed in chapter 3. Using phosphorescence quenching We tested the hypothesis that enhanced vascular control following BR would elevate the skeletal muscle $\dot{Q}O_2$ to $\dot{V}O_2$ ratio (PO_2mv) and raise the PO_2mv during the rest-contractions transition. Chapter 4 then further

builds on the results from chapters 2 and 3 by investigating the impacts of BR on the PO_2mv profile of fast vs. slow twitch muscles revealing additional fiber type specific effects of BR supplementation.

It is now well understood that bioconversion of NO_3^- to NO_2^- is requisite to elicit the physiological effects described above. In fact, when the enterosalivary circulation is interrupted, either with the use of antibacterial mouthwash or by preventing the subject from swallowing, the changes in plasma $[NO_2^-]$ and thus any performance/therapeutic benefits are completely ablated (61, 83). In this regard, it seems logical to postulate that NO_2^- could be supplemented or administered in lieu of NO_3^-, thereby simplifying the route of delivery whilst preserving the positive effects on vascular and metabolic function. This question is addressed in chapter 5 whereby BF during exercise is measured in healthy control rats, and prior to and following direct intra-arterial infusion of $NaNO_2^-$ with NOS blockade elicited via N^G-nitro-L arginine methyl ester (L-NAME). Considering the multiple cardiovascular diseases that impair NOS function (1, 99), therapies that increase $[NO_2^-]$ may have multiple therapeutic applications. This theme is visited routinely in the following chapters and will be mentioned again in in the conclusion (chapter 6).

Chapters 2-5 are self-contained research papers that have been submitted to peer-reviewed journals for publication. All chapters, with the exception of chapter 5, have been previously published and thus reflect the individual formatting style of the journal venue. Chapter 6 provides some concise concluding remarks, potential clinical and therapeutic applications and future research directions. References from this dissertation have been appended to the end of the document and are combined for simplicity.

Chapter 2 - Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats

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Summary

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. Male Sprague-Dawley rats (3-6 months) were administered either NO_3^- (via beetroot juice; 1 mmol $\cdot$ kg^{-1} $\cdot$ 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 $\cdot$ min^{-1}, 5% grade). 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 $\cdot$ min^{-1} $\cdot$ 100 g^{-1}, $P<0.05$) and vascular conductance (control: 0.78 ± 0.05, BR: 1.16 ± 0.10 ml $\cdot$ min^{-1} $\cdot$ 100 g^{-1} $\cdot$ 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). 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.

Introduction

It is now recognized that nitric oxide (NO) functions as a major contributor to skeletal muscle vascular and metabolic control (59). NO is produced endogenously by the reduction of L-arginine to L-citrulline via three distinct NO synthase (NOS) isoforms: constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS), as well as inducible NOS (iNOS, 106). In addition, there is emerging evidence that dietary inorganic nitrate (NO_3^-) delivered, for example, via ingested BR, can be reduced to nitrite (NO_2^-) and, subsequently, NO and other reactive nitrogen intermediates and impact hemodynamic and muscle metabolic function (11, 78). These effects have been divorced from other active BR constituents (i.e. antioxidants, 74) and, crucially, the reduction of NO_2^- to NO is potentiated by hypoxic and acidic conditions (26), 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 (38, 40).

In humans, acute (2-3 hours) and chronic (3-6 days) dietary NO_3^- ingestion via sodium NO_3^- salt (78) or BR (11, 64, 112) reduces blood pressure, lowers submaximal exercising $\dot{V}O_2$, and has been shown to enhance exercise tolerance. In addition, BR ameliorates the muscle metabolic perturbations found during exercise when breathing a hypoxic inspirate (113), improves muscle oxygenation in peripheral artery disease patients (64), and improves human mitochondrial efficiency as measured using the P/O ratio (75).

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 (64, 113).

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* (29) 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) (NO$_3^-$ 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 (11, 64, 82). 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 (91).

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 (94). 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 (41).

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; (91)). 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 (5).

Determination of regional BF and VC

Following the second microsphere infusion, rats were euthanized with a sodium

pentobarbital overdose ($\geq$50 mg $\cdot$ 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 (94) and expressed as ml $\cdot$ 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 $\cdot$ min^{-1} $\cdot$ 100 g^{-1} $\cdot$ 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

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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 and Duan (27). Significance was set at *P*<0.05 and values are expressed as mean ± SEM.

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 $[NO_3^-]$ 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 ($P<0.05$).

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].

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 (11, 64, 86, 112). While there were no differences in resting MAP between groups there was a ~10 mmHg 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 suggesting that dietary NO_3^- reduces basal vasomotor tone and may play a cardioprotective role in renal vascular diseases as proposed previously (19, 84, 111).

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 (11, 26, 64, 86). However, muscle blood volume is not a measurement of BF per say 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. 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 (14, 39, 88). Cosby et al. (26) 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 (12) and the attenuation of skeletal muscle sympathetic vasoconstriction (i.e. functional sympatholysis) within glycolytic muscles during contractions (109) is mediated, at least in part, by NO (28, 109). 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.

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 (11,

74) but not long duration exercise performance of highly trained endurance athletes (21, 118).

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 (48).

BR resulted in substantially higher hindlimb skeletal muscle BF and VC despite no reductions in BF or VC to renal or splanchnic organs during exercise compared to control, 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 (123). 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. (92) 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, (11, 75, 78, 112), the ~38% increase in total hindlimb BF 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 (10, 113) may have been mediated, in part, by elevated O_2 driving pressures in the microvasculature which reduce PCr breakdown (47, 114) and speed PCr recovery kinetics during hypoxia (45). This mechanism is consistent with the lower blood [lactate] found herein with the BR group during exercise but remains to be tested specifically.

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 (25) and skeletal muscle microvascular function (e.g., PO_{2mv}, (14)), 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; (99)). A prime example illustrating the potential clinical benefits of BR has already been demonstrated by Kenjale et al. (64) 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 (6) as well as a comparison between the present control data and previous data from our laboratory (23) 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 (10, 11, 64, 75, 113).

Table 2.1 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 2.2 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 2.3 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 2.4 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
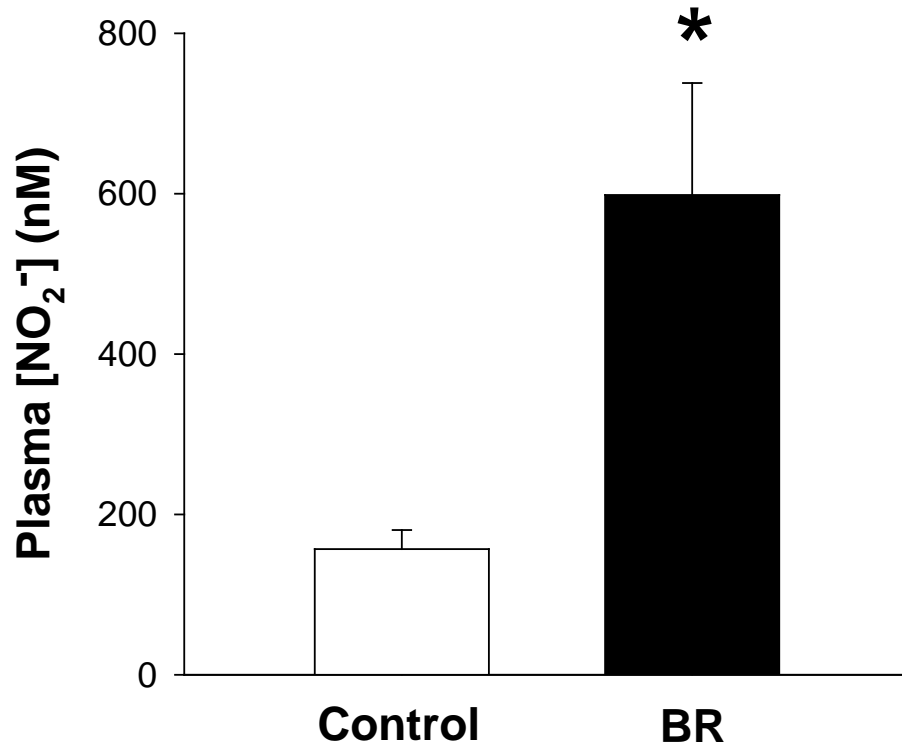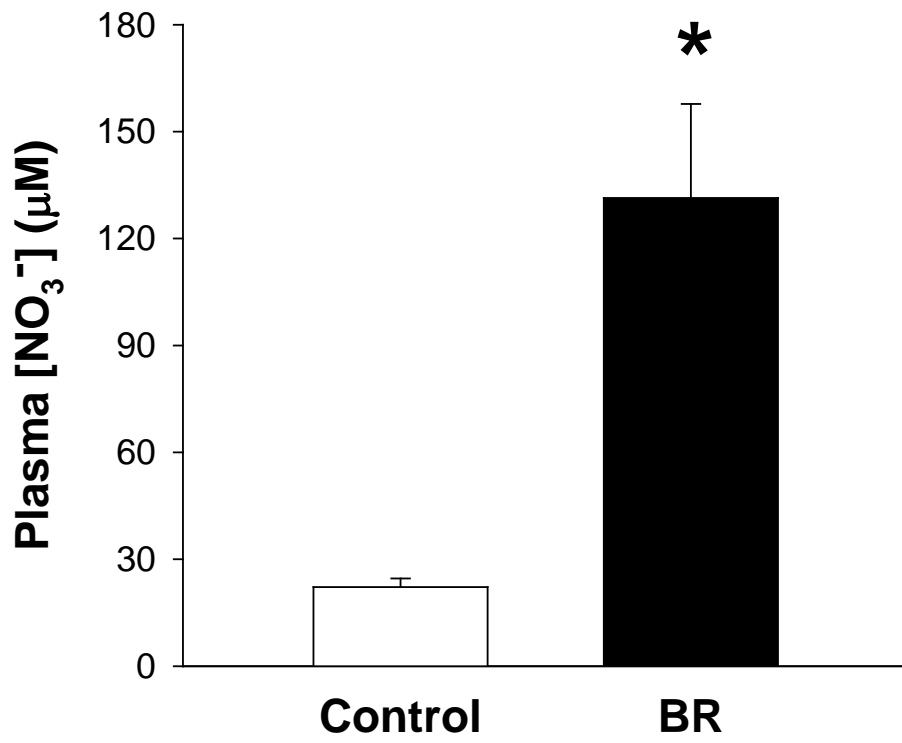
Figure 2.1 Effects of dietary NO$_3^-$ supplementation with BR on plasma [NO$_3^-$] and [NO$_2^-$]. * *P*<0.05 vs. control.
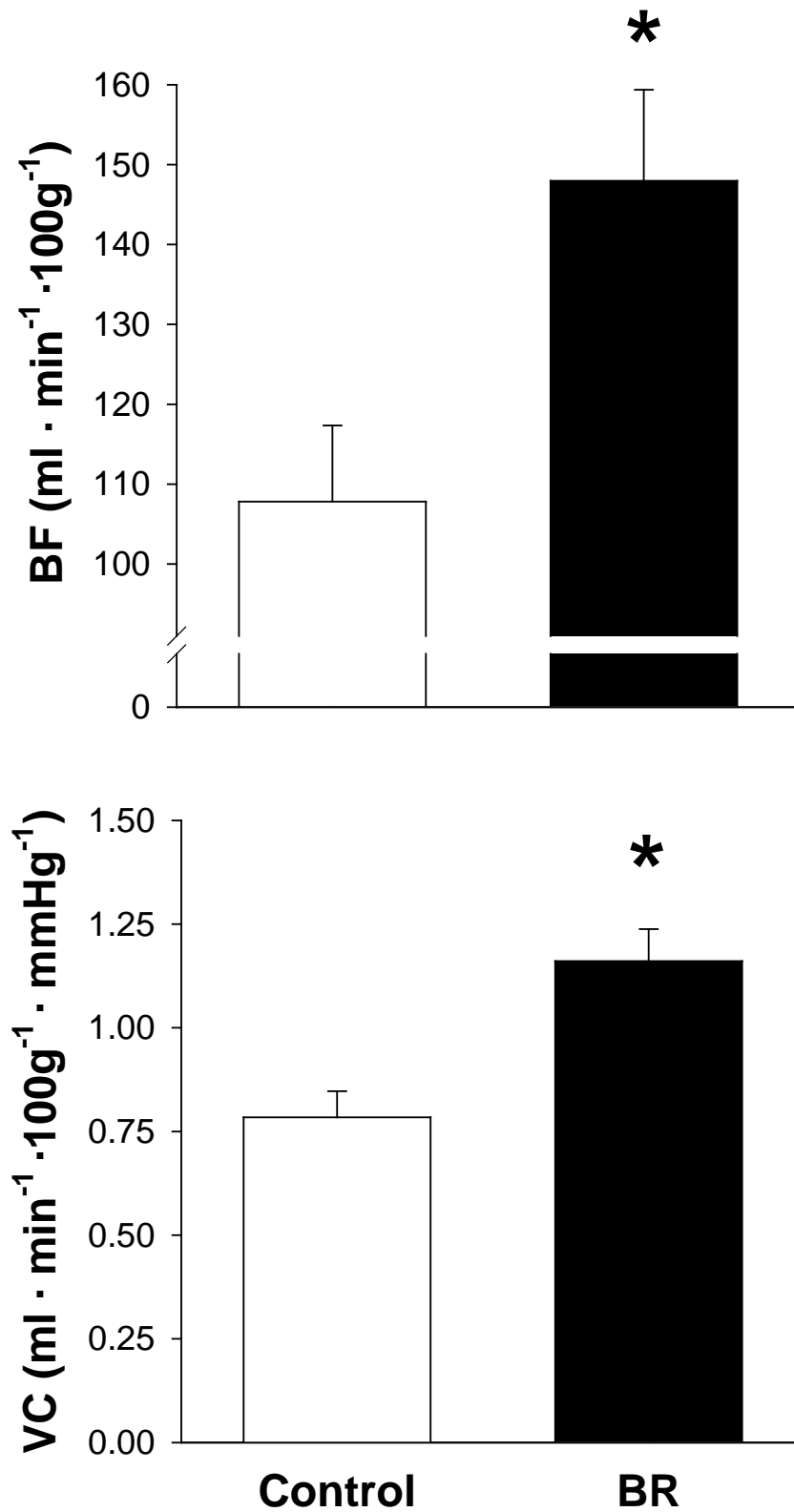
Figure 2.2 Effects of dietary NO$_3^-$ supplementation with BR on total hindlimb muscle BF and VC during submaximal locomotory exercise. * *P*<0.05 vs. control.
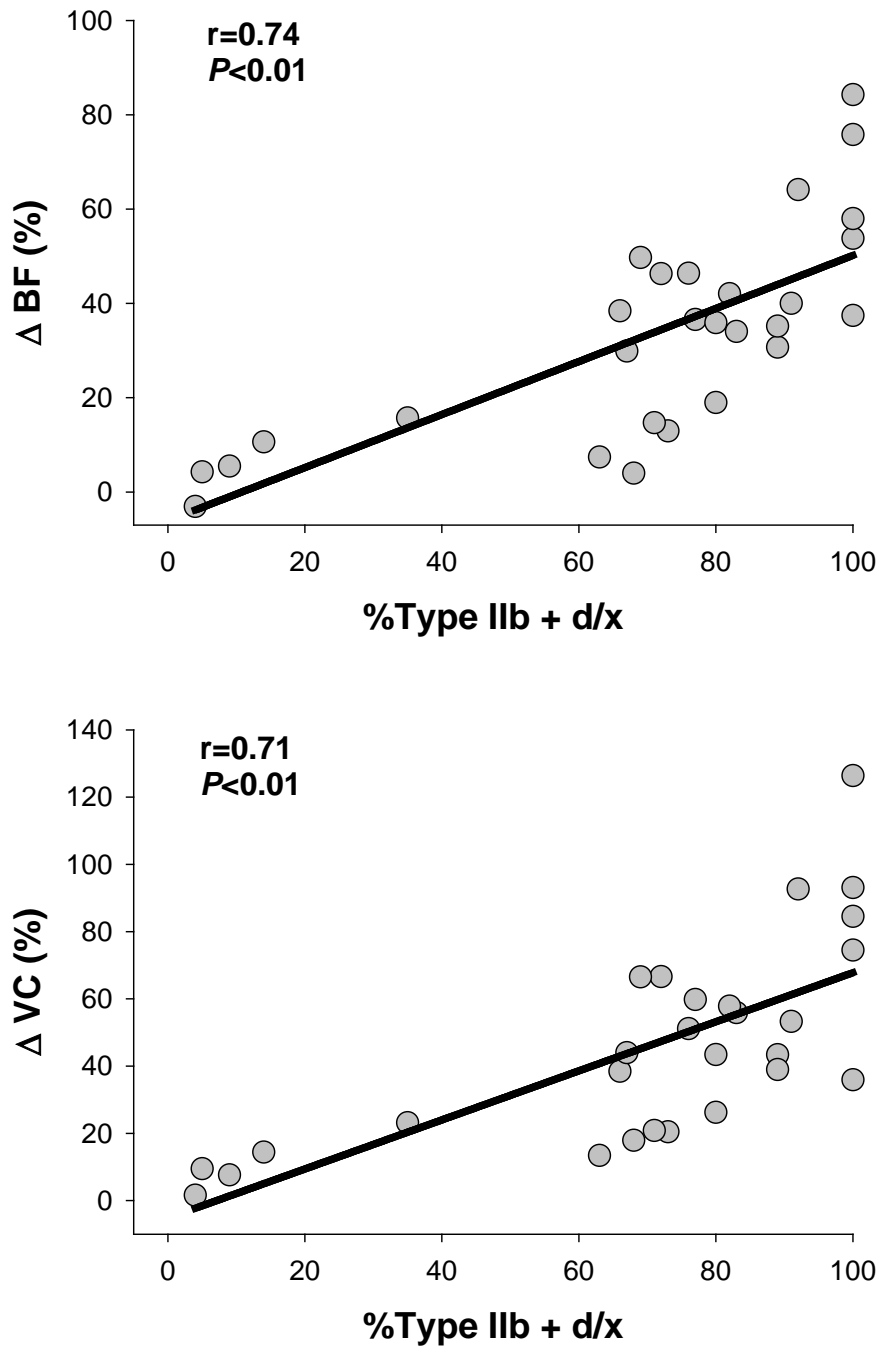
Figure 2.3 Relationship between the relative changes in total hindlimb muscle BF and VC (% Δ BF and VC, respectively) with dietary NO_3^- supplementation with BR during submaximal locomotory exercise and the percentage of type IIb + d/x fibres found in the individual muscles and muscle parts of the rat hindlimb according to Delp & Duan (27).
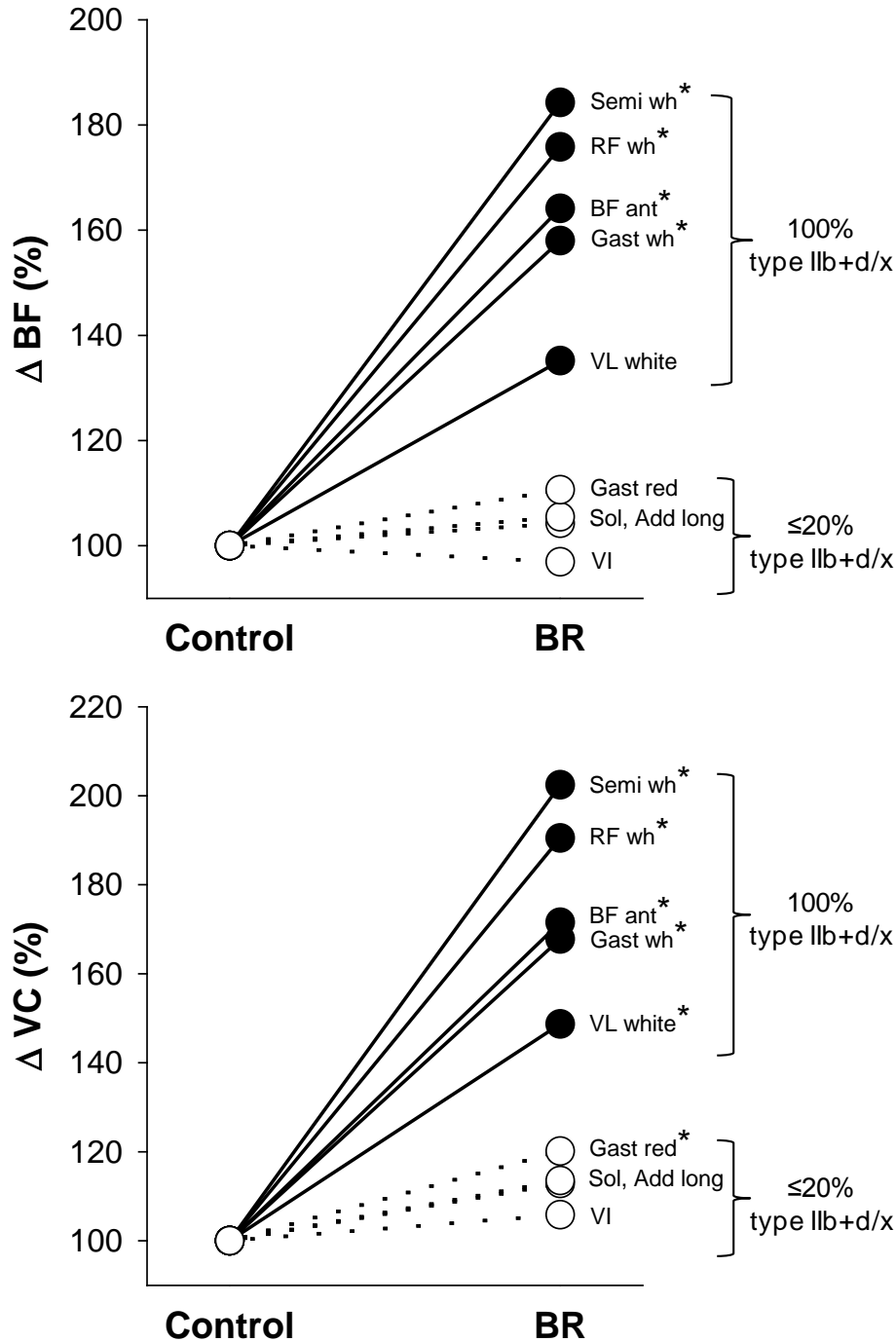
Figure 2.4 Relative changes in BF and VC (% Δ BF and VC, respectively) for NO₃⁻ supplemented rats compared to control during submaximal locomotory exercise for all hindlimb muscles and muscle parts comprised of 100% type IIb + d/x fibres (solid lines and symbols) and ≤20% type IIb + d/x fibres (dashed lines and open symbols) according to Delp & Duan (1996). * *P*<0.05 vs. control. Semi wh, white portion of the semitendinosus; RF wh, white portion of the rectus femoris; BF ant, anterior portion of the biceps femoris; Gast

wh, white portion of the gastrocnemius; VL white, white portion of the vastus lateralis; Gast red, red portion of the gastrocnemius; Sol, soleus; Add long, adductor longus; VL, vastus lateralis.

Chapter 3 - Effects of nitrate supplementation via beetroot juice on contracting rat skeletal muscle microvascular oxygen pressure dynamics

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Summary

NO_3^- supplementation via beetroot juice (BR) augments exercising skeletal muscle blood flow subsequent to its reduction to NO_2^- then NO. We tested the hypothesis that enhanced vascular control following BR would elevate the skeletal muscle O_2 delivery/O_2 utilization ratio (microvascular PO_2, $PmvO_2$) 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 (MRT_1; 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 O_2 driving pressure during the crucial rest-contractions transients thereby providing a potential mechanism by which BR supplementation may improve metabolic control.

Introduction

At exercise onset, skeletal muscle blood flow ($\dot{Q}m$) is elevated via a complex array of mechanical (muscle pump) as well as vasodilatory controllers (60) in an effort to balance O_2 delivery ($\dot{Q}O_2$)-to-utilization ($\dot{V}O_2$). Across species, for small and large muscle mass exercise $\dot{Q}O_2$ increases between 5 and 6 L per L $\dot{V}O_2$ irrespective of muscle fiber-type composition (39, 57, 100, 116) serving to meet the rising metabolic demands during the rest-contraction transient. However, owing to a lower intercept of the $\dot{Q}O_2$-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 $\dot{Q}O_2$ than their slow-twitch (type I) counterparts such that their fractional O_2 extraction at rest is higher and thus their microvascular PO_2 ($PmvO_2$) lower (14, 39, 88). 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 $PmvO_2$ values. This is particularly important given that low $PmvO_2$ values lead to reduced intramyocyte $PmvO_2$ (46, 52, 65, 102) 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 $\dot{Q}O_2$ (59) and also increases muscle mitochondrial oxidative (76) and contractile (4) 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* (10, 11, 36, 48, 78). In humans, acute NO_3^- supplementation via BR has been linked to improvements in muscle tissue oxygenation during exercise in a hypoxic environment (86, 113) 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 (64).

Recently, our laboratory demonstrated that BR supplementation in rats (NO_3^- dose 1 mmol/kg/day for 5 days) elevates $\dot{Q}O_2$ preferentially in muscles comprised of fast-twitch fibers during treadmill running (36). The resultant $\dot{Q}m$ increase would presumably elevate the $\dot{Q}O_2/\dot{V}O_2$ ratio and thus $PmvO_2$, thereby improving metabolic control, which may help explain mechanistically the lowered arterial [lactate] seen in the running rat following BR supplementation (36).

To our knowledge there have been no reports on the effects of NO_3^- supplementation on the $PmvO_2$ profile of contracting skeletal muscle. We hypothesized that 5 days of BR supplementation (NO_3^- dose: ~0.8 mmol/kg/day) would increase $PmvO_2$ across the rest/contraction transient (i.e. slow $PmvO_2$ on kinetics) and raise the subsequent steady-state $PmvO_2$.

Methods

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.

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. (19) and accounts for the resting metabolic rate of the Sprague Dawley rat (~7x faster than humans, (91)). Moreover, we have reported recently that this identical BR dose and administration period elevates plasma $[NO_3^-]$ and $[NO_2^-]$ to levels similar to those seen in humans and improves skeletal muscle O_2 delivery in exercising rats (36).

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

(9). 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 (27,

80).

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 ($\geq$50 mg/kg administered into

the carotid artery catheter).

$PmvO_2$ 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 (103):

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

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 mmHg^{-1}·s^{-1} and

τ° is 601 μs (81) 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 (81, 103).

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 (98).

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:

One component: $PmvO_{2(t)} = PmvO_{2(BL)} - \Delta PmvO_2(1 - e^{-(t-TD)/\tau})$

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 (Δtotal $PmvO_2$) was determined.

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 heparnized 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 [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 [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, 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].

Statistical analysis

Data are presented as mean±SEM. Results were compared with mixed 2-way ANOVAs (plasma [NO_3^-], MAP, and HR) with Student-Newman-Keuls *post hoc* tests where appropriate or unpaired Student's t-tests ([NO_2^-], blood gases, PmvO$_2$ kinetics parameters). Significance was accepted at $p<0.05$.

Results

Plasma [NO$_3^-$] and [NO$_2^-$]

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

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±1% mmHg, p>0.05) were not different between control and BR rats.

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 PmvO$_{2(BL)}$. 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, Δ_{total}PmvO$_2$) amplitudes, and slower PmvO$_2$ kinetics (i.e., longer MRT$_1$ and lower ΔPmvO$_2$/τ) 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).

Discussion

The primary novel finding of the present investigation was that, relative to control, 5 days of BR supplementation elevated plasma $[NO_2^-]$ and $[NO_3^-]$ 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 $\dot{Q}O_2/\dot{V}O_2$ 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.

Effects of BR on the PmvO2 kinetics profile

The principal novel finding of this investigation was the slower $PmvO_2$ kinetics in BR supplemented rats. Behnke *et al.* (14) 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; (27)) 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.* (49) 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, in part, by elevations in NO bioavailability (49). 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

$\dot{Q}O_2$ substantially (36), the higher $\dot{Q}O_2/\dot{V}O_2$ ratio seen herein may be due to a faster rate of $\dot{Q}O_2$

increase following the onset of contractions. In addition, BR supplementation may reduce $\dot{V}O_2$

due to improvements in mitochondrial and/or muscle contractile efficiency (10, 11, 48, 76, 78,

113). 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 (10, 74, 113).

Moreover, elevations in O_2 pressures within the microvasculature reduce PCr breakdown (47,

113) and speed PCr recovery in hypoxic conditions (45), which may also help explain the

improvements in exercise tolerance seen in peripheral artery disease patients following acute BR

supplementation (64). In this regard, BR supplementation may have vital implications for other

diseases hallmarked by reduced $\dot{Q}O_2$ and elevated metabolic perturbation during exercise (e.g.,

chronic heart failure, reviewed by (99)) 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 (36)

and contractile function (48). 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 (48). Considering that the

spinotrapezius muscle utilized presently is composed of approximately 52% type IIb+d/x fibers

(27) and that the elevations in blood flow demonstrated by Ferguson *et al.* (36) 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 (57, 100)). 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, (62)).

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 (43). 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 (87) thereby providing further insights into the potential metabolic effects of NO$_3^-$ supplementation.

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 $\dot{Q}$O$_2$/$\dot{V}O_2$ ratio) following the onset of muscle contractions. Muscle $\dot{Q}$O$_2$/$\dot{V}O_2$ 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.

Table 3.1 MAP and HR prior to and during the steady-state of electrically-induced muscle contractions for control and BR rats.

	Control	BR
Rest (pre-contractions)		
MAP (mmHg)	123±8	110±9
HR (bpm)	360±11	342±22
Contracting steady-state		
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 3.2 Microvascular partial pressure of O_2 ($PmvO_2$) kinetics parameters during contractions for control and BR rats.

	Control	BR
$PmvO_{2(BL)}$, mmHg	30.2±2.0	28.6±2.3
$\Delta_1 PmvO_2$, mmHg	16.8±1.4	12.0±1.5*
$\Delta_2 PmvO_2$, mmHg	4.1±0.7	5.6±1.9
$\Delta_{total} PmvO_2$, mmHg	13.5±1.4	9.2±1.3*
$PmvO_{2(steady-state)}$, mmHg	17.4±1.6	18.5±1.0
TD_1, s	6.9±1.4	11.8±1.8*
TD_2, s	46.7±10.2	26.0±3.5
τ_1, s	9.9±1.1	12.6±1.9
τ_2, s	88.0±16.2	76.6±12.9
MRT_1, s	16.8±1.9	24.4±2.7*
T_{63}, s	16.2±1.6	23.8±3.2*
$\Delta_1 PmvO_2/\tau_1$, 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 $PmvO_2$ data (control: n=8 of 10; BR: n=5 of 10). $PmvO_{2(BL)}$, pre-contracting $PmvO_2$; $\Delta_1 PmvO_2$, amplitude of the first component; $\Delta_2 PmvO_2$, amplitude of the second component; $\Delta_{total} PmvO_2$; overall amplitude regardless of one- or two-component model fit; $PmvO_{2(steady-state)}$, contracting steady-state $PmvO_2$; 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, mean response time describing the overall kinetics response; T_{63}, time to reach 63% of the overall response determined independent of modeling procedures; $\Delta_1 PmvO_2/\tau_1$, parameter describing the relative rate of $PmvO_2$ fall. *p<0.05 versus control.
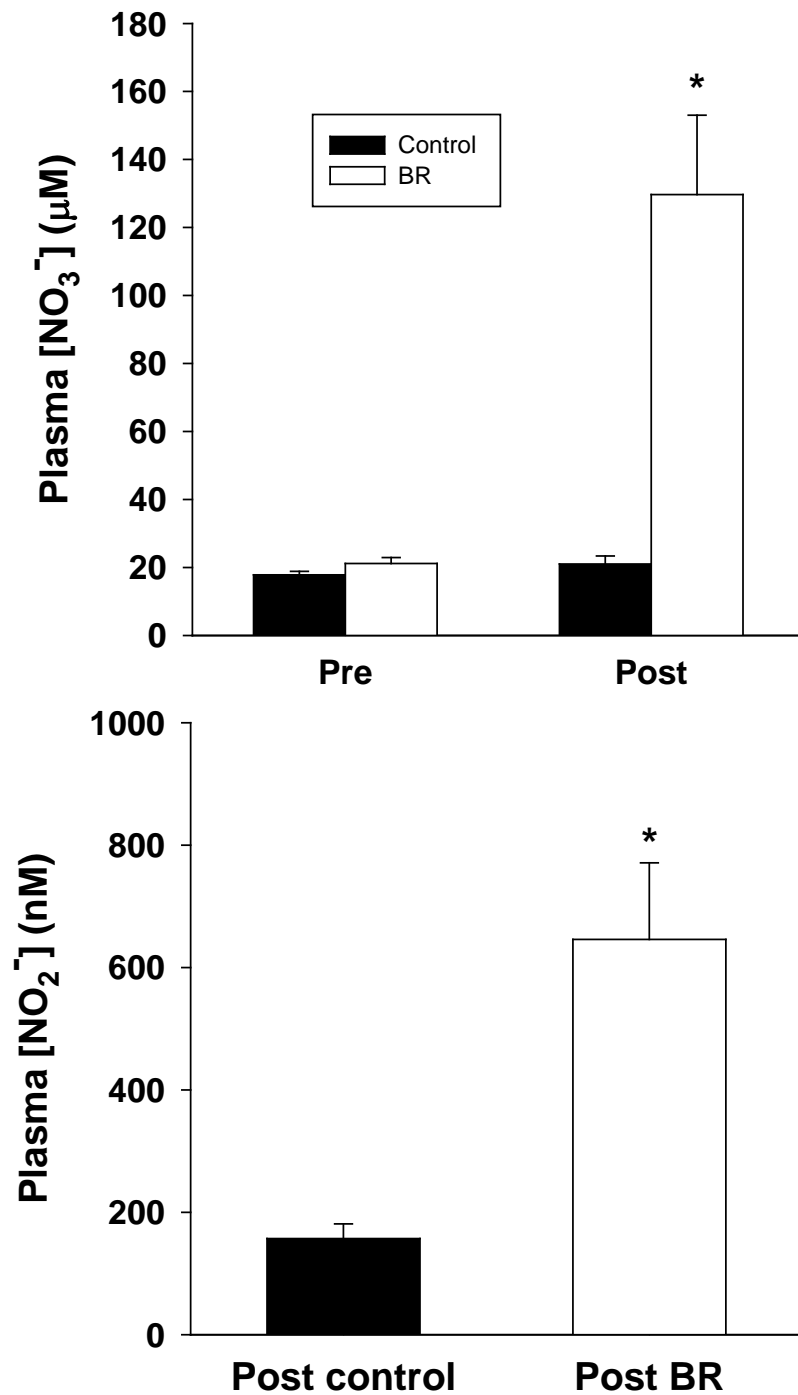
Figure 3.1 Top panel: Pre- and post-supplemention plasma [NO$_3^-$] for control and BR rats. Bottom panel: Post-supplemention plasma [NO$_2^-$] for control and BR rats. *p<0.05 versus control.
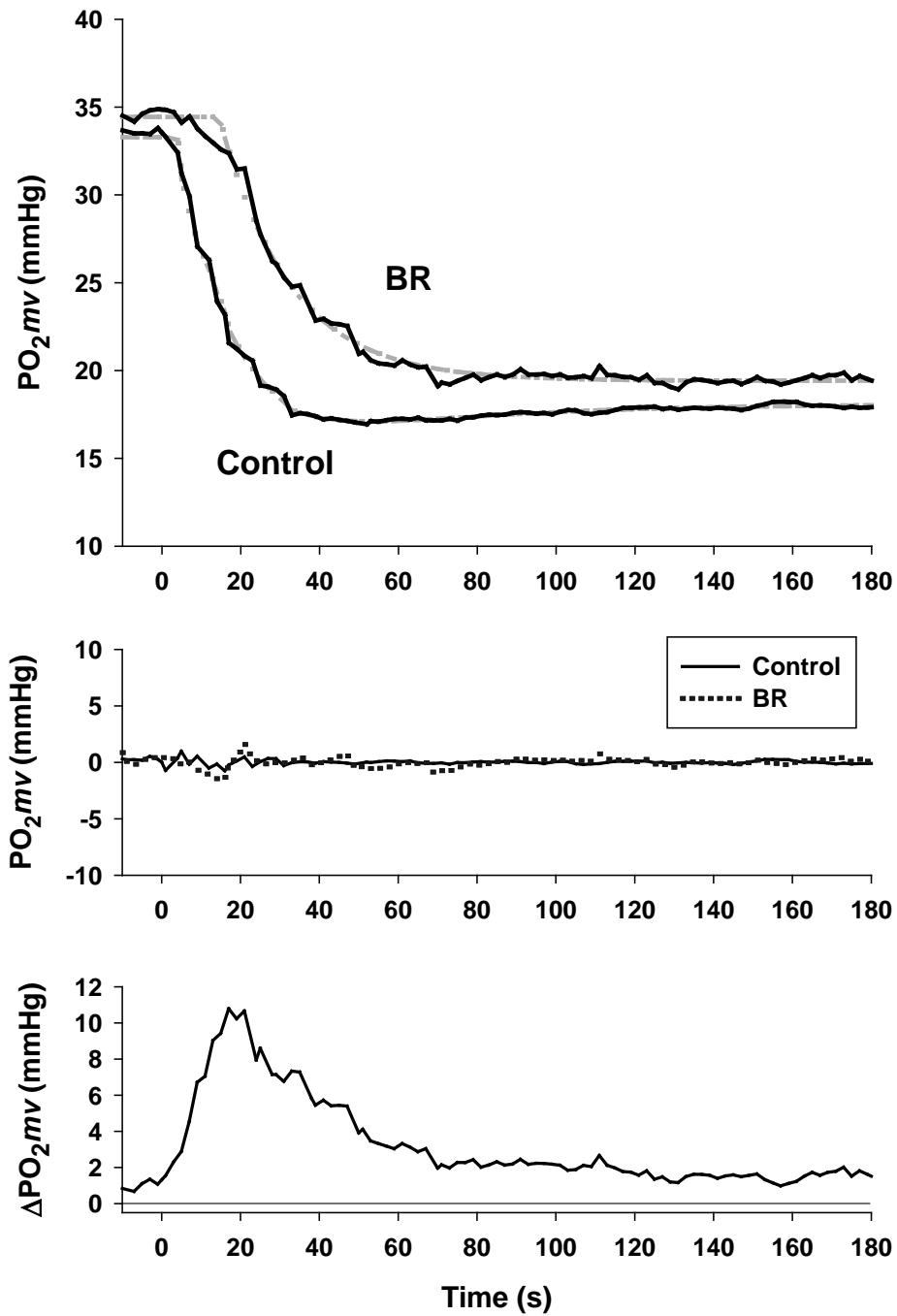
Figure 3.2 Top panel: Representative P*mv*O₂ profiles (black lines) and their model fits (gray lines) for one control and one BR rat. Middle panel: P*mv*O₂ residuals demonstrate excellent model fits. Bottom panel: Absolute P*mv*O₂ difference (BR-control) for responses shown in top panel. Time "0" represents the onset of contractions. Note greatest effect of BR across the initial transient (i.e. 0-60 s).

Chapter 4 - Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation

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Summary

Nitrate (NO_3^-) supplementation via beetroot juice (BR) preferentially improves vascular conductance and O_2 delivery to contracting skeletal muscles comprised predominantly of type IIb + d/x (i.e. highly glycolytic) fibers following its reduction to nitrite and nitric oxide (NO). To address the mechanistic basis for NO_3^- to improve metabolic control we tested the hypothesis that increased NO bioavailability via BR supplementation would elevate microvascular PO_2 (PO_2mv) in fast twitch but not slow twitch muscle. Twelve young adult male Sprague-Dawley rats were administered BR ([NO_3^-] 1 mmol/kg/day, n=6) or water (control, n=6) for 5 days. PO_2mv (phosphorescence quenching) was measured at rest and during 180s of electrically induced 1-Hz twitch contractions (6-8 V) of the soleus (9% type IIb +d/x) and mixed portion of the gastrocnemius (MG, 91% type IIb + d/x) muscles. In the MG, but not the soleus, BR elevated contracting steady state PO_2mv by ~43% (control: 13.7 ± 0.5, BR: 19 ± 1.6 mmHg, ($P<0.05$). This higher PO_2mv represents a greater blood-myocyte O_2 driving force during muscle contractions thus providing a potential mechanism by which NO_3^- supplementation via BR improves metabolic control in fast twitch muscle. Recruitment of higher order type II muscle fibers is thought to play a role in the development of the $\dot{V}O_2$ slow component which is inextricably linked to the fatigue process. These data therefore provide a putative mechanism for the BR-induced improvements in high-intensity exercise performance seen in humans.

Introduction

At exercise onset, the immediate increase in ATP turnover within contracting skeletal muscle mandates an elevated rate of O_2 delivery ($\dot{Q}O_2$), such that capillary blood flow is rapidly increased to meet the rising O_2 demand of contracting myocytes (i.e. O_2 uptake; $\dot{V}O_2$) (66). This augmented capillary flow is accomplished via elevated cardiac output and blood flow redistribution (neurohumoral activation) as well as local mechanical and vasomotor mechanisms (60). Of the local controllers, the powerful signaling molecule nitric oxide (NO) promotes vasodilation of terminal arterioles within skeletal muscle, helping to facilitate this hyperemic response and better match $\dot{Q}O_2$ to the elevated $\dot{V}O_2$ demands (51, 59, 67).

It is now understood that nitrate (NO_3^-) and nitrite (NO_2^-) can be converted to NO and other reactive nitrogen species *in vivo* following a stepwise reduction (83). In humans, dietary NO_3^- supplementation has been shown to enhance muscle contractile (10) and mitochondrial (P/O ratio; (75)) efficiency, both of which are associated with a reduction in the O_2 cost of submaximal exercise (10, 11, 20, 74, 75, 77, 112) and improvements in tolerance to high intensity exercise (10, 11, 16, 63, 74, 77, 90, 120).

What is particularly interesting is that the improvements in performance have been seen predominantly during severe-intensity exercise (16, 121) rather than long term endurance exercise (21, 118). Recent studies performed in murine models suggest that this phenomenon may be due to a fiber type selective enhancement in skeletal muscle vascular and metabolic control following NO_3^- ingestion (36, 48). Specifically, our laboratory has demonstrated that rats supplemented with BR (NO_3^- concentration 1 mmol/kg/day) for five days had higher exercising blood flow and vascular conductance in muscles comprised principally of type II muscle fibers (36). BR also raised the pressure head for capillary-myocyte O_2 flux during the crucial transition period from

rest to muscle contractions (i.e., ~20-60 s) in the rat spinotrapezius muscle, which is composed of approximately 50% Type IIb+d/x muscle fibers (27, 35). Moreover, Hernandez *et al.* (48) demonstrated improved calcium handling and rate of force development in type II but not type I muscles of NO_3^- supplemented mice.

Given the lower contracting PO_2mv reported in fast twitch muscles (88) and the evidence that NO_2^- reduction to NO is potentiated in environments with low PO_2 (26) the physiological effects of BR on the PO_2mv profile may be intensified in fast twitch muscles. Therefore the purpose of the present investigation was to examine the effects of 5 days of NO_3^- supplementation via BR (NO_3^- concentration 1 mmol/kg/day) on the PO_2mv profile of rat muscles comprised of predominantly type I (slow twitch) and type IIb+d/x (fast twitch) muscle fibers. We tested the hypothesis that BR would attenuate the fall in PO_2mv in the fast twitch mixed portion of the gastrocnemius (MG) across the rest-contraction transition with either a lesser or no effect in the slow twitch soleus muscle.

Methods

Animal selection and care

Twelve young adult male Sprague-Dawley rats (average body mass = 521±20 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 Institutes of Health guidelines.

Supplementation protocol

Rats were randomly assigned to receive 5 days of BR supplementation with a NO_3^- dose of 1 mmol/kg/day (BR; n=6, Beet it™, James White Drinks, Ipswich UK, diluted with 100 ml of tap water) or NO_3^- depleted BR (control; n=6, Beet it™ placebo, diluted with 100 ml of tap water) with consumption monitored. This NO_3^- dose (1 mmol/kg/day) represents a NO_3^- concentration similar to that used in humans by Jones and colleagues (11, 74, 112, 120) after accounting for the resting metabolic rate of rats [~7x that of humans, 28]. In addition, this dose was used in our laboratory previously with significant vascular effects observed following supplementation (35, 36).

Surgical instrumentation

Rats were anaesthetized with a 5% isoflurane-O_2 mixture and maintained subsequently on 3% isoflurane-O_2 mixture. 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 mean arterial pressure (MAP) and heart rate (HR), arterial blood sampling (Nova State Profile M, Waltham, MA, USA) and, infusion of the phosphorescent probe (see below). A second catheter was also

placed in the caudal artery. Incisions were then closed and the rats were transitioned to pentobarbital sodium anesthesia (administered to effect and subsequently maintained via the caudal artery catheter) with level of anesthesia monitored continuously via the toe pinch and blink reflexes. If indicated, additional pentobarbital sodium was administered in supplemental dosage (5-10 mg/kg) as needed. Rats were then transferred onto a heating pad to maintain core body temperature at ~38˚C (measured via rectal probe thermometer) and the carotid artery catheter was connected to a pressure transducer (Digi-Med BPA model 200, Louisville, KY, USA) for measurement of MAP and HR.

The muscles chosen for the present experiment (soleus and mixed portion of the gastrocnemius, MG) were selected based on their fiber type composition (27) and represent the spectrum of slow twitch (type I/IIa) and fast twitch (type IIb+d/x) muscle fiber types. The highly oxidative soleus (84% type I, 7% type IIa and 9% type IIb+d/x, (27)) serves as a postural muscle whose primary functions are ankle stabilization and plantar flexion while the MG functions in plantar flexion and is comprised predominantly of highly glycolytic fast-twitch muscle fibers (3% type I, 6% type IIa, 91% type IIb+d/x, (27)). Each muscle was exposed for PO_2mv experiments in the following manner. Overlaying skin and fascia along the sagittal plane on the right hindlimb were reflected carefully to expose the muscles of the 'calf'. For measurements made in the MG, silver wire electrodes were sutured (6-0 silk) to the proximal (cathode) and distal (anode) portions of the muscle. Following measurements of PO_2mv in the MG the soleus muscle was exposed by carefully reflecting overlaying tissue covering the peroneal muscle group (cornal plane) and silver wire electrodes were sutured (6-0 silk) in the same manner as for the MG. Measurement order was randomized to avoid the potential influences of an ordering effect caused by NO_3^- metabolism. The exposed muscles were continuously superfused with 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). This method has been used previously in our laboratory and facilitates access to the MG and soleus muscles whilst minimizing perturbation caused by surgery (14).

Experimental protocol

The phosphorescent probe palladium meso-tetra (4 carboxyphenyl)tetrabenzoporphyrin-dendrimer (G2: 1–5 mg/kg 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 lateral surface of the exposed muscle (either MG or soleus) of the right hindlimb 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). As an indicator of preserved vasomotor function, it was ensured that PO_2mv returned to baseline values following the contraction period. Rats were euthanized via pentobarbital sodium overdose (≥50 mg/kg administered into the carotid artery catheter). Power analysis based on a known sample variability of PO_2mv and anticipated supplementation effects (35, 36) indicate that six rats per group would be sufficient to demonstrate a statistical difference, if present.

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 (103):

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

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 G2, k_Q is 273 mmHg/s and τ°

is 251 µs at 38°C [31] 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 (81, 103).

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

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. The two

component model was only used when the PO_2mv increased above its initial nadir during

contractions. 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 and prior to 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.

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

Post-supplementation blood samples were collected following the experiment via the

caudal artery catheter to assess: 1) plasma $[NO_3^-]$ and $[NO_2^-]$; and 2) pH; PO_2; and $\%O_2$

saturation. For measurements of 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 blood [lactate], 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]. Signals obtained using potassium iodide were then subtracted from those with vanadium chloride to provide a clearer representation of the NO_3^- concentrations.

Statistical analysis

Data are presented as mean ± SEM. Results were compared within and between groups using mixed 2-way ANOVAs (MAP and HR) with Student-Newman-Keuls *post hoc* tests where appropriate or unpaired student's t-test (PO_2mv kinetics parameters, blood gasses, [NO_3^-], [NO_2^-]). Significance was accepted at *P*<0.05.

Results

Plasma [NO$_3^-$] and [NO$_2^-$]

BR rats had significantly higher plasma [NO$_3^-$] compared to control (control: 29 ± 6, BR: 79 ± 17 µmol/l, *P*=0.02).). Relative to control, plasma [NO$_2^-$] tended to be higher in BR rats, however these changes did not reach significance (control: 156 ± 66, BR: 216 ± 46 nmol/l, *P*=0.22).

MAP, HR, and arterial blood gases

There were no between group differences in arterial PO$_2$, PCO$_2$, or pH (data not shown, *P*>0.05 for all). Resting (control: 1.6 ± 0.1, BR: 1.1 ± 0.2 mmol/l, *P*>0.05) arterial blood [lactate] was not different between groups. There were no differences in resting or contracting steady-state MAP for the soleus (resting control: 122 ± 7, resting BR: 114 ± 6, contracting control: 124 ± 6, contracting BR: 117 ± 8 mmHg, *P*>0.05 for all) or MG (resting control: 114 ± 5, resting BR: 106 ± 6, contracting control: 116 ± 6, contracting BR: 110 ± 6 mmHg, *P*>0.05 for all). Furthermore, there were no differences in resting or contracting steady-state HR for the soleus (resting control: 364 ± 16, resting BR: 345 ± 25, contracting control: 366 ± 17, contracting BR 381 ± 45 mmHg, *P*>0.05 for all) or MG (resting control: 377 ± 16, resting BR: 372 ± 35, contracting control: 391 ± 19, contracting BR: 354 ± 52 mmHg, *P*<0.05 for all).

PO$_2$mv baseline, contracting steady-state and kinetics parameters

Baseline and contracting steady-state: As expected control PO$_2$mv$_{(base line)}$ and contracting PO$_2$mv$_{(steady-state)}$ (evident over the final ~30 s of contractions) were higher in the soleus compared to MG (Table 1, Figure 1). However, during contractions BR supplemented rats demonstrated an elevated PO$_2$mv$_{(steady-state)}$ in the MG when compared to control (Table 1, *P*=0.01) with no significant difference evident in the soleus (Table 1, *P*=0.31). It was noteworthy that BR raised PO$_2$mv$_{(steady-state)}$ in the MG to that found in the control soleus.

PO$_2$mv kinetics parameters: The kinetics following the onset of contractions for the soleus were adequately fit by a one-component model in all control and BR rats. In contrast, for the MG, the more complex two-component model was necessary for 1 of 6 control and all BR rats. Indeed, ~80% of the increased PO$_2$*mv*$_{(steady-state)}$ for the BR group resulted from the secondary component increase of PO$_2$*mv* (Table 1). The coefficient of determination (r^2) for soleus and MG ($\geq$0.98 for control and BR) and low sum of squared residuals (RSS <20) for both groups suggested the appropriateness of the respective model fits.

Table 1 presents the mean PO$_2$*mv* kinetics parameters. The time delay and time constant for the first component as well as the mean response time and T$_{63}$ were greater in the soleus versus the MG (for both control and BR, *P*<0.05 for all, Table 1). There were no between-group differences in any kinetic parameter following the onset of contractions for the soleus or MG (Table 1, *P*>0.05 for all). Importantly, within the control and BR groups the model-dependent MRT$_1$ and model-independent T$_{63}$ were not different (Table 1) providing additional confidence in the model parameters.

Discussion

The present investigation provides the first demonstration that NO_3^- supplementation-induced (via BR) elevation of PO_2mv occurs preferentially in muscles comprised of fast twitch (MG) rather than slow twitch (soleus) fibers. This finding coheres with the presence of lower PO_2mv levels in fast twitch muscles (14, 88) and a physicochemical milieu (lower pH, higher lactate) that favors the reduction of NO_2^- to NO (24). It is also true that the low PO_2 environment extant in these muscles which favors NO_2^- reduction will suppress the endogenous production of NO from the neuronal and endothelial nitric oxide synthase (nNOS, eNOS) pathways; of which nNOS is the most important in fast twitch muscles (24, 49, 70, 101). NO_3^- supplementation (BR) raises PO_2mv (i.e., $QO_2/\dot{V}O_2$ ratio) by simultaneously increasing O_2 delivery (QO2, (36)) and reducing the O_2 cost ($\dot{V}O_2$) of exercise via changes in mitochondrial and contractile function (48, 75, 78, 83). Increases in PO_2mv in-and-of themselves have the capacity to improve blood-myocyte O_2 flux, increase intramyocyte PO_2 and consequently enhance mitochondrial oxidative phosphorylation whilst suppressing glycolysis. This behavior may underlie the reduced arterial [lactate] levels found during heavy intensity exercise in running rats (36).

Effects of BR on the PO₂mv profile

As also shown in the present investigation, previous studies have reported pronounced fiber type differences in both kinetics parameters and the magnitude of the overall change in PO_2mv following the onset of contractions. Specifically, Behnke *et al.* (14) found a longer time delay, MRT, slower rate of PO_2mv fall, and a lower overall amplitude of PO_2mv fall (e.g. Δ PO_2mv) in the soleus (slow-twitch) vs. peroneal (fast-twitch) muscles during electrically induced contractions. Additionally, McDonough and colleagues (88) reported a higher PO_2mv (steady-state) in soleus versus MG muscles. In the present investigation, despite BR raising the MG PO_2mv(steady-

state) to levels commensurate with those observed in the soleus muscle of control rats herein ($PO_2mv_{(steady-state)}$ ↑43%, Table 1) the kinetics profile was unchanged. This situation does not mean that the kinetics profile is intransigent. Indeed, changes in the PO_2mv kinetics profile can be driven by both NOS-dependent and independent mechanisms invoked by 6-8 weeks of exercise training (49). Interestingly, the training-induced adaptation of the PO_2mv kinetics profile occurred in the absence of an elevated $PO_2mv_{(steady-state)}$. Thus, exercise training raises PO_2mv across the dynamic transition at a time when $\dot{V}O_2$ is increasing most rapidly. In marked contrast, the elevation of PO_2mv in the MG after BR seen in the present investigation is delayed and appears to consist of a secondary effect that only becomes apparent after ~60 s of contractions (Figure 1). Such an augmented $PO_2mv_{(steady-state)}$ is expected to facilitate fatigue resistance consequent to decreased metabolic perturbations during exercise (e.g.↓PCr breakdown, (47, 113)).

Relationship to existing literature

BR supplementation elevates QO_2 preferentially in muscles comprised of ≥66% type IIb+d/x muscle fibers (36). Those fiber type selective elevations in QO_2 observed during treadmill exercise, if present herein, would help explain the greater PO_2mv seen in the MG (Figure 1). As mentioned above, one potential explanation for the fiber type specific elevations in QO_2 (and thus PO_2mv) is that NO_2^- reduction is facilitated to a greater extent in fast twitch muscles as a result of lower contracting PO_2mv in type II vs. type I fibers (14, 39, 88). This is supported by Cosby *et al.* (26) who demonstrated that NO_2^- reduction is potentiated in environments with low PO_2 and pH, such that may exist in skeletal muscle during exercise. Furthermore, activity of the nitric oxide synthase family of enzymes (nNOS and eNOS rather than iNOS being relevant here) may be reduced under such conditions (84) allowing the NO_3^-

NO_2^--NO pathway to serve a complimentary role in the local regulation of NO bioavailability. In this respect also, there is a fiber type specificity: The Michaelis-Menten constant (Km) for nNOS (350 μm) is over 15-fold greater than eNOS (23 μm) (107). Thus, the sensitivity of nNOS to the changes in PO_2 (and PO_2mv) in the MG evoked by BR may potentiate the overall NO bioavailability by allowing nNOS, the predominant NOS in fast twitch fibers, to function more effectively.

In addition to the beneficial vascular impacts of NO_3^- supplementation, there may be fiber type specific improvements in metabolic control brought about via improvements in contractile function. For example the improvements in rate of force development and tetanic contractile force reported by Hernandez *et al.* (48), consequent to improvements in intracellular calcium handling (↑ expression of calsequestrin 1 and the dihydropyridine receptor), may serve to reduce $\dot{V}O_2$ in the face of elevated QO_2, further raising the $QO_2/\dot{V}O_2$ ratio. Furthermore, NO_3^- supplementation reportedly increases mitochondrial efficiency in the human vastus lateralis muscle (comprised of ~58% Type IIa/IIb+dx fibers, (44)) in proportion to the reduced pulmonary $\dot{V}O_2$ response to submaximal exercise (75). Collectively, these studies support that simultaneous increases in muscle(s) QO_2 combined with reductions in $\dot{V}O_2$ may account for the observed fiber type-specific elevations in contracting $PO_2mv_{(steady-state)}$.

With regards to performance, the fiber type-selective impacts of BR supplementation may delay the onset of fatigue given that phosphocreatine and glycogen degradation is greater in Type II vs. Type I muscles during maximal exercise (72). Therefore, an elevated $PO_2mv_{(steady-state)}$ has the means to contribute to BR-induced improvements in intense intermittent exercise (121) especially when considering that type II muscle fibers are heavily recruited during the transition from low to high metabolic rates (71, 73). In addition, higher exercise intensities result in a

greater muscle $\dot{V}O_2$/ QO_2 ratio exacerbating intramyocyte hypoxia and accumulation of [ADP], [Pi], [K$^+$] and [H$^+$] each of which may play a role in the fatigue process (18, 64). That these perturbations may be ameliorated, at least in part, by BR-induced elevations in PO$_2mv_{\text{(steady-state)}}$ carries significant implications for individuals suffering from diseases where derangements in skeletal muscle O$_2$ delivery/utilization balance (e.g. chronic heart failure, peripheral artery disease) expedite fatigue.

Experimental considerations

That the elevated PO$_2mv_{\text{(steady-state)}}$ occurred in the absence of elevated plasma [NO$_2^-$] suggests that alternate pools of NO$_2^-$, perhaps within muscle tissue, contributed to this effect. None-the-less, an improved QO$_2$/$\dot{V}O_2$ ratio in the highly glycolytic muscles and muscle parts would presumably delay the onset of fatigue and thus, may be the mechanism responsible for the improvements in high intensity exercise seen in humans following BR supplementation (16, 121). Contrary to our original hypothesis, BR did not impact PO$_2mv$ during the immediate rest-contraction transition (i.e. rapid PO$_2mv$ kinetics) as shown previously in the mixed fiber type spinotrapezius muscle (35). The absence in effect may be due to the length of the experimental procedure utilized herein which, given the relatively short half-life of plasma NO$_2^-$ (~45 minutes in humans, (105, 121)) may have allowed any significant elevation of circulating plasma [NO$_2^-$] to subside reducing/abolishing any effect on PO$_2mv$ kinetics. In this regard, it is important to note that muscle [NO$_2^-$] can remain elevated after plasma [NO$_2^-$] has returned to normal (see Calvert *et al.* (18)). The robust changes in PO$_2mv_{\text{(steady-state)}}$ seen in BR supplemented rats herein combined with the results reported by Calvert *et al.* (18) suggests that high plasma [NO$_2^-$] may not be obligatory to elicit beneficial physiological responses. Elevated tissue [NO$_2^-$] may be the

consequence of prolonged periods (i.e., several days to weeks) of high exposure for example with BR supplementation or exercise training.

It is important to note that $PO_2mv_{(steady\text{-}state)}$ was numerically (~20%), but not significantly, higher in in the contracting soleus of BR supplemented rats (Table 1). This finding may be due to the very small proportion of fast twitch fibers in the soleus (~10%) and/or the reduced effect of BR on slow twitch fibers. Post-hoc power analysis revealed that 19 additional animals would be needed to achieve significance.

Potential limitations, future directions, and conclusions

Five days of BR-induced NO_3^- supplementation raised substantially the contracting $PO_2mv_{(steady\text{-}state)}$ in the fast twitch MG with no significant changes evoked in the predominantly slow twitch soleus. That this effect occurred in the presence of unchanged plasma $[NO_2^-]$ seen in BR supplemented rats highlights the complex nature of NO_3^-/NO_2^- bioactivation and suggest that potentially other storage pools of NO_2^- (i.e., within skeletal muscle) may impact skeletal muscle vascular and metabolic function. Importantly, the elevated $PO_2mv_{(steady\text{-}state)}$ in the MG reflects an improved ability to maintain QO_2 relative to $\dot{V}O_2$ and thus, is expected to ameliorate fatigue during high intensity exercise as demonstrated in humans (16, 121). Future measurements of QO_2 and calculation of $\dot{V}O_2$ will provide valuable insight into the relative contribution of vascular versus intramyocyte (mitochondrial, contractile machinery) mechanisms responsible for this effect.

In addition, while the lower PO_2mv in the gastrocnemius offers one putative mechanism for NO_2^- reduction to NO *in vivo* (i.e. ↓PO_2 ↑NO_2^- reduction) experiments in which tissue pH and/or mitochondrial function are manipulated may offer further insight into the precise mechanism(s) responsible for the fiber type preferential effect observed herein. Indeed, fiber type

differences in tissue pH may afford enhanced bioactivation of NO_2^- particularly if tissue, rather than plasma, $[NO_2^-]$ is responsible for the effects on PO_2mv. In this regard, investigations into the impacts of tissue pH on NO_2^- bioactivation are warranted.

Considering the preponderance of fast twitch fibers in human locomotory muscles and the increased reliance on these fibers in diseased states (e.g. heart failure) NO_3^- supplementation via BR may constitute a novel and powerful "bench-to-bedside" therapeutic modality. Furthermore, considering that traditional organic NO_3^- therapies employing isosorbide mononitrate or nitroglycerine eventually lead to tachyphylaxis (31) it is plausible that utilizing the NO_3^--NO_2^--NO pathway will provide a viable NO-based treatment strategy for various disease conditions.

Table 4.1 Microvascular O₂ partial pressure (PO₂mv) kinetics parameters during soleus and MG contractions in control and BR rats.

	Soleus		MG	
	Control	BR	Control	BR
$PO_2mv_{(Base\ line)}$ (mmHg)	32 ± 3	33 ± 3	24 ± 2†	25 ± 2†
$\Delta 1PO_2mv$ (mmHg)	11 ± 1	9 ± 2	11 ± 2	10 ± 1
$\Delta 2PO_2mv$ (mmHg)			1 ± 0	4 ± 1
$\Delta totalPO_2mv$ (mmHg)	11 ± 1	9 ± 2	9 ± 1	5 ± 1
$PO_2mv_{(steady-state)}$ (mmHg)	20 ± 3	24 ± 2	14 ± 1†	20 ± 2*
Time delay 1 (s)	12 ± 1	8 ± 2	6 ± 2†	2 ± 1†
Time delay 2 (s)				51 ± 14
Time constant 1 (s)	25 ±5	26 ± 6	13 ± 2†	15 ± 4†
Time constant 2 (s)				58 ± 12
Mean response time (s)	37 ± 4	34 ± 6	19 ± 3†	17 ± 3†
T_{63} (s)	39 ± 5	34 ± 6	19 ± 3†	13 ± 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 PO₂mv data (control: n=1 of 6 BR: n=6 of 6 MG profiles). *$P<0.05$ vs. control. †$P<0.05$ vs. soleus.
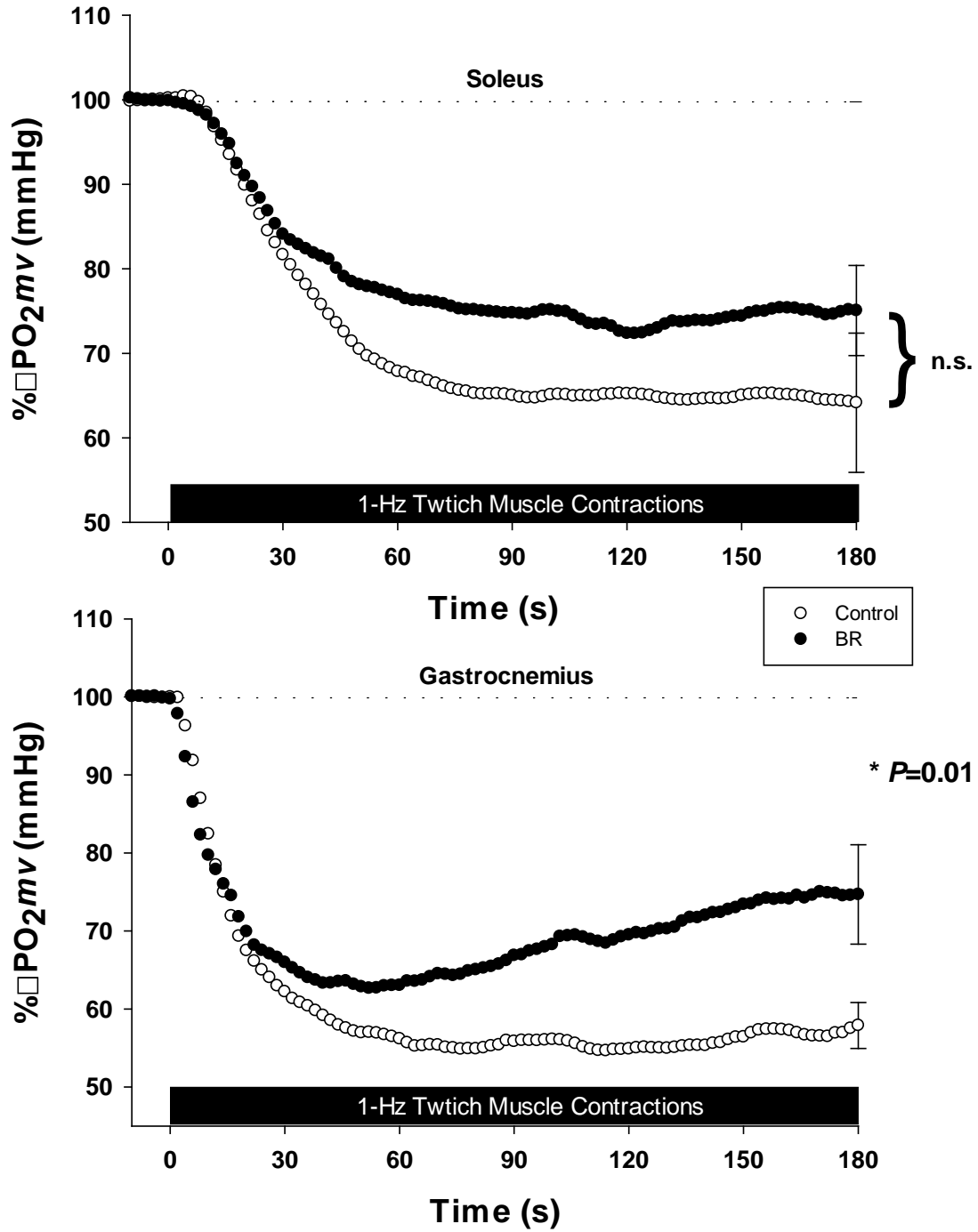
Figure 4.1 Mean percent delta PO$_2$$mv$ profiles for the soleus (top panel) and MG (bottom panel) muscles of control and BR rats. Time "0" represents the onset of contractions. *P<0.05 versus control.

Chapter 5 - Skeletal muscle vascular control during exercise: impact of nitrite infusion during nitric oxide synthase inhibition in healthy rats

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Summary

The nitric oxide synthase (NOS) independent pathway of nitric oxide (NO) production in which nitrite (NO_2^-) is reduced to NO may have therapeutic applications for those with cardiovascular diseases in which the NOS pathway is downregulated. We tested the hypothesis that NO_2^- infusion would reduce mean arterial pressure (MAP) and increase skeletal muscle blood flow (BF) and vascular conductance (VC) during exercise in the face of NOS blockade via L-NAME. Male Sprague-Dawley rats (3-6 months) exercised without (control, n=11) and after (n=8) infusion of L-NAME (10 mg $\cdot$ kg^{-1}: L-NAME) and sodium NO_2^- (7 mg $\cdot$ kg^{-1}: L-NAME + NO_2^-). MAP and hindlimb skeletal muscle BF (radiolabeled microsphere infusions) were measured during submaximal treadmill running (20 m $\cdot$ min^{-1}, 5% grade). Following an L-NAME induced increase in exercising MAP, NO_2^- infusion restored MAP to levels observed in healthy control animals (control: 137 ± 3 L-NAME: 157 ± 7, L-NAME + NO_2^-: 136 ± 5 mmHg). Also, NO_2^- infusion restored VC to levels observed in control animals (control: 0.78 ± 0.05, L-NAME: 0.57 ± 0.03, L-NAME + NO_2^-: 0.69 ± 0.04 ml $\cdot$ min^{-1} $\cdot$ 100 g^{-1} $\cdot$ $mmHg^{-1}$) with no apparent fiber type preferential effect. Overall hindlimb BF was decreased significantly by L-NAME: however, in L-NAME+NO_2^- BF was not significantly different when compared to healthy controls (control: 108 ± 8, L-NAME: 88 ± 3, L-NAME + NO_2^-: 94 ± 6 ml $\cdot$ min^{-1} $\cdot$ 100 g^{-1}, P=0.38 L-NAME vs. L-NAME + NO_2^-). Individuals with diseases that impair NOS activity, and thus vascular function, may benefit from a NO_2^- based therapy in which NO bioavailability is elevated in a NOS-independent manner.

Introduction

The cardiovascular response to exercise is characterized by a multitude of neural, humoral and mechanical components serving to elevate cardiac output and redistribute blood flow (BF), and thus O_2 delivery (QO_2), to contracting myocytes. Of the humoral regulators, the ubiquitous signaling molecule nitric oxide (NO) plays a fundamental role in the hyperemic response to exercise and, as a result, its bioavailability is key to elicit the changes in QO_2 necessary to meet the rapidly rising O_2 demand ($\dot{V}O_2$) of the skeletal muscle (reviewed by 59). Indeed, disease states hallmarked by reduced NO bioavailability (i.e. chronic heart failure, CHF, reviewed by 99) demonstrate a robust disruption in spatial and temporal skeletal muscle QO_2, resulting in perturbed metabolic function and compromised exercise tolerance.

NO is synthesized endogenously in a reaction catalyzed by the NO synthase (NOS) family of enzymes or the one-step reduction of nitrite (NO_2^-) to NO; the latter being a NOS-independent pathway (reviewed by 83). Recent evidence from murine models suggests that the bioactivity of NO_2^- may be upregulated via ingestion of nitrate (NO_3^-) rich food stuffs (i.e. beetroot juice), thus likely elevating NO bioavailability (following the reduction of NO_3^- to NO_2^- and finally NO) resulting in improved skeletal muscle vascular, metabolic (34, 35, 75), and contractile (48) function. These results extend to humans as several laboratories have demonstrated ergogenic effects of dietary NO_3^- supplementation in healthy (10, 11, 90, 112, 113, 121) and diseased (2, 15, 64, 122) populations. Interestingly, while these studies employ a dietary means of increasing endogenous $[NO_2^-]$, vasoactivity of the directly infused anion is evident in humans (26, 54, 85, 97) and animals (3, 42, 96, 104) suggesting that bolus delivery may afford an expedited method of augmenting vascular and metabolic control *in vivo*.

Bearing in mind the beneficial impacts of dietary NO_3^- supplementation on exercise performance, and the vascular effects of NO_2^- infusion highlighted above it is logical to consider that direct infusion with NO_2^- may also impact skeletal muscle vascular control during exercise. Furthermore, when considering that NO_2^- reduction to NO is potentiated in low PO_2 and/or pH environments (26), bioactivity of NO_2^- may be further facilitated (or relied upon) when NOS function is reduced or completely abolished and O_2 transport is impaired (as is the case in many pathological conditions). If direct NO_2^- infusion augments exercising skeletal muscle vascular function independent of NOS, NO_2^- therapy could emerge as an attractive means of restoring NO bioavailability in various cardiovascular diseases in which NOS function is compromised.

Despite these prospects, there are no investigations into the effects of NO_2^- infusion on exercising skeletal muscle vascular control under conditions of NOS blockade. Therefore, the purpose of this investigation was to determine the impact(s) of NO_2^- infusion on skeletal muscle vascular control during exercise in rats with NOS blockade elicited via L-NAME. We tested the hypothesis that, relative to the L-NAME condition, treatment with NO_2^- would restore exercising mean arterial pressure (MAP) and total exercising hindlimb skeletal muscle BF and vascular conductance (VC) to values observed in healthy young-adult rats (with intact NOS function).

Methods

Ethical approval

All procedures employed in this investigation were approved by the Institutional Animal Care and Use Committee of Kansas State University and were conducted under the guidelines established by *The Journal of Physiology* (29). Sixteen young adult male Sprague-Dawley rats (~3 months of age, Charles River Laboratories, Wilmington, MA, USA) were maintained at accredited animal facilities at Kansas State University on a 12:12-hr light-dark cycle with food and water provided *ad libitum*. All rats were familiarized with running on a custom-built motor-driven treadmill for 5 min $\cdot$ day^{-1} at a speed of 20 m $\cdot$ min^{-1} up a 5% grade for ~5 days. In an effort to minimize the unnecessary utilization of additional animals, control BF, VC, blood gas, [lactate], and plasma $[NO_2^-]/[NO_3^-]$ values reported herein represent animals from recently published work ($n=11$, (36)) and followed the same experimental procedures as detailed below.

Surgical instrumentation

On the day of the experiment, rats were anaesthetized initially with a 5% isoflurane-O_2 mixture and maintained subsequently on 3% isoflurane/O_2 mixture. A catheter (PE-10 connected to PE-50, Intra-Medic polyethylene tubing, Clay Adams Brand, Becton, Dickinson and Company, Sparks, MD, USA) was placed in the ascending aorta via the right carotid artery. A second catheter was surgically placed in the caudal (tail) artery as described previously (94). Both catheters were tunneled subcutaneously through the dorsal aspect of the cervical region and exteriorized via a puncture wound in the skin. The incisions were closed, anesthesia was terminated and the rats were given a minimum of 60 min to recover (41).

L-NAME infusion

Rats were then placed on the treadmill and, following a ~5 minute resting period, N^G-nitro-L arginine methyl ester (10 mg $\cdot$ kg^{-1}, L-NAME; $n=8$, Sigma Chemical, St. Louis, MO,

USA) was administered to each rat via the caudal artery catheter to inhibit NOS. This dose has been used extensively in our laboratory and has demonstrated inhibition of NOS via attenuation of acetylcholine induced reductions in MAP (23, 50).

Exercise protocol and measurement of hindlimb skeletal muscle BF

Following L-NAME infusion, the caudal artery catheter was connected to a 1 ml syringe chambered in a Harvard infusion/withdrawal pump (model 907, Cambridge, MA, USA) and 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. Approximately 3 min post-L-NAME infusion, exercise was initiated and 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; (91)). The rats 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 · min^{-1}. Simultaneously, HR and MAP were measured and recorded. 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 (L-NAME condition). Following the microsphere infusion, ~0.3 ml of blood was sampled from the carotid artery catheter for the determination of blood [lactate] (Nova Stat Profile M, Nova Biomedical, Waltham, MA, USA) and exercise was terminated.

NO_2^- infusion

Following a 30 min recovery period a bolus infusion of sodium NO_2^- (7 mg · kg^{-1} body mass, L-NAME + NO_2^-; *n*=8, Sigma Chemical, St. Louis, MO, USA) was administered to each rat via the caudal artery catheter. The exercise and microsphere infusion

protocols (radio-labeled differently from the first) were then repeated (condition L-NAME + NO_2^-).

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

Immediately following microsphere infusion but prior to the termination of exercise, a ~0.3 ml blood sample was drawn from the carotid artery catheter for determination of blood pH, PO_2, and %O_2 saturation (Nova Stat Profile M, Nova Biomedical, Waltham, MA, USA). For plasma $[NO_3^-]$ and $[NO_2^-]$, following the termination of exercise ~0.8 ml of blood was drawn into heparinized tubes and rapidly centrifuged at 5000 g at 4°C for 6 minutes. Plasma was then extracted and frozen immediately at -80°C for later analysis via chemiluminescence as described previously (34-37).

Determination of BF and VC

Rats were euthanized via pentobarbital sodium overdose ($\geq$50 mg $\cdot$ kg^{-1}). The thorax of each rat was opened and accurate placement of the carotid artery catheter was confirmed before the internal organs and 28 individual muscles and muscle parts of the hindlimb were excised.

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 (94) and expressed as ml $\cdot$ min^{-1} $\cdot$ 100g^{-1}. VC was then calculated by normalizing BF to MAP and expressed as ml $\cdot$ min^{-1} $\cdot$ 100g^{-1} $\cdot$ mmHg^{-1}.

Statistical analysis

Results were compared among (control vs. L-NAME and control vs. L-NAME + NO_2^-) and within (L-NAME vs. L-NAME + NO_2^-) groups using *a priori* unpaired and paired one-tail Student's *t* tests, respectively, corrected for multiple comparisons. Values are expressed as mean $\pm$ SEM.

Results

MAP, HR, plasma [NO$_3^-$] and [NO$_2^-$] and blood gases

Relative to control, post NO$_2^-$ infusion plasma [NO$_2^-$] (control: 0.17 ± 0.2, L-NAME + NO$_2^-$: 306.8 ± 38.7 μMol, $P<0.01$) and [NO$_3^-$] (control: 17.8 ± 1, L-NAME + NO$_2^-$: 152.5 ± 35 μMol, $P<0.01$) were significantly elevated. Relative to control, MAP was significantly higher in the L-NAME condition (Figure 1, $P<0.03$). Following NO$_2^-$ infusion, MAP was reduced significantly when compared to the L-NAME condition ($P<0.03$). Exercising MAP was not different between control and L-NAME+NO$_2^-$ groups ($P=0.36$). Relative to the control and L-NAME+NO$_2^-$ conditions, exercising HR was significantly lower in the L-NAME condition (control: 528 ± 12, L-NAME: 493 ± 37, L-NAME + NO$_2^-$: 520 ± 33 beats · min^{-1}, $P<0.01$).

There were no differences in arterial PO$_2$, PCO$_2$, or %O$_2$ saturation during exercise. Arterial blood [lactate] during exercise was greater following NO$_2^-$ infusion (3.8 ± 0.5 mM) compared to control (2.7 ± 0.4 mM) and L-NAME only (2.1 ± 0.3 mM) conditions, ($P<0.016$).

BF and VC

L-NAME significantly reduced exercising total hindlimb skeletal muscle BF and VC (Figure 2, $P<0.03$). Following NO$_2^-$ infusion total hindlimb skeletal muscle VC was restored to levels observed in control rats (Figure 2, $P<0.03$ L-NAME vs. L-NAME+NO$_2^-$, $P>0.10$ control vs. L-NAME+NO$_2$). There were no differences in total hindlimb skeletal muscle BF during exercise in L-NAME vs. L-NAME + NO$_2^-$ or control vs. L-NAME + NO$_2^-$ conditions (Figure 2 bottom panel, $P>0.03$).

Relative to control, L-NAME treated rats had lower BF in 5 and VC in 15 of the 28 individual hindlimb muscles and muscle parts, whereas this was the case for only 3 muscles (BF and VC) in the L-NAME+NO$_2^-$ condition (Table 1, $P<0.03$ for all). Moreover, following NO$_2^-$

infusion, VC in 19 of the 28 individual hindlimb muscles and muscle parts was increased significantly when compared to the L-NAME condition (P<0.03, Table 1).

Relative to control, BF and VC were lower in the adrenals and pancreas while VC was lower in the kidneys, stomach, and small intestine in rats treated with L-NAME (P<0.03, Table 2). Following NO_2^- infusion, renal and adrenal BF and VC were lower when compared to control animals while renal and adrenal BF was reduced when compared to L-NAME (P<0.03, Table 2).

Discussion

The principal original finding of this investigation is that, in the face of NOS blockade, NO_2^- infusion restored exercising MAP and hindlimb skeletal muscle VC to levels observed in young-adult healthy rats with intact NOS function. While NO_2^- infusion did not increase BF when compared to the L-NAME condition, it did abolish the lower BF induced by L-NAME. Elevations in VC and reductions in MAP could serve to reduce afterload and thus reduce the work of the heart during exercise. These results demonstrate that NO_2^- may serve as a powerful modulator of vascular control *in vivo*, independent of NOS function and thus may hold promising therapeutic potential, particularly in diseases with impaired NOS function and chronically elevated MAP.

Effects of inorganic NO_2^- infusion on skeletal muscle BF and VC and MAP

An abundance of research has focused on defining the vasoactive/cardioprotective role(s) of NO_2^- with many studies suggesting that the reduction of NO_2^- to NO compliments the well understood NOS pathway of NO production, particularly when NOS function becomes uncoupled or otherwise impaired ((reviewed by 1, 8)). The vascular responses to NO_2^- infusion presented herein support this notion. Similar to what has been reported previously in our laboratory (51, 93), infusion with the comprehensive NOS blocker L-NAME increased MAP ~15% and decreased skeletal muscle VC ~26% during exercise. Consistent with our hypothesis, infusion with NO_2^- (7mg $\cdot$ kg^{-1}) restored MAP and VC to levels similar to those observed in healthy control animals. One potential explanation for these effects of NO_2^- could be the lower PO_2/pH environment present within the skeletal muscle following NOS inhibition (37). Such environments facilitate (or uninhibit) NO_2^- reduction to NO *in vivo* (26, 33), which may allow local NO_2^- to support the blood-myocyte PO_2 gradient (via $\uparrow QO_2$ and microvasculature PO_2,

PO$_2$*mv*) that, when compromised, leads to tissue hypoxia and exacerbates intracellular perturbations (7).

One striking aspect of this investigation, in which acute NO$_2^-$ infusion was employed, was that the augmented skeletal muscle VC was observed in muscles and muscle parts that span the full spectrum of fast and slow twitch fibre types (Table 1). This is in contrast to investigations utilizing short-term dietary NO$_3^-$ supplementation as a means of increasing circulating [NO$_2^-$]. Specifically, there is a fibre type preferential effect of dietary NO$_3^-$ supplementation as rats given NO$_3^-$ rich beetroot juice for 5 days exhibited elevated skeletal muscle BF and VC exclusively in muscles and muscle portions comprised of $\geq$ 66% type IIb + d/x muscle fibres (36). Moreover, beetroot juice elevates PO$_2$*mv* during muscle contractions in the gastrocnemius (fast twitch) but not soleus (slow twitch) muscles (37). The substantial array of muscles and muscle portions exhibiting a vasoactive response to NO$_2^-$ infusion herein suggests that the fibre type preferential effects observed following dietary NO$_3^-$ supplementation may be conferred via changes in protein expression which require a longer period of elevated NO$_2^-$ exposure to manifest. This idea is supported by evidence from Hernandez, Schiffer, Ivarsson, Cheng, Bruton, Lundberg, Weitzberg and Westerblad (48) in which the improvements in fast twitch skeletal muscle force production evoked by NO$_3^-$ supplementation were attributed to elevations in calcium handling proteins (i.e. calsequestrin 1 and the dihydropyridine receptor) which were present following multiple days of dietary NO$_3^-$ supplementation.

Additionally, the discrepancies in the vascular responses to NO$_3^-$ vs. NO$_2^-$ treatment could be related to the relative impacts of NOS inhibition in fast vs. slow twitch muscles. Skeletal muscles comprised predominantly of slow twitch fibres demonstrate the greatest deficits in BF and VC following L-NAME infusion (51) likely due to a greater expression of endothelial

NOS (eNOS) within these tissues (119). These slow twitch muscles may exhibit much greater

BF and $\dot{V}O_2$ than their fast twitch counterparts both at rest and during exercise (~100% greater for

both BF and $\dot{V}O_2$ (14)). Consequently, NOS inhibition may have crippled O_2 delivery in these

muscles sufficiently enough to produce an environment ripe for NO_2^- bioactivation (i.e. very low

PO_2 and pH). This effect could place more emphasis on NO_2^- as the primary source of NO in

these specific tissues when vascular function is impaired, as it is in many disease states (13). In

this regard, the spatial changes in VC seen following NO_2^- infusion herein may mimic closely

what would be observed in individuals with diseases that compromise NOS function. However,

these questions require further investigation using specific models of vascular disease.

Clinical and Therapeutic implications

In healthy individuals eNOS is the primary endogenous source for NO_2^- and NO (68).

Endothelial dysfunction becomes evident early on in many diseases including CHF ((reviewed

by 99)) and peripheral artery disease ((reviewed by 17)) and thus likely limits vascular and

metabolic function via attenuated NO production from both NOS dependent and independent

pathways (68, 69). As evidenced by Hirai *et al.* (38, 40), reduced NO from NOS dramatically

impairs the matching of skeletal muscle QO_2 to $\dot{V}O_2$ such that superfusion of L-NAME in the

contracting rat spinotrapezius muscle transforms the healthy PO_2mv profile into one resembling

CHF (38). In this regard, the blockade of NOS induced by L-NAME infusion performed in the

present investigation presents a challenge that mimics the consequences of CHF, and potentially

other diseases. Therefore, from the present findings, a therapy in which systemic $[NO_2^-]$ is

elevated (via endogenous or exogenous sources) may provide beneficial vascular responses

independent of NOS function. Even small improvements in vascular function may enhance

metabolic control during dynamic exercise; potentially improving adherence to rehabilitation

programs (1), which in-and-of themselves would upregulate eNOS function and endogenous NO_2^- production.

Experimental considerations and Potential limitations

A surprising result of the present investigation was the rise in exercising blood [lactate] following NO_2^- infusion (~41% and 81% greater vs. control and L-NAME respectively). Lower levels of NO may act as a useful brake on mitochondrial activity via competitive binding to complex IV of the respiratory chain (32). In contrast, high concentrations of NO have been associated with adverse effects on cell respiration via nitrosylation of mitochondrial electron chain complexes, specifically complex I (22). In addition NO works to inhibit complex IV (cytochrome oxidase) thereby reducing cellular O_2 consumption. Both of these effects may prove beneficial in certain environments or situations when O_2 delivery becomes reduced as reductions in tissue $\dot{V}O_2$ work to extend the PO_2 gradient across a larger tissue area, effectively sharing the available O_2 (108). However, in the current study it is possible that the rate of NO_2^- reduction to NO became high enough to overwhelm mitochondrial respiration, thus leading to impaired oxidative metabolism and an increased reliance on glycolytic means of ATP production. In addition, while the current dose of NO_2^- raised plasma $[NO_3^-]$ to levels very similar to what has been reported following dietary NO_3^- supplementation in humans (11, 64) and animals (34, 36) the plasma $[NO_2^-]$ were much greater than that achieved via NO_3^- supplementation, and thus may have contributed to the aforementioned effect on metabolism. In this regard a comprehensive dose-response relationship will need to be determined before NO_2^- can be used as an effective therapeutic.

Furthermore, considering that NOS was acutely inhibited in the present investigation, the impacts of NO_2^- infusion may differ when administered to specific models of vascular diseases

that have been developed chronically, as this would more closely mimic specific etiologies. Additionally, due to the relatively long half-life and bioactivity of L-NAME metabolites (~20 hours in rats (115)) the experimental design was limited to a fixed sequence and therefore, an ordering effect cannot be ruled out. Future investigations in which NO_2^- is employed in healthy control animals would also provide further insight into the bioactivity of NO_2^- in animals with intact NOS function and could shed light on how a NO_2^- based intervention may impact healthy cardiovascular function.

Conclusions

These data highlight the potential for NO_2^- to act independently of NOS and improve skeletal muscle vascular control during exercise. Considering the multiple cardiovascular diseases that impair NOS function, therapies that increase $[NO_2^-]$ may result in improved skeletal muscle vascular control during exercise. However, the NO_2^- induced changes in blood [lactate] seen during exercise herein suggests that the reduction of NO_3^- to NO_2^-, accomplished via facultative anaerobes in the mouth following dietary NO_3^- consumption, may provide the controlled release of NO_2^- needed to elicit the most beneficial vascular and metabolic changes during exercise. It is anticipated that future investigations into the vascular impacts of both NO_2^- and NO_3^- based therapies will provide crucial insight into the potential benefits, and limitations, of both interventions.

Table 5.1 Effects NO$_2^-$ infusion (7 mg $\cdot$ kg^{-1}) on exercising hindlimb skeletal muscle BF (ml $\cdot$ min^{-1} $\cdot$ 100g^{-1}) and VC (ml $\cdot$ min^{-1} $\cdot$ 100g^{-1} $\cdot$ mmHg^{-1}) in rats with NOS blockade (L-NAME).

	BF			VC		
	Control	**L-NAME**	**L-NAME+NO$_2^-$**	**Control**	**L-NAME**	**L-NAME+NO$_2^-$**
Ankle extensors						
Soleus (9%)	295 ± 42	242 ± 71	285 ± 36	2.14 ± 0.30	1.56 ± 0.17	2.06 ± 0.23†
Plantaris (80%)	207 ± 15	144 ± 8*	173 ± 15	1.50 ± 0.10	0.93 ± 0.06*	1.27 ± 0.08†
Gastrocnemius, red (14%)	452 ± 44	333 ± 59	362 ± 65	3.27 ± 0.30	2.18 ± 0.02*	2.63 ± 0.44†
Gastrocnemius, white (100%)	42 ± 7	26 ± 3	37 ± 4†	0.30 ± 0.05	0.17 ± 0.02*	0.27 ± 0.03†
Gastrocnemius, mixed (91%)	149 ± 12	120 ± 5	141 ± 8	1.08 ± 0.08	0.77 ± 0.04*	1.04 ± 0.04†
Tibialis posterior (73%)	118 ± 17	81 ± 12	91 ± 13	0.85 ± 0.12	0.51 ± 0.07*	0.66 ± 0.09†
Flexor digitorum longus (68%)	99 ± 14	60 ± 7*	69 ± 9	0.71 ± 0.09	0.38 ± 0.04*	0.51 ± 0.06†
Flexor halicus longus (71%)	75 ± 10	68 ± 8	99 ± 14†	0.54 ± 0.06	0.44 ± 0.06	0.74 ± 0.11†
Ankle flexors						
Tibialis anterior, red (63%)	343 ± 35	209 ± 10*	219 ± 20*	2.48 ± 0.23	1.36 ± 0.10*	1.62 ± 0.14*
Tibialis anterior, white (80%)	119 ± 14	83 ± 6*	89 ± 12	0.86 ± 0.09	0.54 ± 0.05*	0.66 ± 0.09†
Extensor digitorum longus (76%)	54 ± 7	75 ± 20	77 ± 17	0.39 ± 0.05	0.50 ± 0.14	0.57 ± 0.13†
Peroneals (67%)	128 ± 11	72 ± 14*	91 ± 13*	0.93 ± 0.08	0.46 ± 0.09*	0.67 ± 0.09*†
Knee extensors						
Vastus intermedius (4%)	359 ± 39	257 ± 25	302 ± 39	2.60 ± 0.27	1.66 ± 0.17*	2.20 ± 0.25†
Vastus medialis (82%)	114 ± 18	137 ± 13	144 ± 14	0.82 ± 0.12	0.89 ± 0.08	1.06 ± 0.08†
Vastus lateralis, red (35%)	388 ± 43	310 ± 35	281 ± 25	2.82 ± 0.29	2.02 ± 0.26	2.08 ± 0.52
Vastus lateralis, white (100%)	33 ± 5	26 ± 8	31 ± 7	0.24 ± 0.03	0.16 ± 0.04	0.23 ± 0.04†
Vastus lateralis, mixed (89%)	167 ± 21	123 ± 12	127 ± 13	1.22 ± 0.14	0.81 ± 0.09*	0.94 ± 0.09†
Rectus femoris, red (66%)	224 ± 33	181 ± 15	204 ± 17	1.62 ± 0.23	1.17 ± 0.10	1.50 ± 0.11†
Rectus femoris, white (100%)	101 ± 13	81 ± 7	91 ± 8	0.73 ± 0.09	0.52 ± 0.05	0.67 ± 0.06†
Knee flexors						
Biceps femoris anterior (100%)	50 ± 8	33 ± 4	36 ± 4	0.36 ± 0.05	0.21 ± 0.03*	0.27 ± 0.03†
Biceps femoris posterior (92%)	79 ± 8	65 ± 3	71 ± 5	0.58 ± 0.06	0.42 ± 0.02*	0.53 ± 0.04†
Semitendinosus (83%)	56 ± 6	34 ± 3*	37 ± 4*	0.40 ± 0.04	0.22 ± 0.02*	0.28 ± 0.03*
Semimembranosus, red (72%)	119 ± 14	86 ± 7	83 ± 14	0.87 ± 0.09	0.56 ± 0.05*	0.62 ± 0.11
Semimem., white (100%)	33 ± 6	38 ± 7	40 ± 11	0.24 ± 0.04	0.25 ± 0.05	0.30 ± 0.09
Thigh adductors						
Adductor longus (5%)	315 ± 38	263 ± 26	231 ± 31†	2.28 ± 0.26	1.71 ± 0.21	1.68 ± 0.22
Adductor magnus & brevis (89%)	83 ± 8	80 ± 7	80 ± 9	0.60 ± 0.05	0.52 ± 0.05	0.60 ± 0.06
Gracilis (77%)	42 ± 4	37 ± 4	34 ± 5	0.30 ± 0.03	0.24 ± 0.02	0.26 ± 0.04
Pectineus (69%)	54 ± 8	40 ± 6	46 ± 11	0.39 ± 0.06	0.25 ± 0.03	0.34 ± 0.08

Data are mean ± SEM. Values in parentheses indicate % type IIb + d/x according to Delp & Duan (1996).

Control: $n=11$, L-NAME: $n=8$, L-NAME + NO_2^-: $n=8$. *$P<0.03$ vs. control. †$P<0.03$ vs. L-NAM

Table 5.2 Effects of NO_2^- infusion (7 mg · kg^{-1}) on exercising BF (ml · min^{-1} · 100g^{-1}) and VC (ml · min^{-1} · 100g^{-1} · mmHg^{-1}) in the kidneys and organs of the splanchnic region.

	BF			VC		
	Control	L-NAME	L-NAME + NO_2	Control	L-NAME	L-NAME + NO_2
Kidney	421 ± 42	338 ± 28	267 ± 31*†	3.05 ± 0.28	2.22 ± 0.25*	1.96 ± 0.22*
Stomach	67 ± 13	38 ± 3	35 ± 4	0.49 ± 0.10	0.25 ± 0.02*	0.25 ± 0.03
Adrenals	353 ± 72	128 ± 17*	100 ± 66*	2.87 ± 0.44	0.85 ± 0.14*	0.72 ± 0.15*
Spleen	61 ± 14	102 ± 21	48 ± 7†	0.44 ± 0.10	0.68 ± 0.16	0.35 ± 0.06
Pancreas	110 ± 15	72 ± 8*	93 ± 22	0.80 ± 0.11	0.47 ± 0.06*	0.67 ± 0.15
Sm. intestine	240 ± 27	177 ± 24	211 ± 26	1.74 ± 0.18	1.17 ± 0.19*	1.55 ± 0.17
Lg. intestine	127 ± 16	123 ± 20	140 ± 42	0.92 ± 0.10	0.82 ± 0.15	1.01 ± 0.28
Liver**	16 ± 4	15 ± 2	13 ± 3	0.12 ± 0.02	0.10 ± 0.01	0.09 ± 0.02

Data are mean ± SEM. **Indicates arterial, not portal, BF and VC. Control: *n*=11, L-NAME: *n*=8, L-NAME + NO_2^-: *n*=8. *$P<0.03$ vs. control. †$P<0.03$ vs. L-NAME.
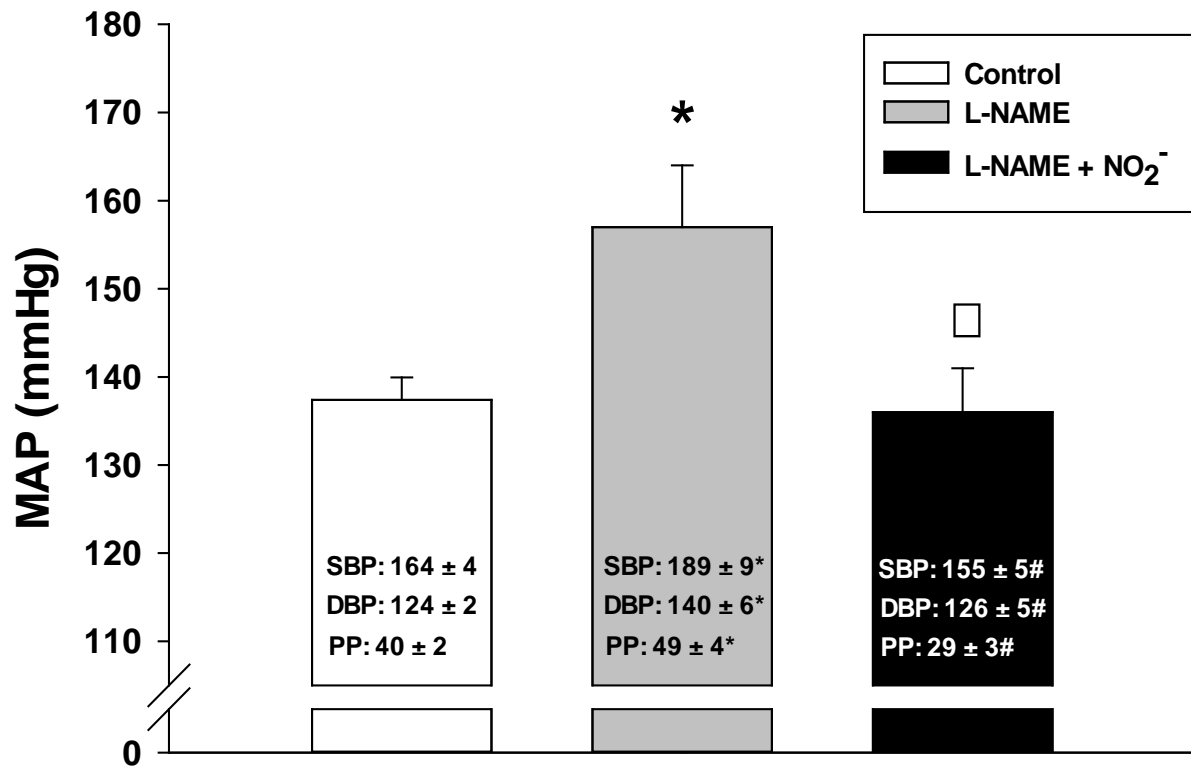
Figure 5.1 Exercising MAP, systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP) values for control, L-NAME and L-NAME+NO$_2^-$ conditions. *P<0.03 vs. control, #P<0.03 vs. L-NAME. Note: control values represented are from previously published data.
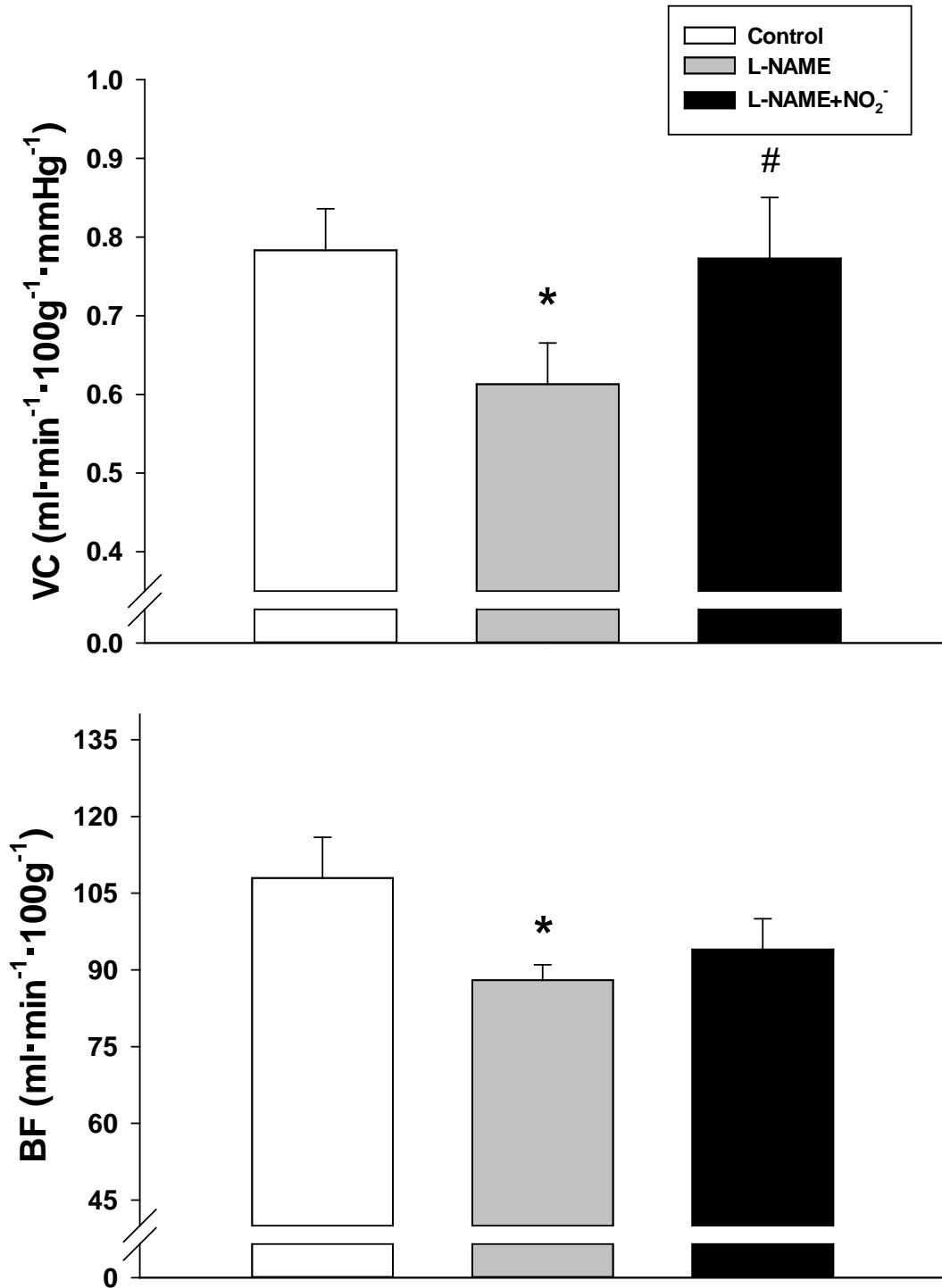
Figure 5.2 Total hindlimb skeletal muscle BF and VC for control, L-NAME and L-NAME+NO₂⁻ conditions in rats during submaximal locomotory exercise. *$P<0.03$ vs. control, #$P<0.03$ vs. L-NAME. Note: control values represented are from previously published data.

Chapter 6 - Conclusion

In conclusion, the NO_3^-- NO_2^--NO pathway plays a significant role in the cardiovascular response to exercise. Dietary NO_3^- supplementation reduces exercising MAP, preferentially elevates skeletal muscle BF during exercise, lowers exercising blood [lactate] and constrains the fall in the PO_2mv during the rest-contraction transition (in the mixed fiber type spinotrapezius) and throughout the duration of muscle contractions (in fast but not slow twitch muscle). These fiber type preferential effects are likely due to the lower PO_2/pH environment within fast twitch muscles and may explain the reduced blood [lactate] during exercise and are summarized in figure 6.1. In addition, following an L-NAME induced rise in MAP, arterial infusion with NO_2^- restored MAP and VC to levels observed in healthy control animals highlighting the ability for NO_2^- to work independently of NOS. Furthermore, considering the evidence in humans that NO_3^- supplementation improves muscle metabolic function in hypoxia, and cognitive function during high intensity exercise, BR supplementation could improve the functionality of those living at altitude (i.e. military personnel, miners, those operating astronomical observatories…etc.).

These three questions remain to be answered before NO_3^-/NO_2^- therapy can be used most effectively in patient and/or healthy populations: 1) Given the large amount of studies on the subject, no standardized NO_3^- delivery method has been developed, what mode of delivery/supplementation provides the most efficacious means of elevating circulating NO_3^-/NO_2^-? Failure to collect experimental data at the correct time (e.g. at peak [NO_2^-/NO_3^-]) could introduce substantial variability to the results. This subject is discussed at length in a recent review by (55). 2) Does NO_3^-/NO_2^- supplementation ameliorate the vascular dysfunction evident in models of disease (e.g. CHF, diabetes, aging, pulmonary hypertension)? A recent study by Zamani et al (122) demonstrated improvements in $\dot{V}O_2$max following an acute dose of BR,

however the effects of BR on the peripheral vascular dysfunction in CHF remains to be determined. 3) The long term effects (i.e. months, years) of elevated NO_3^-/NO_2^- must be determined to address questions regarding potential toxicity and effects on longevity.

1. Figure one shows the PO$_2$*mv* response to muscle contractions in the fast twitch white (WG) and mixed portion of the gastrocnemius (MG) vs. the slow twitch soleus (sol) muscle in rats. Notice that fast twitch muscles exhibit a much lower PO$_2$*mv* at rest and during muscle contractions when compared to their slow twitch counterparts.
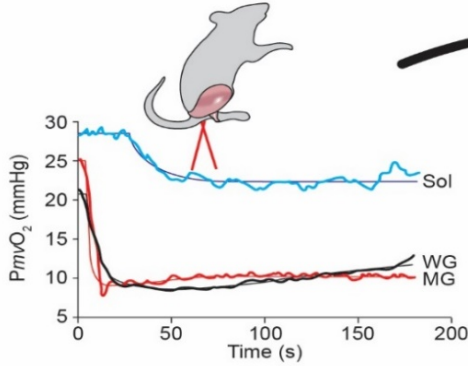
2. The lower PO$_2$*mv* in fast twitch muscles likely facilitates the reduction of tissue and/or plasma NO$_2^-$ to NO preferentially within these tissues, potentially leading to the targeted increases in blood flow seen within fast twitch but not slow twitch muscles (figure 2).
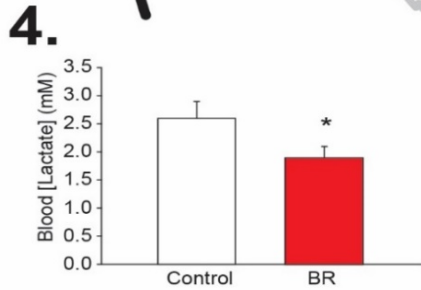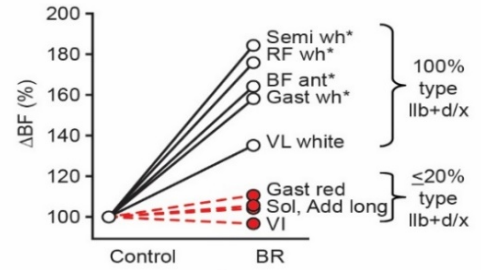
4. Figure 4 shows the significantly lower blood [lactate] observed during submaximal exercise in rats. This effect likely reflects the impact of NO$_3^-$ supplementation on fast but not slow twitch muscles (figures 2 and 3) and ultimately could play a significant role in the improved tolerance to high-intensity exercise observed in humans.
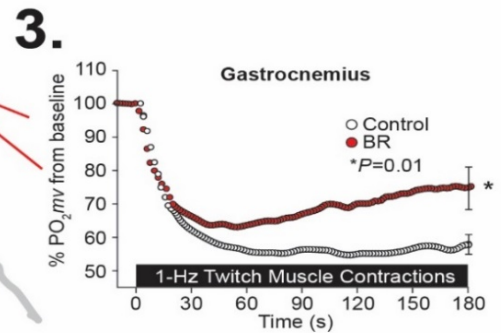
3. Figure 3 shows the impact of NO$_3^-$ supplementation on the PO$_2$*mv* of the mixed gastrocnemius in response to electrically stimulated muscle contractions. 5 days of NO$_3^-$ supplementaiton via BR resulted in a significantly higher contracting PO$_2$*mv* durnig the steady state of contractions when compared to control. This effect was not evident in the slow witch soleus muscle, further suggesting a fiber type preferential effect.

Figure 6.1 Mechanisms likely responsible for the improved exercise performance reported following BR supplementation. Lower PO$_2$ environments in fast vs. slow twitch muscles likely facilitate reduction of NO$_2^-$ to NO, resulting in preferential increases in BF and PO$_2$*mv* within these tissues. This could explain the reduced blood [lactate] observed during exercise in rats (chapter 2). These effects may be exacerbated at high altitude and thus remains to be investigated.

References

1.   **Allen JD, Giordano T, and Kevil CG**. Nitrite and nitric oxide metabolism in peripheral artery disease. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 26: 217-222, 2012.

2.   **Allen JD, Stabler T, Kenjale A, Ham KL, Robbins JL, Duscha BD, Dobrosielski DA, and Annex BH**. Plasma nitrite flux predicts exercise performance in peripheral arterial disease after 3months of exercise training. *Free radical biology & medicine* 49: 1138-1144, 2010.

3.   **Alzawahra WF, Talukder MA, Liu X, Samouilov A, and Zweier JL**. Heme proteins mediate the conversion of nitrite to nitric oxide in the vascular wall. *American journal of physiology Heart and circulatory physiology* 295: H499-508, 2008.

4.   **Andrade FH, Reid MB, Allen DG, and Westerblad H**. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *The Journal of physiology* 509 (Pt 2): 577-586, 1998.

5.   **Armstrong RB, Hayes DA, and Delp MD**. Blood flow distribution in rat muscles during preexercise anticipatory response. *Journal of applied physiology* 67: 1855-1861, 1989.

6.   **Armstrong RB, and Laughlin MH**. Metabolic indicators of fibre recruitment in mammalian muscles during locomotion. *The Journal of experimental biology* 115: 201-213, 1985.

7.   **Arthur PG, Hogan MC, Bebout DE, Wagner PD, and Hochachka PW**. Modeling the effects of hypoxia on ATP turnover in exercising muscle. *Journal of applied physiology* 73: 737-742, 1992.

8.   **Bailey JC, Feelisch M, Horowitz JD, Frenneaux MP, and Madhani M**. Pharmacology and therapeutic role of inorganic nitrite and nitrate in vasodilatation. *Pharmacology & therapeutics* 2014.

9.   **Bailey JK, Kindig CA, Behnke BJ, Musch TI, Schmid-Schoenbein GW, and Poole DC**. Spinotrapezius muscle microcirculatory function: effects of surgical exteriorization. *American journal of physiology Heart and circulatory physiology* 279: H3131-3137, 2000.

10.   **Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N, and Jones AM**. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *Journal of applied physiology* 109: 135-148, 2010.

11.   **Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, Tarr J, Benjamin N, and Jones AM**. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *Journal of applied physiology* 107: 1144-1155, 2009.

12.   **Behnke BJ, Armstrong RB, and Delp MD**. Adrenergic control of vascular resistance varies in muscles composed of different fiber types: influence of the vascular endothelium. *American journal of physiology Regulatory, integrative and comparative physiology* 301: R783-790, 2011.

13.   **Behnke BJ, Delp MD, McDonough P, Spier SA, Poole DC, and Musch TI**. Effects of chronic heart failure on microvascular oxygen exchange dynamics in muscles of contrasting fiber type. *Cardiovascular research* 61: 325-332, 2004.

14.   **Behnke BJ, McDonough P, Padilla DJ, Musch TI, and Poole DC**. Oxygen exchange profile in rat muscles of contrasting fibre types. *The Journal of physiology* 549: 597-605, 2003.

15.   **Berry MJ, Justus NW, Hauser JI, Case AH, Helms CC, Basu S, Rogers Z, Lewis MT, and Miller GD**. Dietary nitrate supplementation improves exercise performance and decreases blood pressure in COPD patients. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2014.

16.   **Breese BC, McNarry MA, Marwood S, Blackwell JR, Bailey SJ, and Jones AM**. Beetroot juice supplementation speeds O2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. *American journal of physiology Regulatory, integrative and comparative physiology* 305: R1441-1450, 2013.

17.   **Brevetti G, Silvestro A, Schiano V, and Chiariello M**. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation* 108: 2093-2098, 2003.

18.   **Calvert JW, Condit ME, Aragon JP, Nicholson CK, Moody BF, Hood RL, Sindler AL, Gundewar S, Seals DR, Barouch LA, and Lefer DJ**. Exercise protects against myocardial ischemia-reperfusion injury via stimulation of beta(3)-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. *Circulation research* 108: 1448-1458, 2011.

19.   **Carlstrom M, Persson AE, Larsson E, Hezel M, Scheffer PG, Teerlink T, Weitzberg E, and Lundberg JO**. Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. *Cardiovascular research* 89: 574-585, 2011.

20.   **Cermak NM, Gibala MJ, and van Loon LJ**. Nitrate supplementation's improvement of 10-km time-trial performance in trained cyclists. *International journal of sport nutrition and exercise metabolism* 22: 64-71, 2012.

21.   **Cermak NM, Res P, Stinkens R, Lundberg JO, Gibala MJ, and van Loon LJ**. No improvement in endurance performance after a single dose of beetroot juice. *International journal of sport nutrition and exercise metabolism* 22: 470-478, 2012.

22.   **Clementi E, Brown GC, Feelisch M, and Moncada S**. Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proceedings of the National Academy of Sciences of the United States of America* 95: 7631-7636, 1998.

23.   **Copp SW, Hirai DM, Hageman KS, Poole DC, and Musch TI**. Nitric oxide synthase inhibition during treadmill exercise reveals fiber-type specific vascular control in the rat hindlimb. *American journal of physiology Regulatory, integrative and comparative physiology* 298: R478-485, 2010.

24.   **Copp SW, Holdsworth CT, Ferguson SK, Hirai DM, Poole DC, and Musch TI**. Muscle fibre-type dependence of neuronal nitric oxide synthase-mediated vascular control in the rat during high speed treadmill running. *The Journal of physiology* 591: 2885-2896, 2013.

25.   **Copp SW, Poole DC, and Musch TI**. Valid and reproducible endurance protocols underlie data interpretation, integration, and application. *Journal of applied physiology* 108: 224-225; author reply 226, 2010.

26.   **Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, 3rd, and Gladwin MT**. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature medicine* 9: 1498-1505, 2003.

27.   **Delp MD, and Duan C**. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *Journal of applied physiology* 80: 261-270, 1996.

28.   **Dinenno FA, and Joyner MJ**. Combined NO and PG inhibition augments alpha-adrenergic vasoconstriction in contracting human skeletal muscle. *American journal of physiology Heart and circulatory physiology* 287: H2576-2584, 2004.

29.   **Drummond GB**. Reporting ethical matters in the Journal of Physiology: standards and advice. *The Journal of physiology* 587: 713-719, 2009.

30.   **Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, and Benjamin N**. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nature medicine* 1: 546-551, 1995.

31.   **Elkayam U, Kulick D, McIntosh N, Roth A, Hsueh W, and Rahimtoola SH**. Incidence of early tolerance to hemodynamic effects of continuous infusion of nitroglycerin in patients with coronary artery disease and heart failure. *Circulation* 76: 577-584, 1987.

32.   **Erusalimsky JD, and Moncada S**. Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. *Arteriosclerosis, thrombosis, and vascular biology* 27: 2524-2531, 2007.

33.   **Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, and Ashrafian H**. Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. *The Journal of biological chemistry* 283: 33927-33934, 2008.

34.   **Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, and Poole DC**. Dose dependent effects of nitrate supplementation on cardiovascular control and microvascular oxygenation dynamics in healthy rats. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 39: 51-58, 2014.

35.   **Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, and Poole DC**. Effects of nitrate supplementation via beetroot juice on contracting rat skeletal muscle microvascular oxygen pressure dynamics. *Respiratory physiology & neurobiology* 187: 250-255, 2013.

36.   **Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, and Poole DC**. Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *The Journal of physiology* 591: 547-557, 2013.

37.   **Ferguson SK, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, Musch TI, and Poole DC**. Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2014.

38.   **Ferreira LF, Hageman KS, Hahn SA, Williams J, Padilla DJ, Poole DC, and Musch TI**. Muscle microvascular oxygenation in chronic heart failure: role of nitric oxide availability. *Acta physiologica* 188: 3-13, 2006.

39.   **Ferreira LF, McDonough P, Behnke BJ, Musch TI, and Poole DC**. Blood flow and O2 extraction as a function of O2 uptake in muscles composed of different fiber types. *Respiratory physiology & neurobiology* 153: 237-249, 2006.

40.   **Ferreira LF, Padilla DJ, Williams J, Hageman KS, Musch TI, and Poole DC**. Effects of altered nitric oxide availability on rat muscle microvascular oxygenation during contractions. *Acta physiologica* 186: 223-232, 2006.

41.   **Flaim SF, Nellis SH, Toggart EJ, Drexler H, Kanda K, and Newman ED**. Multiple simultaneous determinations of hemodynamics and flow distribution in conscious rat. *Journal of pharmacological methods* 11: 1-39, 1984.

42.   **Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ, and Ahluwalia A**. Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension* 61: 1091-1102, 2013.

43.   **Gollnick PD, Piehl K, and Saltin B**. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *The Journal of physiology* 241: 45-57, 1974.

44.   **Greenhaff PL, Nevill ME, Soderlund K, Bodin K, Boobis LH, Williams C, and Hultman E**. The metabolic responses of human type I and II muscle fibres during maximal treadmill sprinting. *The Journal of physiology* 478 (Pt 1): 149-155, 1994.

45.   **Haseler LJ, Hogan MC, and Richardson RS**. Skeletal muscle phosphocreatine recovery in exercise-trained humans is dependent on O2 availability. *Journal of applied physiology* 86: 2013-2018, 1999.

46.   **Haseler LJ, Kindig CA, Richardson RS, and Hogan MC**. The role of oxygen in determining phosphocreatine onset kinetics in exercising humans. *The Journal of physiology* 558: 985-992, 2004.

47.   **Haseler LJ, Richardson RS, Videen JS, and Hogan MC**. Phosphocreatine hydrolysis during submaximal exercise: the effect of FIO2. *Journal of applied physiology* 85: 1457-1463, 1998.

48.   **Hernandez A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E, and Westerblad H**. Dietary nitrate increases tetanic [Ca2+]i and contractile force in mouse fast-twitch muscle. *The Journal of physiology* 590: 3575-3583, 2012.

49.   **Hirai DM, Copp SW, Ferguson SK, Holdsworth CT, McCullough DJ, Behnke BJ, Musch TI, and Poole DC**. Exercise training and muscle microvascular oxygenation: functional role of nitric oxide. *Journal of applied physiology* 113: 557-565, 2012.

50.   **Hirai DM, Copp SW, Hageman KS, Poole DC, and Musch TI**. Aging alters the contribution of nitric oxide to regional muscle hemodynamic control at rest and during exercise in rats. *Journal of applied physiology* 111: 989-998, 2011.

51.   **Hirai T, Visneski MD, Kearns KJ, Zelis R, and Musch TI**. Effects of NO synthase inhibition on the muscular blood flow response to treadmill exercise in rats. *Journal of applied physiology* 77: 1288-1293, 1994.

52.   **Hogan MC, Arthur PG, Bebout DE, Hochachka PW, and Wagner PD**. Role of O2 in regulating tissue respiration in dog muscle working in situ. *Journal of applied physiology* 73: 728-736, 1992.

53.   **Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G**. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 84: 9265-9269, 1987.

54.   **Ingram TE, Pinder AG, Bailey DM, Fraser AG, and James PE**. Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. *American journal of physiology Heart and circulatory physiology* 298: H331-339, 2010.

55.   **James PE, Willis GR, Allen JD, Winyard PG, and Jones AM**. Nitrate pharmacokinetics: Taking note of the difference. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2015.

56.   **Jones AM**. Dietary nitrate supplementation and exercise performance. *Sports medicine* 44 Suppl 1: S35-45, 2014.

57.   **Jones AM, Krustrup P, Wilkerson DP, Berger NJ, Calbet JA, and Bangsbo J**. Influence of exercise intensity on skeletal muscle blood flow, O2 extraction and O2 uptake on-kinetics. *The Journal of physiology* 590: 4363-4376, 2012.

58.   **Jones AM, and Poole DC**. Oxygen uptake dynamics: from muscle to mouth--an introduction to the symposium. *Medicine and science in sports and exercise* 37: 1542-1550, 2005.

59.   **Joyner MJ, and Tschakovsky ME**. Nitric oxide and physiologic vasodilation in human limbs: where do we go from here? *Canadian journal of applied physiology = Revue canadienne de physiologie appliquee* 28: 475-490, 2003.

60.   **Joyner MJ, and Wilkins BW**. Exercise hyperaemia: is anything obligatory but the hyperaemia? *The Journal of physiology* 583: 855-860, 2007.

61.   **Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, and Ahluwalia A**. Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free radical biology & medicine* 55: 93-100, 2013.

62.   **Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, and Jones AM**. Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. *American journal of physiology Regulatory, integrative and comparative physiology* 304: R73-83, 2013.

63.   **Kelly J, Vanhatalo A, Wilkerson DP, Wylie LJ, and Jones AM**. Effects of nitrate on the power-duration relationship for severe-intensity exercise. *Medicine and science in sports and exercise* 45: 1798-1806, 2013.

64.   **Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim E, Kraus WE, and Allen JD**. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *Journal of applied physiology* 110: 1582-1591, 2011.

65.   **Kindig CA, Howlett RA, and Hogan MC**. Effect of extracellular PO2 on the fall in intracellular PO2 in contracting single myocytes. *Journal of applied physiology* 94: 1964-1970, 2003.

66.   **Kindig CA, Richardson TE, and Poole DC**. Skeletal muscle capillary hemodynamics from rest to contractions: implications for oxygen transfer. *Journal of applied physiology* 92: 2513-2520, 2002.

67.   **King CE, Melinyshyn MJ, Mewburn JD, Curtis SE, Winn MJ, Cain SM, and Chapler CK**. Canine hindlimb blood flow and O2 uptake after inhibition of EDRF/NO synthesis. *Journal of applied physiology* 76: 1166-1171, 1994.

68.   **Kleinbongard P, Dejam A, Lauer T, Jax T, Kerber S, Gharini P, Balzer J, Zotz RB, Scharf RE, Willers R, Schechter AN, Feelisch M, and Kelm M**. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free radical biology & medicine* 40: 295-302, 2006.

69.   **Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Godecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, and Kelm M**. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free radical biology & medicine* 35: 790-796, 2003.

70.   **Kobzik L, Reid MB, Bredt DS, and Stamler JS**. Nitric oxide in skeletal muscle. *Nature* 372: 546-548, 1994.

71.   **Krustrup P, Mohr M, Amstrup T, Rysgaard T, Johansen J, Steensberg A, Pedersen PK, and Bangsbo J**. The yo-yo intermittent recovery test: physiological response, reliability, and validity. *Medicine and science in sports and exercise* 35: 697-705, 2003.

72.   **Krustrup P, Soderlund K, Mohr M, and Bangsbo J**. The slow component of oxygen uptake during intense, sub-maximal exercise in man is associated with additional fibre recruitment. *Pflugers Archiv : European journal of physiology* 447: 855-866, 2004.

73.   **Krustrup P, Soderlund K, Relu MU, Ferguson RA, and Bangsbo J**. Heterogeneous recruitment of quadriceps muscle portions and fibre types during moderate intensity knee-extensor exercise: effect of thigh occlusion. *Scandinavian journal of medicine & science in sports* 19: 576-584, 2009.

74.   **Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, and Jones AM**. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *Journal of applied physiology* 110: 591-600, 2011.

75.   **Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, and Weitzberg E**. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell metabolism* 13: 149-159, 2011.

76.   **Larsen FJ, Schiffer TA, Weitzberg E, and Lundberg JO**. Regulation of mitochondrial function and energetics by reactive nitrogen oxides. *Free radical biology & medicine* 53: 1919-1928, 2012.

77.   **Larsen FJ, Weitzberg E, Lundberg JO, and Ekblom B**. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free radical biology & medicine* 48: 342-347, 2010.

78.   **Larsen FJ, Weitzberg E, Lundberg JO, and Ekblom B**. Effects of dietary nitrate on oxygen cost during exercise. *Acta physiologica* 191: 59-66, 2007.

79.   **Laughlin MH, Davis MJ, Secher NH, van Lieshout JJ, Arce-Esquivel AA, Simmons GH, Bender SB, Padilla J, Bache RJ, Merkus D, and Duncker DJ**. Peripheral circulation. *Comprehensive Physiology* 2: 321-447, 2012.

80.   **Leek BT, Mudaliar SR, Henry R, Mathieu-Costello O, and Richardson RS**. Effect of acute exercise on citrate synthase activity in untrained and trained human skeletal muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 280: R441-447, 2001.

81.   **Lo LW, Vinogradov SA, Koch CJ, and Wilson DF**. A new, water soluble, phosphor for oxygen measurements in vivo. *Advances in experimental medicine and biology* 428: 651-656, 1997.

82.   **Lundberg JO, and Govoni M**. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free radical biology & medicine* 37: 395-400, 2004.

83.   **Lundberg JO, and Weitzberg E**. NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. *Archives of pharmacal research* 32: 1119-1126, 2009.

84.   **Lundberg JO, Weitzberg E, and Gladwin MT**. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature reviews Drug discovery* 7: 156-167, 2008.

85.   **Maher AR, Arif S, Madhani M, Abozguia K, Ahmed I, Fernandez BO, Feelisch M, O'Sullivan AG, Christopoulos A, Sverdlov AL, Ngo D, Dautov R, James PE, Horowitz JD,**

and Frenneaux MP. Impact of chronic congestive heart failure on pharmacokinetics and vasomotor effects of infused nitrite. *British journal of pharmacology* 169: 659-670, 2013.

86.   **Masschelein E, Van Thienen R, Wang X, Van Schepdael A, Thomis M, and Hespel P**. Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. *Journal of applied physiology* 113: 736-745, 2012.

87.   **McDonough P, Behnke BJ, Kindig CA, and Poole DC**. Rat muscle microvascular PO2 kinetics during the exercise off-transient. *Experimental physiology* 86: 349-356, 2001.

88.   **McDonough P, Behnke BJ, Padilla DJ, Musch TI, and Poole DC**. Control of microvascular oxygen pressures in rat muscles comprised of different fibre types. *The Journal of physiology* 563: 903-913, 2005.

89.   **Moncada S, and Higgs EA**. The discovery of nitric oxide and its role in vascular biology. *British journal of pharmacology* 147 Suppl 1: S193-201, 2006.

90.   **Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, and Easton C**. The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers. *International journal of sport nutrition and exercise metabolism* 23: 498-506, 2013.

91.   **Musch TI, Bruno A, Bradford GE, Vayonis A, and Moore RL**. Measurements of metabolic rate in rats: a comparison of techniques. *Journal of applied physiology* 65: 964-970, 1988.

92.   **Musch TI, Eklund KE, Hageman KS, and Poole DC**. Altered regional blood flow responses to submaximal exercise in older rats. *Journal of applied physiology* 96: 81-88, 2004.

93.   **Musch TI, McAllister RM, Symons JD, Stebbins CL, Hirai T, Hageman KS, and Poole DC**. Effects of nitric oxide synthase inhibition on vascular conductance during high speed treadmill exercise in rats. *Experimental physiology* 86: 749-757, 2001.

94.   **Musch TI, and Terrell JA**. Skeletal muscle blood flow abnormalities in rats with a chronic myocardial infarction: rest and exercise. *The American journal of physiology* 262: H411-419, 1992.

95.   **Palmer RM, Ferrige AG, and Moncada S**. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524-526, 1987.

96.   **Pinheiro LC, Montenegro MF, Amaral JH, Ferreira GC, Oliveira AM, and Tanus-Santos JE**. Increase in gastric pH reduces hypotensive effect of oral sodium nitrite in rats. *Free radical biology & medicine* 53: 701-709, 2012.

97.   **Pluta RM, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, Devroom HL, Nahavandi M, Woo S, Figg WD, and Lonser RR**. Safety and feasibility of long-term intravenous sodium nitrite infusion in healthy volunteers. *PloS one* 6: e14504, 2011.

98.   **Poole DC, Behnke BJ, McDonough P, McAllister RM, and Wilson DF**. Measurement of muscle microvascular oxygen pressures: compartmentalization of phosphorescent probe. *Microcirculation* 11: 317-326, 2004.

99.   **Poole DC, Hirai DM, Copp SW, and Musch TI**. Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. *American journal of physiology Heart and circulatory physiology* 302: H1050-1063, 2012.

100.   **Poole DC, and Jones AM**. Oxygen uptake kinetics. *Comprehensive Physiology* 2: 933-996, 2012.

101.   **Reid MB**. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta physiologica Scandinavica* 162: 401-409, 1998.

102.   **Richardson RS, Leigh JS, Wagner PD, and Noyszewski EA**. Cellular PO2 as a determinant of maximal mitochondrial O(2) consumption in trained human skeletal muscle. *Journal of applied physiology* 87: 325-331, 1999.
103.   **Rumsey WL, Vanderkooi JM, and Wilson DF**. Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue. *Science* 241: 1649-1651, 1988.
104.   **Sindler AL, Fleenor BS, Calvert JW, Marshall KD, Zigler ML, Lefer DJ, and Seals DR**. Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. *Aging cell* 10: 429-437, 2011.
105.   **Sperandio PA, Oliveira MF, Rodrigues MK, Berton DC, Treptow E, Nery LE, Almeida DR, and Neder JA**. Sildenafil improves microvascular O2 delivery-to-utilization matching and accelerates exercise O2 uptake kinetics in chronic heart failure. *American journal of physiology Heart and circulatory physiology* 303: H1474-1480, 2012.
106.   **Stamler JS, and Meissner G**. Physiology of nitric oxide in skeletal muscle. *Physiological reviews* 81: 209-237, 2001.
107.   **Stuehr DJ, Santolini J, Wang ZQ, Wei CC, and Adak S**. Update on mechanism and catalytic regulation in the NO synthases. *The Journal of biological chemistry* 279: 36167-36170, 2004.
108.   **Thomas DD, Liu X, Kantrow SP, and Lancaster JR, Jr.** The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2. *Proceedings of the National Academy of Sciences of the United States of America* 98: 355-360, 2001.
109.   **Thomas GD, and Victor RG**. Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. *The Journal of physiology* 506 (Pt 3): 817-826, 1998.
110.   **Thompson C, Wylie LJ, Fulford J, Kelly J, Black MI, McDonagh ST, Jeukendrup AE, Vanhatalo A, and Jones AM**. Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *European journal of applied physiology* 2015.
111.   **Tsuchiya K, Tomita S, Ishizawa K, Abe S, Ikeda Y, Kihira Y, and Tamaki T**. Dietary nitrite ameliorates renal injury in L-NAME-induced hypertensive rats. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 22: 98-103, 2010.
112.   **Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, Benjamin N, Winyard PG, and Jones AM**. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *American journal of physiology Regulatory, integrative and comparative physiology* 299: R1121-1131, 2010.
113.   **Vanhatalo A, Fulford J, Bailey SJ, Blackwell JR, Winyard PG, and Jones AM**. Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia. *The Journal of physiology* 589: 5517-5528, 2011.
114.   **Vanhatalo A, Fulford J, DiMenna FJ, and Jones AM**. Influence of hyperoxia on muscle metabolic responses and the power-duration relationship during severe-intensity exercise in humans: a 31P magnetic resonance spectroscopy study. *Experimental physiology* 95: 528-540, 2010.
115.   **Vitecek J, Lojek A, Valacchi G, and Kubala L**. Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. *Mediators of inflammation* 2012: 318087, 2012.
116.   **Whipp BJ, and Ward SA**. Cardiopulmonary coupling during exercise. *The Journal of experimental biology* 100: 175-193, 1982.

117.   **Whipp BJ, and Wasserman K**. Oxygen uptake kinetics for various intensities of constant-load work. *J Appl Physiol* 33: 351-356, 1972.

118.   **Wilkerson DP, Hayward GM, Bailey SJ, Vanhatalo A, Blackwell JR, and Jones AM**. Influence of acute dietary nitrate supplementation on 50 mile time trial performance in well-trained cyclists. *European journal of applied physiology* 112: 4127-4134, 2012.

119.   **Woodman CR, Schrage WG, Rush JW, Ray CA, Price EM, Hasser EM, and Laughlin MH**. Hindlimb unweighting decreases endothelium-dependent dilation and eNOS expression in soleus not gastrocnemius. *Journal of applied physiology* 91: 1091-1098, 2001.

120.   **Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, and Jones AM**. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *Journal of applied physiology* 115: 325-336, 2013.

121.   **Wylie LJ, Mohr M, Krustrup P, Jackman SR, Ermiotadis G, Kelly J, Black MI, Bailey SJ, Vanhatalo A, and Jones AM**. Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *European journal of applied physiology* 113: 1673-1684, 2013.

122.   **Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, Doulias PT, Ischiropoulos H, Townsend RR, Margulies KB, Cappola TP, Poole DC, and Chirinos JA**. The Effect of Inorganic Nitrate on Exercise Capacity in Heart Failure with Preserved Ejection Fraction. *Circulation* 2014.

123.   **Zhu SG, Kukreja RC, Das A, Chen Q, Lesnefsky EJ, and Xi L**. Dietary nitrate supplementation protects against Doxorubicin-induced cardiomyopathy by improving mitochondrial function. *Journal of the American College of Cardiology* 57: 2181-2189, 2011.

Appendix A - Curriculum Vitae

<u>Scott Kohman Ferguson</u>

Date of Birth: June 29, 1987

Place of Birth: Media, PA, USA

Current Address: *Work*

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Education

2012-present   Doctoral Candidate (Anatomy and Physiology, Kansas State University)

Expected graduation: August 2015

Dissertation title: Skeletal muscle vascular and metabolic control: Impacts of exogenous vs. endogenous nitric oxide synthesis

Mentor: Dr. David C. Poole

Co-mentor: Dr. Timothy I. Musch

Dec. 2012   M.S. (Kinesiology, Kansas State University)

Mentor: Dr. David C. Poole

Dec. 2010   B.S. (Kinesiology, Kansas State University)

Academic Appointments

2014-present Course Facilitator – *Department of Kinesiology Kansas State University (Cardiorespiratory/Comparative Physiology in Health and Disease)* Responsible for administering the online course content, developing exams, and assigning grades.

2013-present Instructor – *Department of Kinesiology Kansas State University (Nutrition and Exercise KIN/HN 635).* Sole instructor for the exercise section (16 lectures) of KIN/HN 635. Lectures focused on basic cardiopulmonary physiology, metabolic pathways, substrate utilization during exercise and implications of a healthy lifestyle vs. "ideal weight."

2010-present Laboratory Teaching Assistant - *Department of Anatomy and Physiology, Kansas State University (Veterinary Physiology II, AP747).* Assist in instructing a laboratory experience for 1st year veterinary students focused on lung structure and function in health and disease. Demonstrations performed include maximal exercise tests and various pulmonary function tests.

2010-present Graduate Teaching Assistant - *Department of Kinesiology, Kansas State University (KIN163, KIN 220, KIN 336, KIN 360, KIN 625).* Instruct both lifetime sport classes as well as exercise physiology and anatomy and physiology laboratory experiences. Developed all course content for Kin 163 Fitness and Conditioning. Served as laboratory coordinator for Kin 625 Exercise Testing and Prescription Laboratory and played an instrumental role in updating the Kin 336 Physiology of Exercise Laboratory Manual and Manual Supplement.

2010-present Graduate Research Assistant – *Cardiorespiratory Exercise Physiology Laboratory, Department of Anatomy and Physiology, Kansas State University.*

Professional Memberships

2013-present   The American College of Sports Medicine

2013-present   The American Physiological Society, Environmental and Exercise Physiology
                Section

2013-present   The American Alpine Club

2015-present   The American Thoracic Society

Peer-reviewed Manuscripts

1.   Copp SW, Hirai DM, **Ferguson SK**, Musch TI, Poole DC. (2011). Role of neuronal nitric
      oxide synthase in modulating microvascular and contractile function in rat skeletal
      muscle. *Microcirculation* 18, 501-511

2.   Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Musch TI & Poole DC. (2012).
      Chronic heart failure impairs nNOS-mediated control of microvascular O_2 pressure in
      contracting rat skeletal muscle. *J Physiol* 590(15): 3585-3596.

3.   Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, McCullough DJ, Behnke BJ, Musch
      TI & Poole DC. (2012). Exercise training and muscle microvascular oxygenation:
      functional role of nitric oxide bioavailability. *J Appl Physiol* 113(4): 557-565.

4.   Hirai DM, Copp SW, Holdsworth CT**, Ferguson SK**, Musch TI & Poole DC. (2012).
      Effects of neuronal nitric oxide synthase inhibition on microvascular and contractile
      function in skeletal muscle of aged rats. *Am J Physiol Heart Circ Physiol* 303(8): H1076-
      1084.

5.   **Ferguson SK**, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI &
      Poole DC. (2013). Impact of dietary nitrate supplementation via beetroot juice on
      exercising muscle vascular control in rats. *J Physiol* 591:547-57.

6.	Copp SW, Inagaki T, White MJ, Hirai DM, **Ferguson SK**, Holdsworth CT, Sims GE, Poole DC & Musch TI. (2013). (-)-Epicatechin administration and exercising skeletal muscle vascular control and microvascular oxygenation in healthy rats. *Am J Physiol Heart Circ Physiol* 304(2):H206-14.

7.	Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, Musch TI & Poole DC. (2013). Superfusion of the NO donor sodium nitroprusside: potential for skeletal muscle vascular and metabolic dysfunction. *Microvascular Res* 85: 104-11.

8.	Copp SW, Holdsworth CT, **Ferguson SK**, Hirai DM, Poole DC, & Musch TI. (2013). Muscle fibre-type dependence of neuronal nitric oxide synthase-mediated vascular control in the rat during high-speed treadmill running. *J Physiol* 591: 2885-2896.

9.	**Ferguson SK**, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, & Poole DC. (2013). Effects of nitrate supplementation via beetroot juice on contracting skeletal muscle microvascular oxygen pressure dynamics. *Resp Phys Neurobiol* 187: 250-255.

10.	Hirai DM, Copp SW, Holdsworth CT, **Ferguson SK**, McCullough DJ, Behnke BJ, Musch TI, Poole DC. (2013). Skeletal muscle microvascular oxygenation dynamics in heart failure: exercise training and nitric oxide-mediated function. *Am J Physiol Heart Circ Physiol* 306(5): H690-H698.

11.	Holdsworth CT, Copp SW, Hirai DM, **Ferguson SK**, Sims E, Hageman KS, Stebbins CL, Poole DC, Musch TI. (2014). The effects of dietary fish oil on exercising skeletal muscle vascular and metabolic control in chronic heart failure rats. *Appl Physiol Nutr Metab* 39(3): 299-307.

12.	Sims GE, Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Poole DC, & Musch TI. (2014). Effects of pentoxifylline on exercising skeletal muscle vascular control in rats with chronic heart failure. *J Cardiol Therap* 2, 32-44.

13. **Ferguson SK**, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, & Poole DC. (2014). Dose dependent effects of nitrate supplementation on cardiovascular control and microvascular oxygenation dynamics in healthy rats. *Nitric Oxide* 39, 51-58.

14. **Ferguson SK**, Holdsworth CT, Fees AJ, Allen JD, Jones AM, Musch TI, & Poole DC. (2014). Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation. *Nitric Oxide* (DOI: 10.1016/j.niox.2014.09.157).

15. Holdsworth CT, Copp SW, **Ferguson SK**, Sims G, Poole DC, & Musch TI. (2015). Acute blockade of ATP-sensitive K^+ channels impairs skeletal muscle vascular control in rats during treadmill exercise. *Am J Physiol Heart Circ Physiol* (accepted).

16. **Ferguson SK**, Glean AA, Holdsworth CT, Wright JL, Fees AJ, Colburn TD, Stabler T, Allen JD, Jones AM, Musch TI, & Poole DC. (2015). Skeletal muscle vascular control during exercise: impact of nitrite infusion during nitric oxide synthase inhibition in healthy rats. *J Cardiovasc Pharmacol Ther* (in second round of reviews).

17. **Ferguson SK**, Holdsworth CT, Colburn TD, Wright JL, Jones AM, Allen JD, Musch TI, & Poole DC. (2015). Dietary nitrate supplementation: Impact on skeletal muscle vascular control in exercising rats with heart failure. *Am J Physiol Heart Circ Physiol* (in preparation).

Peer-reviewed Review Articles

1. Poole DC, Copp SW, **Ferguson SK**, Musch TI. (2013). Skeletal muscle capillary function: contemporary observations and novel hypotheses. *Exp Physiol* 98(12): 1645-1658

Journals Reviewed For

Journal of Applied Physiology

Nitric Oxide: Biology and Chemistry

Journal of Cardiovascular Pharmacology and Therapeutics

Media Publications

1. **Ferguson SK**. (2015) How nitrate supplementation can help improve your climbing performance. *Climbing Magazine* (in press).

National Presentations

ACSM National Meeting. San Francisco, CA. May 2011
"Impact of dietary nitrate supplementation via beetroot juice on exercising skeletal muscle vascular control in rats"

ACSM National Meeting. Indianapolis, IN. May 2012
Featured Science Session *"O$_2$ delivery/utilization balance and dietary nitrate supplementation: Can a juice improve metabolic control?"*

ACSM National Meeting. Indianapolis, IN. May 2012
"Effects Of Low Dose Nitrate Supplementation On Contracting Rat Skeletal Muscle Microvascular Oxygen Pressure"

Invited Speaking Engagements

ACSM National Meeting. Indianapolis, IN. May 2012
Featured Science Session *"O$_2$ delivery/utilization balance and dietary nitrate supplementation: Can a juice improve metabolic control?"*

Tri-Delta sorority (Kansas State University Chapter). Manhattan, KS. October 2014
Healthy Body Image Week presenter *"Body weight and your health: can one really predict the other?"*

Departmental Seminars

Kansas State University College of Veterinary Medicine. March 2013

Department of Anatomy and Physiology Seminar Series

"O_2 delivery/utilization balance and dietary nitrate supplementation: Can a juice improve metabolic control?"

Kansas State University College of Veterinary Medicine. September 2013

Department of Anatomy and Physiology Seminar Series

"Therapeutic potential of dietary nitrate supplementation: impact on skeletal muscle O_2 delivery/utilization balance"

Awards and Honors

Kansas State University Graduate Student Council Travel Award, Fall 2012

Kansas State University Department of Kinesiology Outstanding Graduate Student Award, Spring 2013

Kansas State University College of Veterinary Medicine Travel Award, Spring 2014

American Physiological Society: Caroline Tum Suden/Frances Hellebrandt Professional Opportunity Award, Spring 2014

Phi Zeta Honor Society: A.S.R Ganta Graduate Student Award, Spring 2014

Kansas State University College of Veterinary Medicine's Dr. Albert L. Burroughs Memorial Award, Spring 2015

Kansas State University College of Veterinary Medicine's Marguerite L. Richards Graduate Student Scholarship Award, Spring 2015

Abstracts

1.   Copp SW, Hirai DM, **Ferguson SK**, Poole DC & Musch TI. (2011). Effects of neuronal nitric oxide synthase (nNOS) inhibition on microvascular O_2 pressures during contractions in rat skeletal muscle. *FASEB J* 25:814.6.

2.   Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, Musch TI & Poole DC. (2012). Exercise training and muscle microvascular oxygenation: role of nitric oxide bioavailability. *FASEB J* 26:860.18.

3.   Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, Poole DC & Musch TI. (2012). Exercise training skeletal muscle blood flow: functional role of neuronal nitric oxide synthase (nNOS). *Med Sci Sports Exerc* 44: S583.

4.   Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Poole DC & Musch TI. (2012). Chronic heart failure (CHF) alters nNOS-mediated control of skeletal muscle contractile function. *FASEB J* 26:860.19.

5.   Holdsworth CT, Copp SW, Hirai DM, **Ferguson SK**, Hageman KS, Stebbins CL, Poole DC & Musch TI. (2012). Effects of dietary fish oil on exercising muscle blood flow in chronic heart failure rats. *Med Sci Sports Exerc* 44; S388.

6.   Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Musch TI & Poole DC. (2012). Chronic heart failure alters nNOS-mediated control of skeletal muscle microvascular O_2 delivery and utilization. *Med Sci Sports Exerc* 44: S734.

7. **Ferguson SK**, Hirai DM, Copp SW**,** Holdsworth CT, Hageman KS, Jones AM, Musch TI & Poole DC. (2012). Acute dietary nitrate supplementation on resting and exercising hemodynamic control in the rat. *Med Sci Sports Exerc* 44: S879.

8. Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Poole DC & Musch TI. (2012). Efficacy of nitric oxide based treatments to improve peripheral vascular function: implications for chronic heart failure. *KSU Graduate Res Forum.*

9. Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, Jones AM, Musch TI & Poole DC. (2012). Diet impacts blood flow control and matching muscle O_2 delivery-to-utilization. *Med Sci Sports Exerc* 44.

10. Hirai DM, Copp SW, **Ferguson SK**, Holdsworth CT, Sims GE, Musch TI, Poole DC. Chronic heart failure and muscle microvascular oxygenation: effects of exercise training. (*2012 APS Intersociety Meeting: Integrative Biology of Exercise VI*)

11. Copp SW, Hirai DM, Inagaki T, White MJ, Sims GE, Holdsworth CT, **Ferguson SK**, Poole DC, Musch TI. Chronic oral (-)-epicatechin does not affect rat hindlimb skeletal muscle vascular function during exercise. (*2012 APS Intersociety Meeting: Integrative Biology of Exercise VI*)

12. Holdsworth CT, Copp SW, Inagaki T, Hirai DM, **Ferguson SK**, Sims GE, White MJ, Poole DC, Musch TI. Chronic (-)-epicatechin administration does not affect contracting skeletal muscle microvascular oxygenation. (*2012 APS Intersociety Meeting: Integrative Biology of Exercise VI*)

13. **Ferguson SK**, Hirai DM, Copp SW, Holdsworth CT, Musch TI, Poole DC. The effects of acute dietary nitrate supplementation on muscle microvascular oxygenation in contracting rat skeletal muscle. (*2012 APS Intersociety Meeting: Integrative Biology of Exercise VI*)

14. Holdsworth, CT, Sims, GE, **Ferguson SK**, Copp SW, Hirai DM, White MJ, Hageman SK, Poole DC, Musch TI. Effects of pentoxifylline on contracting skeletal muscle microvascular oxygenation in chronic heart failure rats. *ACSM Annual Meeting, 2013.*

15.   Sims GE, Hageman KS, Copp SW, Hirai DM, **Ferguson SK**, Holdsworth CT, Poole DC, Musch TI. Effects of pentoxifylline on skeletal muscle vascular control in rats with chronic heart failure. *ACSM Annual Meeting, 2013.*

16.   **Ferguson SK**, Hirai DM, Copp SW, Holdsworth CT, Sims GE, Musch TI, Poole DC. Effects of low dose nitrate supplementation on contracting rat skeletal muscle microvascular oxygen pressure. *ACSM Annual Meeting, 2013.*

17.   **Ferguson SK**, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, Musch TI, Poole DC. Impact of dietary nitrate supplementation on capillary hemodynamics in rats with chronic heart failure. *APS Experimental Biology Meeting*, 2014.

18.   **Ferguson SK**, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, Musch TI, Poole DC. Impact of dietary nitrate supplementation on microvascular oxygen pressures in muscles comprised of different fiber types. *ACSM Annual Meeting, 2014.*

19.   **Ferguson SK**, Holdsworth CT, Wright JL, Hageman KS, Musch TI, and Poole DC. Chronic heart failure and nitrate supplementation: Impact on skeletal muscle vascular control in exercising rats. *APS Experimental Biology Meeting*, 2015.

20.   **Ferguson SK**, Holdsworth CT, Wright JL, Hageman KS, Musch TI, and Poole DC. Exercising Skeletal Muscle Vascular Control: Impacts of nitrite infusion during NOS blockade In rats. *ACSM Annual Meeting, 2015.*