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

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ABSTRACT

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

1. Introduction

A fundamental tenet of exercise physiology is that blood flow (BF) increases following exercise onset to meet the rising skeletal muscle energetic demands. This hyperemic response is mediated by a host of vasodilatory controllers [19] and it is now widely accepted that nitric oxide (NO) plays a deterministic role in regulating not only O_2 delivery ($\dot{Q}O_2$) ([17], reviewed by Joyner and Tschakovsky [18]), but also O_2 utilization ($\dot{V}O_2$) within the skeletal muscle [1,26]. A growing body of evidence suggests that ingestion of inorganic nitrate (NO_3^-), for example via beetroot juice (BR), can, following a step-wise reduction, elevate NO bioavailability and thus impact skeletal muscle hemodynamic and metabolic function during exercise [25,5,43,20,22,23,9,10].

In humans, NO_3^- supplementation via BR reduces blood pressure and enhances exercise tolerance in both healthy [5,43,22,23,7,46] and patient populations (i.e., peripheral arterial disease [20]). These effects appear to have a dose–dependent response with no additional improvement in exercise tolerance after ingesting BR containing 16.8 compared to 8.4 mmol NO_3^- [45]. The precise mechanisms for these improvements are not yet fully understood.

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However, recent investigations using murine models implicate enhanced exercising muscle BF (i.e., $\uparrow\dot{Q}O_2$ [9]), and $\dot{Q}O_2/\dot{V}O_2$ matching (e.g. microvascular PO_2; PO_2mv [10]), combined with greater contractile efficiency (e.g. $\downarrow\dot{V}O_2$; [16]).

Many disease states impair exercise tolerance and its restoration is a primary therapeutic goal. What is not known is whether lower doses of NO_3^- alter cardiovascular control and muscle oxygenation (i.e. PO_2mv, which sets the pressure head for capillary–myocyte O_2 flux) during exercise. Specifically, one question of paramount ecological importance is whether NO_3^- dosing consistent with an individual eating a diet rich in leafy greens and other NO_3^- sources can achieve the cardiovascular and muscular benefits without the necessity for supplementation *per se*. Thus, we tested the hypotheses that a low dose of NO_3^- supplementation (i.e. consistent with a diet containing NO_3^- rich vegetables, 0.3 mmol/kg/day) would be sufficient to (1) raise plasma [NO_3^-] and [NO_2^-], (2) lower mean arterial pressure (MAP) at rest and during exercise, (3) elevate BF and vascular conductance (VC) in locomotory muscles of the hindlimb and (4) raise the PO_2mv of the mixed fiber-type spinotrapezius muscle during the crucial rest-contractions transient.

2. Methods

2.1. Animal selection and care

Thirty-one young adult male Sprague–Dawley rats (~3–4 months of age, Charles River Laboratories, Wilmington, MA, USA) were used in this investigation. Rats were maintained in accredited animal facilities at Kansas State University on a 12/12 h light–dark cycle with food and water provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and conducted according to National Institutes of Health guidelines. All rats were familiarized with running on a custom-built motor-driven treadmill for 5 min/day at a speed of 20 m/min up a 5% grade for ~5 days.

2.2. Supplementation protocol

Rats were randomly assigned to receive 5 days of BR supplementation with either a low NO_3^- dose of 0.3 mmol/kg/day (BRLD; $n = 14$), a higher NO_3^- dose of 1 mmol/kg/day (BRHD; $n = 6$, Beet it™, James White Drinks, Ipswich, UK) or untreated tap water (control; $n = 11$) with consumption monitored. For both BRLD and BRHD rats, 2 days' worth of BR was diluted in 100 ml of tap water (average daily fluid consumption ~50–60 ml/day). This lower NO_3^- dose (0.3 mmol/kg/day) represents a dose found in a diet containing NO_3^- rich vegetables, while the higher NO_3^- dose (1 mmol/kg/day) represents a dietary supplement with a NO_3^- concentration similar to that used by Jones and colleagues [5,43,22,23] after accounting for the resting metabolic rate of rats (~7× that of humans [15,37]). In an effort to minimize the unnecessary utilization of additional animals, both control and BRHD data presented herein represent a randomly selected subset of animals published recently. The BRHD data represent a NO_3^- dose of 1 mmol/kg/day and demonstrate a significant vascular effect of supplementation [9,10]. Data from the BRLD group were obtained within the same time-frame as control and BRHD groups presented in Ferguson et al. ([9,10], e.g. within 16 weeks). In this way any potential seasonal differences or variations in rat-chow content were avoided.

2.3. Surgical instrumentation

Rats were anaesthetized with a 5% isoflurane–O_2 mixture and maintained subsequently on 3% isoflurane–O_2. The carotid artery was isolated and cannulated with a catheter (PE-10 connected to PE-50, Intra-Medic polyethylene tubing, Clay Adams Brand, Becton, Dickinson and Company, Sparks, MD) for the measurement of MAP and HR, infusion of the phosphorescent probe (see below), and arterial blood sampling. A second catheter was placed in the caudal artery. The incisions were then closed and rats were given >1 h to recover [11].

2.4. Protocol 1: measurement of hindlimb skeletal muscle blood flow

After recovery, rats were placed on the treadmill and the caudal artery catheter was connected to a 1 ml syringe chambered in a Harvard infusion/withdrawal pump (model 907, Cambridge, MA, USA). The carotid artery catheter was connected to a pressure transducer (Gould Statham P23ID, Valley View, OH, USA) maintained at the same height as the animal. Exercise was initiated and treadmill speed was increased progressively over a ~30 s period to a speed of 20 m/min (5% grade, ~60% $\dot{V}O_2$ max; [37]). The rat continued to exercise for another 2.5 min until a total time of 3 min was reached. At 3 min the Harvard pump was activated and withdrawal was initiated at a rate of 0.25 ml 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 $0.5–0.6 \times 10^6$ 15 μm diameter radiolabeled microspheres (^{57}Co or ^{85}Sr in random order; Perkin Elmer, Waltham, MA, USA) were infused into the aortic arch for determination of regional BF. Following the microsphere infusion ~0.2 ml of blood was sampled from the carotid artery catheter for the determination of blood lactate concentration ([lactate]) (Nova Stat Profile M, Nova Biomedical, Waltham, MA, USA) after which exercise was terminated.

Following a minimum 1 h recovery period, a second microsphere infusion (differently radio-labeled than the first) was performed while the rat sat quietly on the treadmill for the determination of resting BF, HR and MAP. This experimental strategy (i.e. exercise before rest) mitigates potential influences of the pre-exercise anticipatory response on resting skeletal muscle BF measurements [2].

2.5. Protocol 2: measurement of spinotrapezius muscle PO_2mv

Following the second (resting) microsphere infusion, rats were anesthetized progressively using diluted pentobarbital sodium anesthesia (administered into the caudal artery catheter to effect) with the level of anesthesia monitored continuously via the toe-pinch and blink reflexes. Rats were then placed on a heating pad to maintain core temperature at ~38 °C (measured via rectal probe). Overlying skin and fascia were reflected carefully from the mid-dorsal caudal region of each rat and the right spinotrapezius muscle was carefully exposed in a manner that ensured the integrity of the neural and vascular supply to the muscle [3]. 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 [8,29].

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 phosphorometer (PMOD 5000, Oxygen Enterprises, Philadelphia, PA) was positioned ~2–4 mm