Effect of dietary nitrate on sympathetic vasoconstriction in resting and contracting skeletal muscle

by

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Abstract

Dietary nitrate (NO_3^-) may increase nitric oxide (NO) bioavailability and alter the control of blood flow at rest and during exercise. This thesis investigated the effect of dietary NO_3^- supplementation on sympathetic mediated control of skeletal muscle blood flow in resting and contracting skeletal muscle. It was hypothesized that dietary NO_3^-: 1) would not alter plasma norepinephrine (NE) at rest, during exercise and in response to a sympatho-excitatory stimulus; 2) would attenuate blood pressure and sympathetic vasoconstrictor responsiveness at rest and during exercise and enhance functional sympatholysis. In a double-blind randomized crossover design 10 men (22±2.8 yrs.) performed a cold pressor test (CPT) at rest and during moderate- (30% WR_{max}) and heavy-intensity (60% WR_{max}) alternate-leg knee-extension exercise following consumption of either NO_3^--rich beetroot juice (~12.9 mmol NO_3^-) or a NO_3^--depleted placebo (~0.13 mmol NO_3^-). Venous blood was sampled before and after the consumption of beetroot juice and at rest and during exercise and each CPT. Heart rate and BP were measured continuously via ECG and Finometer, and mean blood velocity (MBV) was measured continuously via Doppler ultrasound. Leg Blood flow (LBF; $ml \cdot min^{-1}$) was calculated as MBV ($cm \cdot s^{-1}$)$\cdot \pi r^2 \cdot 60$. Femoral vascular conductance (FVC; $L \cdot min^{-1} \cdot mmHg^{-1}$) was calculated as LBF/MAP. Sympathetic vasoconstrictor responsiveness to a CPT was calculated by determining the magnitude of the decrease in FVC during the CPT at rest and during exercise. The magnitude of functional sympatholysis was calculated as FVC at rest (% change) – FVC during exercise (% change). Plasma [NO_3^-] was significantly elevated following consumption of NO_3^- rich beetroot juice, but not NO_3^--depleted placebo (NO_3^-: 9±4a.u. ;Placebo: 2±1 a.u.). Dietary

NO_3^- did not alter sympathetic vasoconstrictor responsiveness at rest (NO_3^-: -33±10 %; Placebo: -34±11 %), or during moderate- (NO_3^-: -17±10 %; Placebo: -21±10 %) or heavy-intensity (NO_3^-: -12±9 %; Placebo: -11±10 %) exercise. Sympatholysis was also not altered during moderate- (NO_3^-: -16±11 %; Placebo: -13±8 %) or heavy-intensity (NO_3^-: -21±11 %; Placebo: -23±6 %) exercise following NO_3^- supplementation. These data demonstrate that acute dietary NO_3^- supplementation did not alter the regulation of blood pressure at rest and in response to exercise and sympathoexcitation. In conclusion, acute dietary NO_3^- supplementation did not alter sympathetic nervous system mediated vascular control at rest or during exercise in healthy young men.

Preface

This thesis is original work by Christopher de Vries. This research project, of which this thesis is a part, received research ethics approval from the University of Alberta Health Research Ethics Board (Health Sciences Panel), under the name “Dietary Nitrate and Sympathetic Vasoconstriction”, No. Pro 00051247, approved October 23, 2014. All subjects provided written informed consent.

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NO – nitric oxide
NOS – nitric oxide synthase
nNOS – neuronal nitric oxide synthase
iNOS – inducible nitric oxide synthase
eNOS – endothelial nitric oxide synthase
NO_3^- – nitrate
NO_2^- – nitrite
BH_4 – tetrahydrobiopterin
BP – Blood Pressure (mmHg)
SBP – systolic blood pressure (mmHg)
DBP – diastolic blood pressure (mmHg)
O_2 – oxygen
NE – norepinephrine
ATP – adenosine triphosphate
MAP – mean arterial pressure (mmHg)
VO_{2max} – incremental test to volitional exhaustion
VO_2 – oxygen consumption (L/min)
MBV – mean blood velocity (cm/s)
LBF – leg blood flow (L/min)
CPT – cold pressor test
HR – heart rate (bpm)
FVC – femoral vascular conductance (L/min/mmHg)
Q – cardiac output (L/min)
TPR – total peripheral resistance (mmHg/min/mL)
SV – stroke volume (mL/min)
RPP – rate pressure product
ROS – reactive oxygen species

Chapter 1: Introduction

Introduction

Nitric oxide (NO) is a robust signal molecule that is involved in numerous biological processes and contributes to the regulation of several physiological systems in humans (Cooper et al., 2007; Dejam et al., 2004; Stamler et al., 2001). In the vasculature, NO is a potent local vasodilator and has been shown to inhibit sympathetic vasoconstriction (Habler et al., 1997; Hansen et al., 1994; Jendzjowsky et al., 2013c; Johnson, 1998; Nase et al., 1997; Ohyanagi et al., 1992; Sander et al., 2000; Tesfamariam et al., 1987; Thomas et al., 1997; Thomas et al., 1998b) and is therefore involved in the regulation of vascular resistance and arterial blood pressure (BP) (Chavoshan et al., 2002; Forstermann et al., 1986; Lepori et al., 1999; Rapoport et al., 1983; Sartori et al., 2005). Indeed, several chronic cardiovascular diseases, such as hypertension, heart failure and peripheral arterial disease, are characterized by vascular dysfunction and reduced NO bioavailability (Campese et al., 2004; Desjardins et al., 2006; Rajapakse et al., 2012). Furthermore, exercise training has been shown to increase NOS expression and NO bioavailability (Delp et al., 1997; Delp et al., 1993; Sessa et al., 1994; Sun et al., 1994) and to augment endothelium dependent vasodilation in healthy young adults (Atkinson et al., 2015; Dawson et al., 2013; Green et al., 2014; Green et al., 2011; Thijssen et al., 2010; Thijssen et al., 2011; Tinken et al., 2008; Tinken et al., 2010). Collectively, the available scientific literature indicates that NO is an important regulator of vascular function and that there is considerable plasticity in NO mediated vascular control. Therefore, interventions that enhance NO bioavailability may also the enhance control of BP and tissue blood flow in people.

NO is produced in several tissues through the oxidation of L-arginine to L-citrulline in the presence of oxygen (O_2) (Forstermann et al., 2012). The reaction is enzymatically regulated by

NO synthase (NOS), which exists in neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms (Nelson et al., 2003). In the cardiovascular system, the primary NOS isoforms are eNOS and nNOS, which are both constitutively expressed in the skeletal muscle and the endothelium and are involved in the regulation of vascular tone (Forstermann et al., 2012; Melikian et al., 2009). In vascular smooth muscle, NO binds with and activates guanylyl cyclase to form cyclic guanosine monophosphate (cGMP) and activate protein kinase G leading to phosphorylation of myosin light chain kinase and a decrease in intracellular Ca^{2+} (Lincoln et al., 2001). NO has also been shown to inhibit sympathetic vasoconstriction (Johnson, 1998; Thomas et al., 1997; Thomas et al., 1998b). The cellular mechanism of NO mediated inhibition of vasoconstriction has not been identified, however it may involve changes in post-synaptic α-adrenergic receptor-mediated control of extracellular ion flux (Jendzjowsky et al., 2013b; Tateishi et al., 1995; Thomas et al., 1997). Chronic exercise training has been shown to increase nNOS and eNOS expression and augment NO bioavailability in humans and animals (Braga et al., 2015; Jendzjowsky et al., 2014a; McConell et al., 2007; Oltman et al., 1995) and to improve NO mediated vascular function (McAllister et al., 2006; Wang et al., 1993; Wang et al., 1997; Zhao et al., 1997) indicating that NO-dependent vascular signaling is a potential therapeutic target to improve vascular function.

NO may also be produced through a non-NOS regulated pathway, where nitrate (NO_3^-) in food is converted to NO through the NO_3^- - nitrite (NO_2^-) - NO pathway (Figure 1) (Bryan, 2006; Casey et al., 2015; Lundberg et al., 2011; Lundberg et al., 2009; Lundberg et al., 2005; Machha et al., 2011). After swallowing foods that contain high levels of NO_3^- such as celery, spinach, red beetroot and lettuce, NO_3^- enters the stomach (Hord et al., 2009). NO_3^- and NO are absorbed through the stomach wall, and enter the portal circulation. A small amount of NO_3^- enters the

entero-salivary circulation where it is concentrated on the surface of the tongue (Govoni et al., 2008; Lundberg et al., 2004a; Lundberg et al., 2004b) and NO_3^- is reduced to NO_2^- by oral bacteria (Capurso et al., 2014). The acidic conditions of the stomach reduce a portion of NO_2^- to NO and NO and NO_2^- diffuse into the circulation (Capurso et al., 2014). In the circulatory system, NO_2^- is then transported to the resistance vessels of skeletal muscle, where it is reduced to NO. NO produces vasodilation by binding with and activating guanylyl cyclase in vascular smooth muscle to form cyclic guanosine monophosphate (cGMP) which activates protein kinase G leading to phosphorylation of myosin light chain kinase and a decrease in intracellular Ca^{2+} (Lincoln et al., 2001). The reduction of NO_2^- to NO in the blood involves several enzymes and metalloproteins with redox potential, including hemoglobin, deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, vitamin C, and polyphenols in the blood, liver and intestinal tissue (Figure 1) (Alzawahra et al., 2008; Jansson et al., 2008; Minneci et al., 2008). The consumption of NO_3^- salts or NO_3^- rich vegetables such as spinach, or concentrated beetroot juice has been shown to increase plasma $[NO_3^-]$ and $[NO_2^-]$ in a dose-dependent manner, and peak plasma $[NO_3^-]$ and $[NO_2^-]$ concentrations are achieved ~2.5 hours after consumption (Kenjale et al., 2011; Wylie et al., 2013b). Plasma $[NO_3^-]$ and $[NO_2^-]$ are used as an index of total NO bioavailability because NO is rapidly oxidized to NO_3^- and NO_2^- in biological fluids and is chemically stable for several hours (Kelm, 1999). In contrast, NO has a very short biological half-life (a few seconds) and is rapidly converted into other compounds making it is very difficult to accurately measure *in vivo* (Lauer et al., 2002; Lundberg et al., 2005).

To date, research studies that have utilized dietary NO_3^- supplementation to increase NO bioavailability have predominately focused on the effect of NO_3^- on the control of BP and endothelium dependent vasodilation

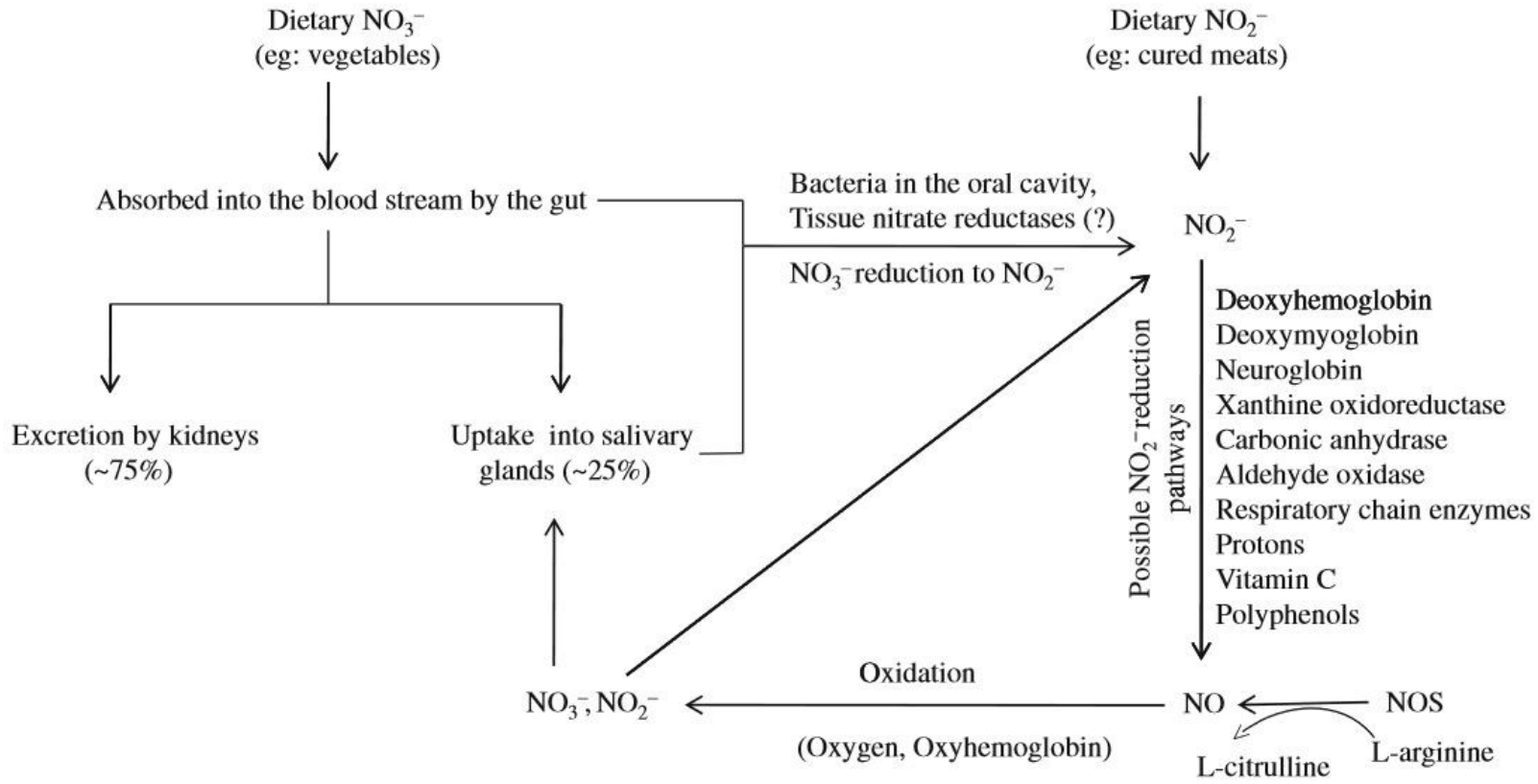

Figure 1: The production of nitric oxide. Nitric oxide (NO) is produced through the oxidation of L-arginine to L-citrulline in presence of oxygen. NO is also produced through a non-NOS regulated pathway, where nitrate (NO_3^-) in food is converted to NO through the NO_3^--nitrite (NO_2^-)-NO pathway. NO_2^- can also be reduced to NO by several enzymes and metalloproteins. Adapted from "Dietary nitrite and nitrate: a review of potential mechanisms for cardiovascular benefits" by A. Machha & A. N. Schechter, 2011, *European Journal of Nutrit*

(Aucouturier et al., 2015; Casey et al., 2015; Gilchrist et al., 2013; Hobbs et al., 2012; Kapil et al., 2010; Kenjale et al., 2011; Kim et al., 2015; Lansley et al., 2011; Lee et al., 2015; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013a). In young healthy adults, Webb et al. (2008) have demonstrated that a single dose of beetroot juice reduced systolic (SBP: -10 mmHg) and diastolic BP (DBP: -8 mmHg) 2.5 following consumption, with DBP returning to baseline levels within 24 hours of consumption, while SBP (-10 mmHg) remained reduced. Kapil et al. (2010) reported that SBP (-5 mmHg) was reduced 2.5 hours following consumption of acute dietary NO_3^- supplementation. Vanhatalo et al. (2010) reported a reduced SBP (-5 mmHg) and DBP (-5 mmHg) 2.5 hours post consumption of beetroot juice. Hobbs et al. (2012) reported the consumption of 2.3, 5.7 and 11.4 mMol of acute dietary NO_3^- reduced SBP (-13, -21 and -22 mmHg) and DBP (-17, -15 and -18 mmHg) in a dose-dependent manner. In contrast, Kim et al. (2015), Casey et al. (2015) and Aucouturier et al. (2015) reported no change in BP with acute dietary NO_3^- supplementation in healthy adults. Studies that investigated the effect of chronic dietary NO_3^- supplementation on BP have also reported reduced BP (Lansley et al., 2011; Vanhatalo et al., 2010). Vanhatalo et al. (2010) reported a sustained reduction of SBP (-6 mmHg) with 15 days of supplementation, and Lansley et al. (2011) reported lower SBP (-5 mmHg) following 6 days of chronic dietary NO_3^- supplementation. In patient populations, Kenjale et al. (2011) reported that acute consumption of dietary NO_3^- reduced DBP (-10 mmHg) in people with peripheral arterial disease, while Gilchrist et al. (2013) reported that two weeks of chronic dietary NO_3^- supplementation did not alter BP in older adults with type 2 diabetes. In summary, the available scientific literature related to the effects of dietary NO_3^- supplementation on the control of BP is conflicting,

however the preponderance of evidence suggests that both acute and chronic dietary NO_3^- supplementation have the potential to reduce BP in healthy adults and in patient populations. The reason for the contrasting findings between the previous studies is not readily apparent. In addition the mechanism responsible for a reduction in BP following dietary NO_3^- supplementation has not been determined. However, Bondonno et al. (2012) reported augmented brachial artery flow mediated vasodilation following dietary NO_3^- supplementation via spinach consumption in young healthy men and women. Similarly, Heiss et al. (2012) reported that sodium NO_3^- supplementation increased flow mediated vasodilation in young healthy males. In contrast, Gilchrist et al. (2013) reported that two weeks of dietary NO_3^- supplementation did not alter brachial artery flow mediated dilation in older adults with type 2 diabetes. Collectively, these results suggest that dietary NO_3^- supplementation may alter the control of BP and enhanced endothelium dependent vasodilation may be mechanistically linked to the reduction in BP.

In the skeletal muscle vasculature, blood flow is regulated by a balance between sympathetic nervous system mediated vasoconstriction and local vasodilation produced by vasoactive paracrine molecules (L. B. Rowell, 1993a). At rest, the skeletal muscle vascular bed receives ~20% of cardiac output and the skeletal muscle is an important vascular bed for the regulation of vascular resistance and BP. The sympathetic nervous system produces tonic vasoconstriction in skeletal muscle blood vessels (Buckwalter et al., 2001) and is the predominant regulator of vascular resistance at rest (L. B. Rowell, 1993b). During exercise, skeletal muscles blood vessels dilate and during intense exercise as much as 80% of cardiac output can be delivered to skeletal muscles (McArdle et al., 2014). During isolated muscle mass exercise (single-leg knee extension) leg blood flows

as high as 10 $L \cdot min^{-1}$ have been reported, suggesting that the vasodilation of skeletal muscle blood vessels could exceed the capacity of the heart to increase cardiac output during large muscle exercise and result in a fall in BP (Saltin et al., 1998). However, sympathetic nervous system activity increases during exercise and produces vasoconstriction in non-active tissues and active skeletal muscle (DiCarlo et al., 1996; O'Leary et al., 1997), which serves to distribute cardiac output to active tissues and balances the robust local vasodilation to maintain BP (Buckwalter et al., 1997; Saltin et al., 1998). Direct measurement of efferent sympathetic nerve activity in active skeletal muscle is not technically feasible; however measures of circulating catecholamines, norepinephrine (NE) and epinephrine (EPI), indicate that sympathetic nerve activity increases in an exercise-intensity dependent manner (DiCarlo et al., 1996; Mancia et al., 1991; Murai et al., 2009; O'Leary et al., 1997). An increase in efferent sympathetic nerve activity directed toward active skeletal muscle tissue during exercise seems counter-intuitive, however vascular responsiveness to sympathetic outflow is attenuated in exercising skeletal muscle (Hansen et al., 1996; Tschakovsky et al., 2002), a phenomenon termed functional sympatholysis (Remensnyder et al., 1962), which allows constriction to occur in inactive vascular beds while maintaining perfusion of active muscle and the maintenance of BP (Buckwalter et al., 2001). The mechanisms for sympatholysis are not well understood, however NO has been shown to inhibit vasoconstriction in resting (Habler et al., 1997; Jendzjowsky et al., 2013a; Nase et al., 1997; Ohyanagi et al., 1992; Tesfamariam et al., 1987) and contracting (Hansen et al., 1994; Jendzjowsky et al., 2013c; Sander et al., 2000; Thomas et al., 1998a; Thomas et al., 1998b) skeletal muscle. Thus, the regulation of sympathetic vasoconstriction in the skeletal muscle vascular bed

may be sensitive to the level of NO bioavailability. Indeed, Jendzjowsky et al. (2014b) have shown that acute supplementation with the essential NOS co-factor tetrahydrobiopterin (BH_4) blunted sympathetic vasoconstrictor responses to sympathetic stimulation in resting and contracting skeletal muscle in rodents. Jendzjowsky et al. (2013c) have also reported that 4 weeks of aerobic exercise increased skeletal muscle nNOS expression and enhanced the inhibition of sympathetic vasoconstriction in contracting skeletal muscle through an NO dependent mechanism in rodents. Collectively, the results of these studies suggest that enhancing NO production may blunt sympathetic vasoconstrictor responsiveness. Therefore changes in sympathetic vascular control many contribute to a decrease in BP and improve tissue blood flow in resting and contracting skeletal muscle following dietary NO_3^- supplementation.

However, a limited number of studies have investigated the effect of supplemental dietary NO_3^- on the control of skeletal muscle blood flow and the available scientific evidence is contradictory. Casey et al., (2015) reported acute dietary NO_3^- supplementation enhanced skeletal muscle blood flow and vasodilation in older adults during rhythmic handgrip exercise at 20% of maximal voluntary contraction during hypoxia, but not normoxia, and acute dietary NO_3^- supplementation did not alter skeletal muscle blood flow or vasodilation during handgrip exercise in young adults during normoxia or hypoxia. Similarly, Kim et al., (2015) reported that acute dietary NO_3^- supplementation did not alter the blood flow response to graded incremental forearm handgrip exercise. In contrast, Ferguson et al. (2013) reported that 5 d of dietary NO_3^- supplementation reduced mean arterial BP (MAP) and increased vascular conductance and hind limb blood flow in rats during incremental treadmill exercise. Interestingly,

dietary NO_3^- supplementation caused the largest increase in blood flow to type II muscles during heavy-intensity exercise. These data suggest that the effects of NO_3^- supplementation may be fiber type specific and/or exercise intensity-dependent and increased NO bioavailability may be particularly important to vascular regulation during heavy-intensity exercise. Whether the increase in vascular conductance was mediated by improved vasodilation or enhanced blunting of sympathetic vasoconstriction was not established.

In summary, the consumption of food containing dietary NO_3^- has been shown to increase plasma [NO_3^-] and [NO_2^-] and reduce arterial BP. Some studies suggest that enhanced endothelium-dependent vasodilation may be mechanistically linked to the reduction, however, to our knowledge the effects of dietary NO_3^- on the regulation of sympathetic nervous system vasoconstriction has not been investigated.

Purpose and Hypothesis

Therefore, the purpose of the proposed study was to investigate the effect of acute dietary NO_3^- supplementation via beetroot juice on plasma catecholamines [NE] and [EPI] (an index sympathetic activity), blood pressure and sympathetic vasoconstrictor responsiveness at rest and during exercise, and in response to sympathoexcitation in humans. It was hypothesized that acute dietary NO_3^- supplementation would: 1) have no effect on plasma catecholamines, at rest and during exercise and in response to a sympatho-excitatory stimulus; 2) attenuate sympathetic vasoconstrictor responsiveness at rest and during exercise and enhance sympatholysis.

Significance

This study investigated the effects of dietary NO_3^- on sympathetic vasoconstriction in resting and contracting skeletal muscle. To my knowledge, this is the first study to investigate the effects of dietary NO_3^- on sympathetic vascular control at rest and during moderate- and heavy-intensity large muscle mass exercise. The findings from this study will advance our understanding of NO mediated sympathetic vascular control at rest and during exercise. Furthermore, the findings from this thesis have implications for the treatment of people with conditions that are characterized by vascular dysfunction, reduced NO bioavailability and elevated sympathetic nerve activity, such as aging, diabetes and hypertension.

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Chapter 2: The effect of dietary nitrate on vasoconstriction in resting and contracting skeletal muscle

Introduction

The regulation of arterial vascular resistance is vital for the control of blood pressure (BP) and tissue blood flow (L. B. Rowell, 1993). Vascular smooth muscle integrates signals from the sympathetic nervous system, circulating factors, local paracrines, etc. to regulate the diameter of arteries and control resistance and blood flow (L. B. Rowell, 1993). In response to exercise, skeletal muscle arteries dilate to increase blood flow and O_2 delivery to meet the increased metabolic demand of exercise (Andersen et al., 1985; Richardson et al., 1993; Saltin et al., 1998). At the same time, sympathetic nervous system activity also increases in response to exercise and this nerve activity produces vasoconstriction in both non-active tissue and active skeletal muscle (Buckwalter et al., 1997; DiCarlo et al., 1996; O'Leary et al., 1997; L. B. Rowell, 1993). The presence of tonic vasoconstriction in active muscle seems contradictory at a time when the demand for blood flow is increasing, however the vasoconstriction is necessary to prevent local vasodilation from increasing to a point where BP cannot be maintained by increases in cardiac output and resistance in other vascular beds. Additionally, the vasoconstrictive effects of sympathetic activity directed to skeletal muscle blood vessels is attenuated during exercise, a phenomenon termed functional sympatholysis (Buckwalter et al., 2001; Remensnyder et al., 1962), and it is believed that this blunting of local vasoconstriction facilitates skeletal muscle blood flow while allowing BP to be maintained during exercise (Dinenno et al., 2004; Habler et al., 1997; Ohyanagi et al., 1992; Thomas et al., 1998b). The mechanism(s) for sympatholysis is not well understood, however the signal molecule nitric oxide (NO) has been shown to inhibit sympathetic vasoconstriction in resting

(Habler et al., 1997; Nase et al., 1997; Ohyanagi et al., 1992; Tesfamariam et al., 1987) and contracting (Hansen et al., 1994; Sander et al., 2000; Thomas et al., 1998a; Thomas et al., 1998b) skeletal muscle.

NO is produced by the oxidation of L-arginine to L-citrulline in several tissues in the body. The reaction is regulated by the enzyme NO synthase (NOS). NOS exists in neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms (Nelson et al., 2003). Endothelial NOS and nNOS isoforms are expressed in skeletal muscle and vascular tissue and have been shown to be involved in the control of vascular resistance and skeletal muscle blood flow. A decline in NOS expression has been linked with vascular dysfunction, whereas interventions that improve NOS expression (such as exercise training) or increase NO bioavailability have been associated with improved vascular function (Jendzjowsky et al., 2013c; Jendzjowsky et al., 2014; McConell et al., 2007; Tatchum-Talom et al., 2000; Wang et al., 1993; Wang et al., 1997; Zhao et al., 1997). Collectively these results suggest that NO is an important signal molecule for the control of vascular resistance, BP and tissue blood flow.

NO may also be produced through the non-enzymatically regulated nitrate (NO_3^-) – nitrite (NO_2^-) - NO pathway where NO_3^- in the diet is converted to NO. Briefly, bacteria on the tongue and the acidic conditions of the stomach convert dietary NO_3^- to NO_2^- which can then be reduced to NO in the circulation. The ingestion of foods high in NO_3^- content, such as celery, spinach, lettuce and beetroot juice has been shown to increase plasma [NO_3^-] and [NO_2^-] and improve NO-dependent vascular function (Bondonno et al., 2012; Heiss et al., 2012; Wylie et al., 2013b).

Several recent studies have shown that acute and chronic supplementation with beetroot juice reduces BP in sedentary and exercise-trained men (Hobbs et al., 2012; Vanhatalo et al., 2010; Webb et al., 2008), women (Kapil et al., 2010), and in patients with peripheral arterial disease (Kenjale et al., 2011). Dietary NO_3^- supplementation has also been shown to enhance brachial artery flow-mediated dilation in healthy adults (Heiss et al., 2012; Webb et al., 2008). In contrast, dietary NO_3^- supplementation did not alter brachial artery flow-meditated dilation in older adults with type 2 diabetes (Gilchrist et al., 2013). While not conclusive, the accumulated evidence from these studies suggests that dietary NO_3^- supplementation may alter the control of BP and enhance endothelium-dependent vasodilation.

A limited number of studies have investigated the effects of dietary NO_3^- on the control of skeletal muscle blood flow at rest and during exercise. Ferguson et al. (2013) reported that 5 d of dietary NO_3^- supplementation augmented hind limb blood flow and vascular conductance during treadmill exercise in rats. In contrast, Kim et al. (2015) reported that dietary NO_3^- had no effect on forearm blood flow at rest or during handgrip exercise. Finally, Casey et al. (2015) have shown that acute dietary NO_3^- supplementation augmented skeletal muscle blood flow during handgrip exercise in older adults during hypoxic, but not normoxic exercise, whereas the blood flow response to handgrip exercise was unaltered in young adults. These studies have provided inconclusive evidence and have largely focused on the effects of dietary NO_3^- supplementation on vasodilation. However, NO_3^- supplementation may also affect the regulation of sympathetic vasoconstriction at rest and during exercise.

Whether dietary NO_3^- alters NO-mediated inhibition of sympathetic vasoconstriction has not been investigated on vascular control. NO has been shown to inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle (Johnson, 1998; Thomas et al., 1997; Thomas et al., 1998b). Several recent studies from our laboratory have demonstrated that aerobic exercise training increases NOS expression and improves NO-mediated sympatholysis in rodents (Jendzjowsky et al., 2013b, 2013c; Jendzjowsky et al., 2014). Furthermore, we have demonstrated that acute treatment with the NO-precursor tetrahydrobiopterin (BH_4) attenuated sympathetic vasoconstrictor responsiveness at rest and during exercise in rodents. Therefore it is conceivable that dietary NO_3^- supplementation may blunt sympathetic vasoconstrictor responsiveness at rest and during exercise by enhancing NO bioavailability.

An increase in NO bioavailability may also alter the control of efferent sympathetic outflow. In the present study, plasma catecholamines [NE] and [EPI] were used as an index of sympathetic nervous system activity, consistent with previous studies (Grassi et al., 1999; Mancia et al., 1991; L. B. Rowell et al., 1987). Several studies have locally inhibited NOS enzyme activity in the cardiovascular control centers of the brainstem and have observed increased sympathetic nerve activity and BP indicating that NO may inhibit efferent sympathetic nerve activity (Bruno et al., 2012; Hirooka et al., 2011; Patel et al., 2001). Consistent with NO-mediated inhibition of sympathetic activity, injection of NO-donors have been shown to decrease sympathetic nerve activity and BP (Bruno et al., 2012; Zanzinger et al., 1995). In the present study, direct measurement of sympathetic nerve activity to active muscle during exercise would have been technically challenging due to active involvement of all limbs in the exercise task or experimental procedures,

however measurement of circulating plasma epinephrine [EPI] and norepinephrine [NE] has been shown to correlate with direct measures of sympathetic nerve activity and can therefore be used as an index of efferent sympathetic nerve activity at rest and during exercise (Grassi et al., 1999; Mancia et al., 1991; L. B. Rowell et al., 1987). The effect of acute dietary NO_3^- supplementation on plasma catecholamines has not been investigated.

Therefore, the purpose of this study was to investigate the effect of acute dietary NO_3^- supplementation via beetroot juice on plasma catecholamines [NE] and [EPI] (an index sympathetic activity), blood pressure and sympathetic vasoconstrictor responsiveness at rest and during exercise, and in response to sympathoexcitation in humans. It was hypothesized that acute dietary NO_3^- supplementation would: 1) have no effect on plasma catecholamines, at rest and during exercise and in response to a sympatho-excitatory stimulus; 2) attenuate sympathetic vasoconstrictor responsiveness at rest and during exercise and enhance sympatholysis.

Methods

Subjects

Ten healthy, young males (22 ± 3 years) volunteered and provided written informed consent to participate in the study. The study was approved by the University of Alberta Health Research Ethics Board. All subjects were non-obese, non-smokers, and did not have any diseases or conditions which may affect the respiratory, cardiovascular, metabolic, neurological, or musculoskeletal disease. Subjects were not taking any medications known to alter the cardiovascular, respiratory or metabolic response to exercise. Subjects were recreationally active, but were not engaged in an exercise training program during the project.

Experimental Protocol

Subjects reported to the Integrative Human Exercise Physiology laboratory (4-246 Van Vliet Complex) in the Faculty of Physical Education & Recreation at the University of Alberta on four separate occasions. Subjects reported to the laboratory in a rested state (no exercise 24 hours prior), after having consumed a light meal two hours prior to testing, and after having abstained from caffeine, alcohol, and ibuprofen for 12 hours prior to testing. Subjects also refrained from using antibacterial mouthwash, toothpaste or chewing gum prior to testing.

Day 1: Subjects completed an incremental exercise test to volitional exhaustion (VO_{2peak}) on a cycle ergometer (Ergoselect 200 K, Ergoline, Bitz, Germany) for determination of maximal aerobic capacity. Testing began with 2 minutes of resting data collection, after which the work rate was progressively incremented in a ramp-like fashion (20 – 30 watts per minute. Criteria used to establish a maximal test included a plateau in VO_2 despite an increase in work rate, a respiratory exchange ratio >1.10, achievement of >90% of age-predicted maximal heart rate (HR) and volitional exhaustion.

Day 2: Subjects performed an alternate-leg incremental exercise test to volitional exhaustion on a knee-extensor ergometer in which the work rate increased by 3 watts every minute until volitional exhaustion. Criteria used to establish a maximal test included a plateau in VO_2 despite an increase in work rate, a respiratory exchange ratio >1.10, and volitional exhaustion. The maximum work rate achieved was used to calculate the work rates for constant load knee-extensor exercise on Day 3 and 4.

Day 3 and Day 4: The timeline for testing and blood samples is illustrated in Figure 2. Testing on days 3 and 4 were performed in a randomized, double-blind crossover manner and separated by at least 48 hours. Subjects were asked to consume the same meal 12 h prior, and the same breakfast and snacks prior to testing on days 3 and 4. Subjects were also provided with a list of foods containing dietary NO_3^-, and were asked to avoid eating the foods on the list 48 hours prior to testing. Following arrival, an indwelling venous catheter was inserted into the antecubital vein of their right arm by a registered nurse or certified phlebotomist. Venous blood (10 ml) was drawn for the measurement of resting plasma [NO_3^-] and [NO_2^-] levels. The subject then consumed either the placebo (~0.13 mmol NO_3^-: Beet It, James White Drinks, Ipswich, U.K.) or NO_3^--rich BR juice (~12.9 mmol NO_3^-). Following 2.5 hours of rest, the subject was seated, and rested quietly on the alternate-leg knee-extension ergometer and another venous blood sample was withdrawn. Data were collected under resting conditions for 7 minutes and then subjects performed a cold-pressor test (CPT) where they submerged their hand in -4°C ice water (CPT) for 3 minutes and another venous blood sample was withdrawn at the midway point of the CPT.

Following a recovery period, subjects performed 10 minutes of constant-load alternate-leg knee-extension at 30% (0.8±0.2 Kg), and at 60% (1.6±0.3 Kg) of peak work rate. Following six minutes of exercise, subjects submerged their hand in -4°C ice water for 3 minutes. Venous blood was sampled during exercise and the CPT. Rest and exercise trials were separated by a recovery period of ~30 minutes.

The CPT was used as the sympatho-excitatory stimulus in the present study. The CPT activates Type 3 and 4 nerves afferents leading to an increase in sympathetic

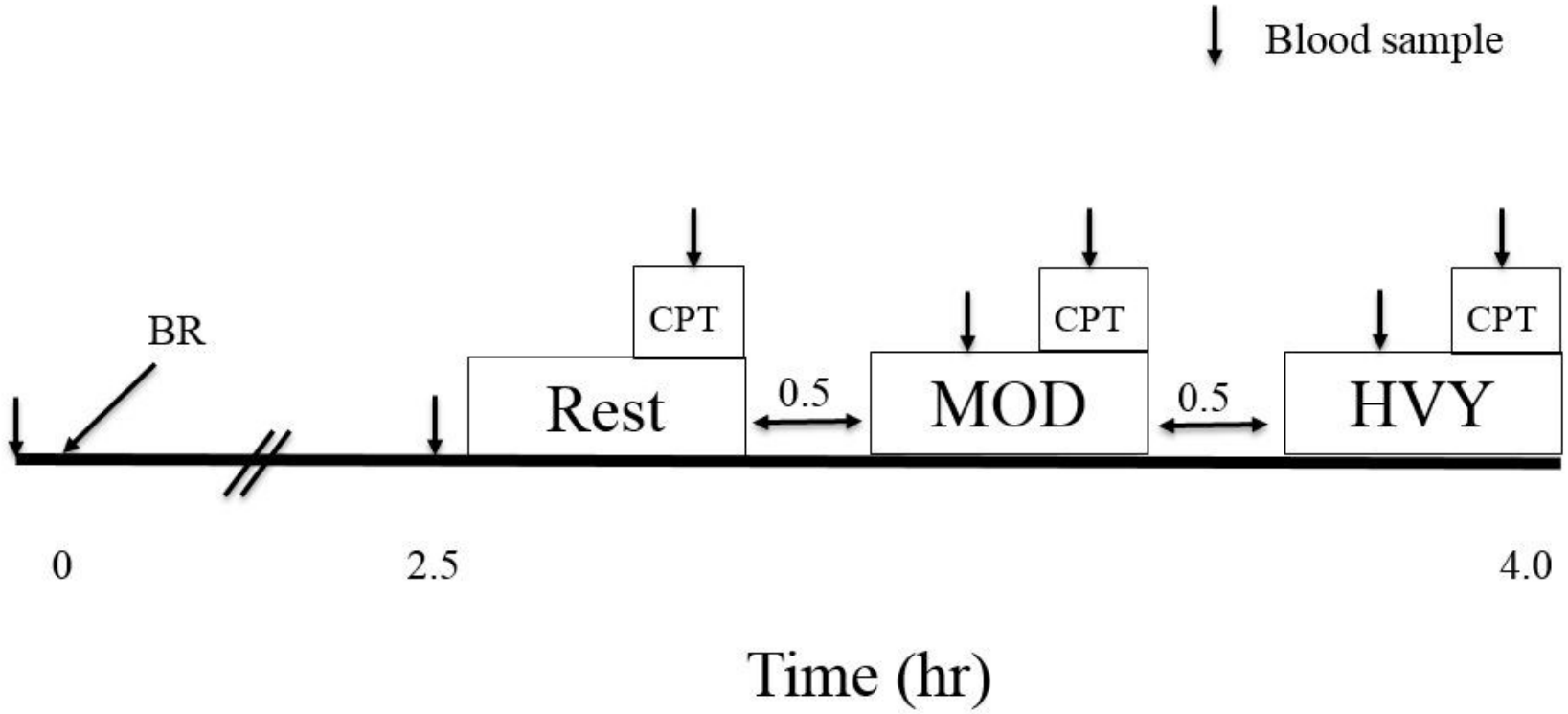

Figure 2. Timeline of testing on days 3 and 4. Following a resting blood sample, beet root juice (BR) containing dietary nitrate (NO_3^-) or placebo was consumed (time 0). After 2.5 hours, a resting blood sample was taken while the subject was seated in the alternate-leg knee-extension ergometer. The resting trial (Rest), moderate- (MOD) and heavy- intensity (HVY) exercise trials were 14 minutes long and separated by ~30 minutes of recovery. Subjects performed a 3 min cold pressor test (CPT) at rest and during each exercise bout. Blood samples were taken at rest and during exercise and during each CPT.

nervous system activity, an increase in BP and vasoconstriction (Seals, 1991; Victor, et al., 1989). Importantly, the CPT augments sympathetic outflow without changing the contribution of central command to sympathetic nerve activity. (Rowell, 1993).

Measurements

Femoral artery mean blood velocity (MBV) was measured using pulsed-Doppler ultrasonography (GE Vivid I, Waukesha WI, USA). Data were acquired continuously with a 7.5 MHz probe with a 45° angle of insonation placed on the skin surface 2–3 cm distal to the inguinal ligament. The diameter of the common femoral artery was measured during diastole in triplicate at rest. Mean blood velocity was measured on a beat-by beat basis. Leg blood flow (LBF) was calculated as LBF ($ml \cdot min^{-1}$) = MBV ($cm \cdot s^{-1}$)$\cdot \pi r^2 \cdot 60$, where r is the radius of the femoral artery. Femoral vascular conductance (FVC) (LBF/MAP, $L \cdot min^{-1} \cdot mmHg^{-1}$) and resistance (MAP/LBF, $mmHg \cdot L^{-1} \cdot min^{-1}$) were calculated.

The percentage change in FVC was used to assess sympathetic vasoconstrictor responsiveness in response to the CPT at rest and during exercise. Downstream changes in vascular resistance in response to sympathetic stimulation are reflected upstream at the femoral artery, and therefore the percentage change in FVC reflects the vasoconstrictor response of the leg vasculature.

Previous studies have reported that the common femoral artery does not dilate during exercise, therefore resting diameters were used to calculate blood flow at rest and during exercise (Radegran et al., 2000; Saltin et al., 1998).

After withdrawal, venous blood samples were immediately mixed with EDTA and centrifuged at 2500 rpm for 10 minutes. Plasma was aliquoted and placed in micro centrifuge tubes and frozen for subsequent analysis of [NE] and [EPI] (2-CAT Plasma ELISA Sensitive Enzyme Immunoassay BA E-4500: Colorado Springs, Colorado) and plasma [NO_3^-] and [NO_2^-] (Abcam Nitrate/Nitrite Colorimetric Assay Kit ab65328: Toronto, Ontario).

VO_2, carbon dioxide production, and ventilation were measured breath-by-breath using a mass flow sensor and metabolic cart (Vmax® 229d; Viasys™ Healthcare, Palm Springs, California).

The ECG was continuously recorded in a three lead configuration (Power Lab 16/30; AD instruments, Colorado Springs, Colorado) and HR was derived was derived from the ECG waveform.

Beat-by-beat arterial BP was measured by finger photo-plethysmography (Finometer™, Amsterdam, Netherlands). BP was also measured by sphygmomanometer and Finometer BP was corrected to manually measured pressures if necessary.

Data Analysis

Data was recorded using a PowerLab 16/30 system and Chart 7™ data acquisition software (AD Instruments, Colorado Springs, CO, USA) at a sampling frequency of 100Hz.

The change in HR, BP and femoral vascular conductance to the CPT was calculated by determining the magnitude of the change in each variable during the CPT at rest and during contraction. The difference between the peak responses (20s average) and

preceding baseline was calculated for each variable and was expressed as an absolute and percentage change. The magnitude of functional sympatholysis was calculated as the difference between the vasoconstrictor response at rest and during exercise (Δ % FVC). Rate pressure product was calculated as SBP x HR.

Statistical Analysis

All data were reported as mean ± standard deviation. The effect of dietary NO_3^- supplementation on resting HR, MAP, SBP, DBP LBF and FVC were determined by paired t-test. The effect of dietary NO_3^- supplementation on sympathetic vasoconstrictor responsiveness, sympatholysis and the increases in HR, MAP, SBP, DBP, LBF, and FVC in response to moderate- and heavy-intensity exercise were analyzed by two-way repeated (exercise intensity x treatment) measures ANOVA. When significant main effects were identified, Student-Newman-Keuls post hoc analyses was performed. Relationships between variables were determined by Pearson-Product moment correlation. A p-value < 0.05 was considered statistically significant. Statistics were performed using the analytical software SigmaPlot (SigmaPlot Version 13.0, Systat Software Inc., San Jose, CA, USA).

Results

Subject Characteristics

Subjects age, height, weight, absolute and relative VO_{2peak} from the maximal cycling test, and maximal work rate achieved during the alternate-leg knee-extension test are reported in Table 1.

Table 1: Participant characteristics.

Age (yrs.)	21.5 ± 2.8
Height (cm)	177.7 ± 8.6
Weight (Kg)	72.2 ± 10.2
Absolute VO_{2max} (L/min.)	3.4 ± 1.2
Relative VO_{2max} (mL/Kg/min.)	45.5 ± 12.8
Peak Work Rate (Kg)	2.6 ± 0.5

Values are mean ± SD.

Effect of Nitrate Supplementation on Cardiovascular Variables at Rest

NO_3^--rich beetroot juice significantly ($p<0.05$) elevated (Figure 2) plasma [NO_2^-] and [NO_3^-] levels, whereas the NO_3^- depleted beetroot juice did not alter ($p>0.05$) plasma [NO_2^-] or [NO_3^-] levels. An original data tracing of HR, MAP and FVC at rest and in response to a CPT in a representative subject are illustrated in Figure 3. Dietary NO_3^- did not alter resting HR, cardiac output, MAP, SBP, DBP, LBF, plasma [NE] or [E] total peripheral resistance or femoral vascular resistance (Table 2).

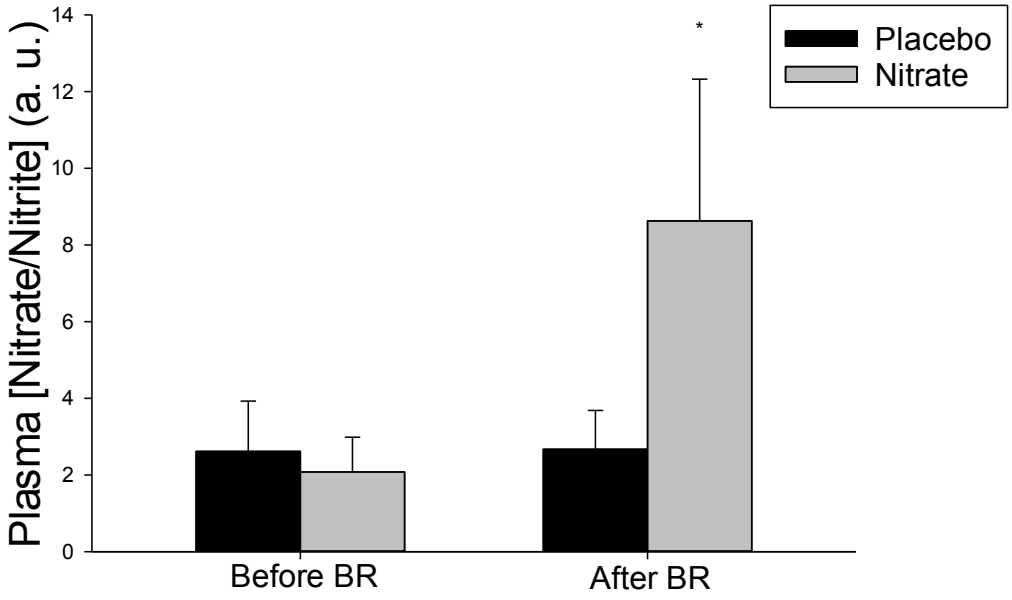

Figure 3. Total plasma nitrate/nitrite at rest, and 2.5 hours following consumption of beetroot juice containing nitrate, or nitrate-depleted beetroot juice. Values are Mean±SD. * indicates significant difference from placebo trial (effect of treatment in the nitrate condition, $p<0.001$).

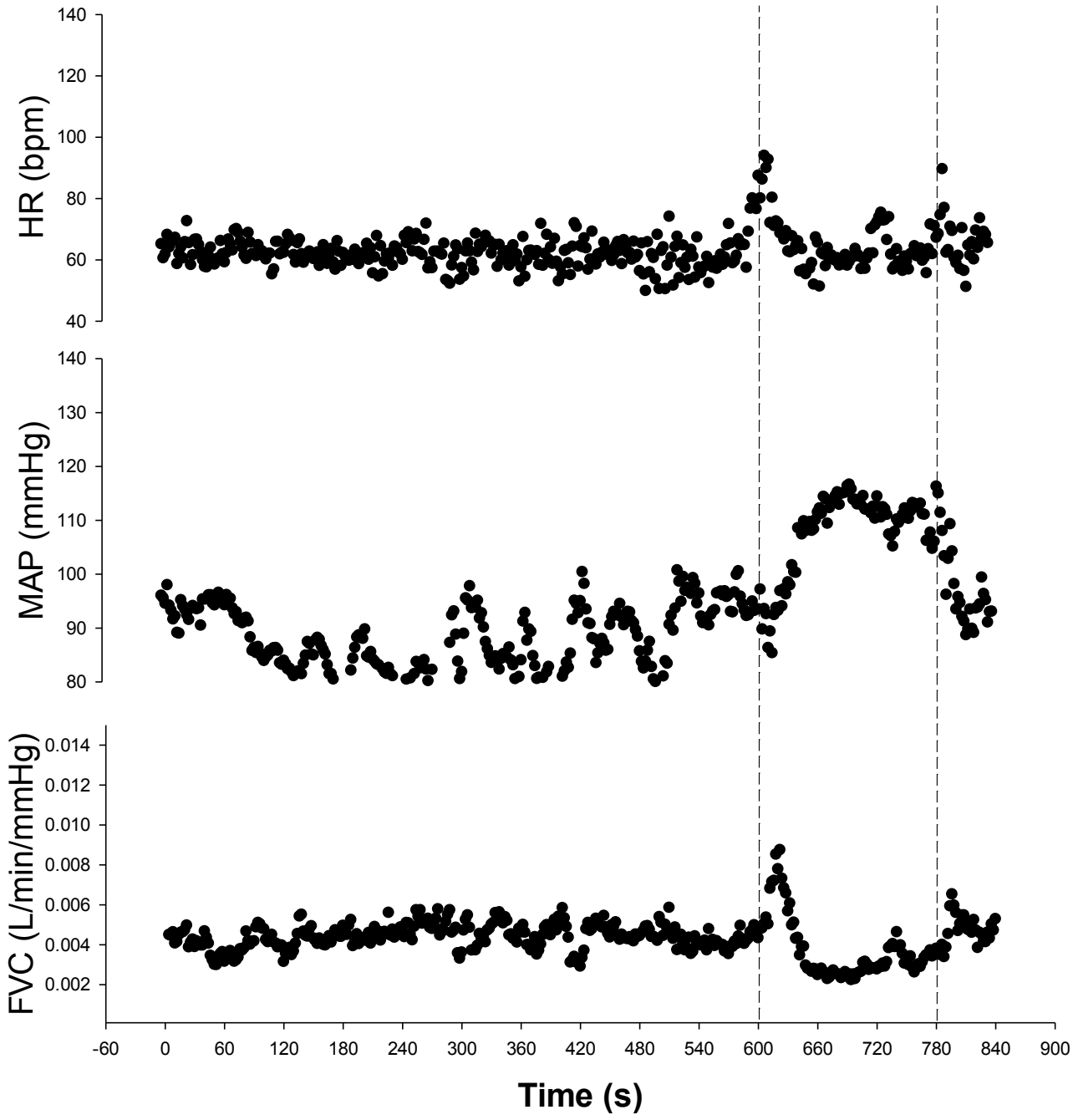

Figure 4: The response of heart rate, HR; mean arterial pressure, MAP; systolic blood pressure, SBP; diastolic blood pressure, DBP to a cold pressor test (CPT) in a representative subject. The CPT was initiated at 600s and maintained until 780s followed by 60s of recovery. Each data point represents a 2s average.

Table 2: Effect of dietary nitrate at rest.

	Placebo	**NO_3^-**
Heart rate (beats·min^{-1})	62.7 ± 10.8	62.2 ± 9.1
Stroke volume (mL·min^{-1})	102.3 ± 8.2	96.7 ± 17.3
Cardiac output (L·min^{-1})	6.4 ± 1.0	6.0 ± 0.9
Mean arterial pressure (mmHg)	83.7 ± 7.5	81.8 ± 5.8
Systolic blood pressure (mmHg)	120.5 ± 12.1	117.3 ± 8.0
Diastolic blood pressure (mmHg)	65.5 ± 5.7	64.3 ± 6.1
Limb blood flow (L·min^{-1})	0.34 ± 0.08	0.32 ± 0.06
Plasma Norepinephrine (pg/mL)	499.6 ± 156.1	375 ± 193.6
Plasma Epinephrine (pg/mL)	31.1 ± 9.80	35.10 ± 6.7
Total peripheral resistance (mmHg·min^{-1}·mL^{-1})	804.9 ± 134.9	856.7 ± 148.3
Femoral vascular conductance (L·min^{-1}mmHg^{-1})	0.004 ± 0.001	0.004 ± 0.058

Values are mean ± SD.

Effect of Nitrate Supplementation on the Cardiovascular Response to Exercise

An original data tracing of the HR, MAP, and FVC response to exercise and to a CPT from a representative subject is illustrated in Figure 4.

MAP, SBP, DBP, HR, cardiac output, femoral vascular conductance and leg blood flow all increased in response to moderate- and heavy-intensity exercise in all subjects. Total peripheral resistance decreased in response to both moderate- and heavy-intensity exercise.

In response to exercise, dietary NO_3^- did not alter the absolute increase in HR (Figure 5), LBF (Figure 6), cardiac output (Figure 7), femoral vascular conductance (Figure 8), VO_2 (Figure 9), MAP, SBP, DBP (Figure 10), myocardial VO_2 (RPP; RPP=SBP x HR) (Figure 11) or the absolute decrease in total peripheral resistance (Figure 12).

Circulating plasma catecholamines (Figure 13) were not different between placebo and nitrate conditions during moderate- or heavy-intensity exercise. The percentage decrease in femoral vascular conductance (Figure 14) in response to a CPT at rest and during moderate- and heavy-intensity exercise was also not different ($p>0.05$) in the NO_3^- trial as compared to placebo. The magnitude of sympatholysis was not different ($p>0.05$) in the NO_3^- trial as compared to the placebo trial (Figure 15).

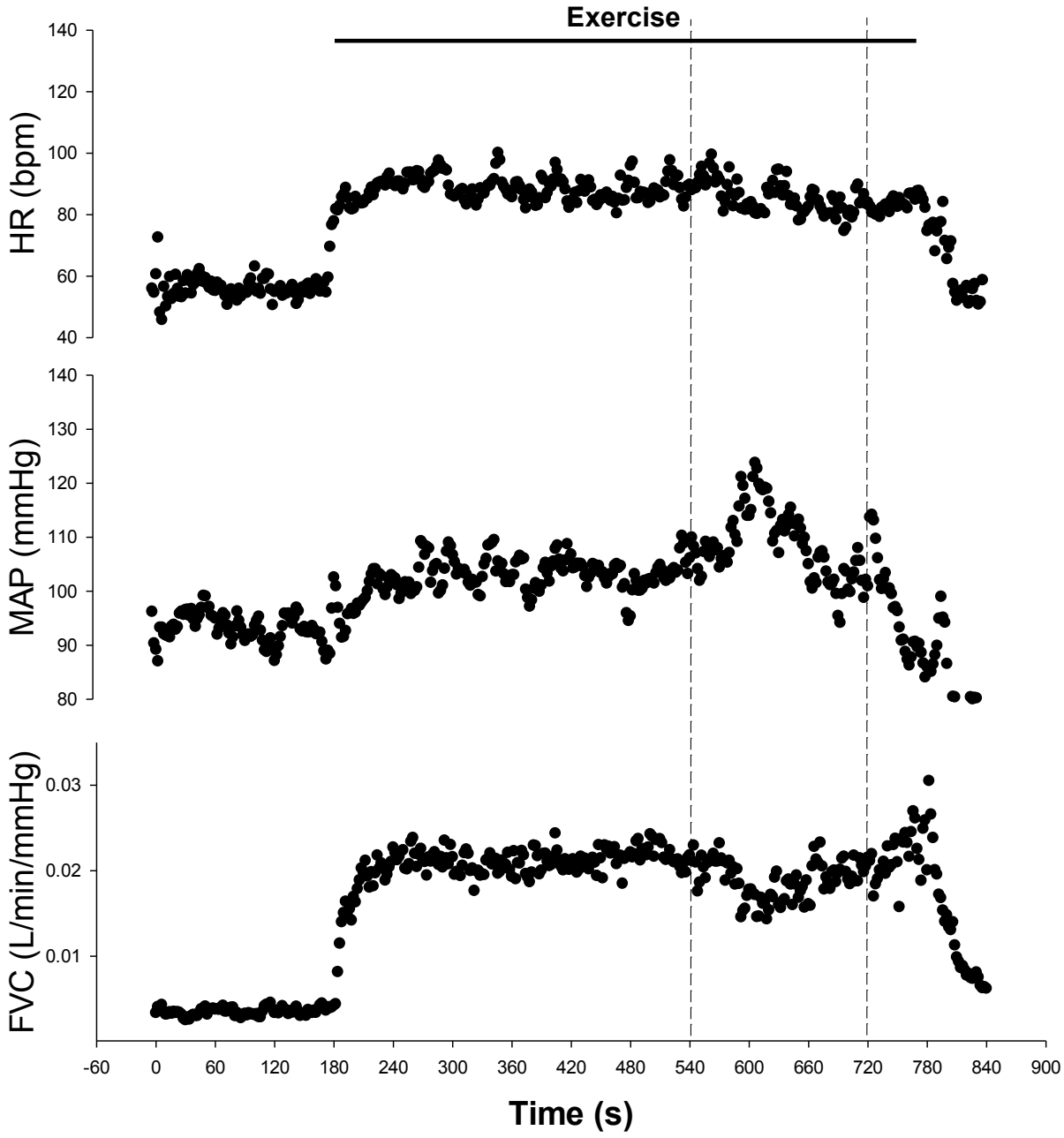

Figure 5: The response of heart rate, HR; mean arterial pressure, MAP; femoral vascular conductance, FVC) response to dynamic exercise, and in response to a cold pressor test (CPT) in a representative subject. Exercise was initiated at 180s, followed by a 3 minute CPT at 540s. The CPT was maintained until 720s followed by exercise until 780s, and 60s of recovery. Each data point represents a 2s average.

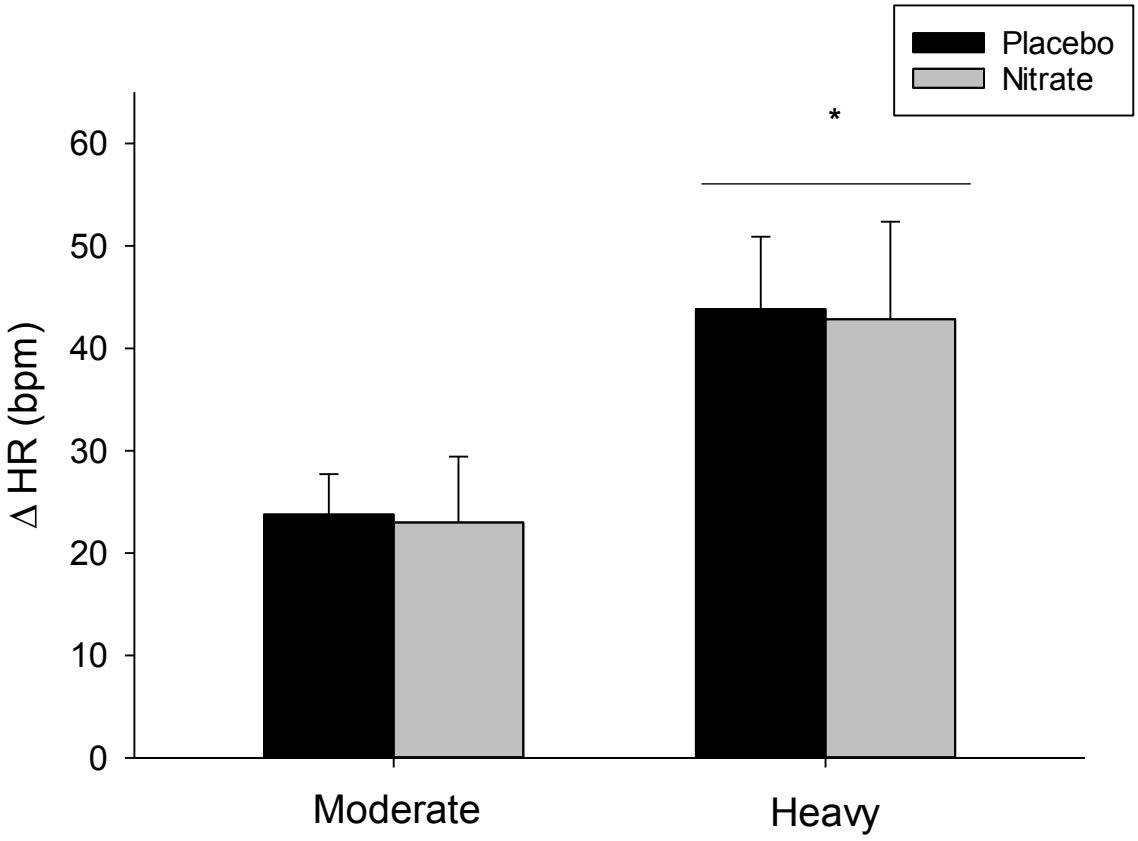

Figure 6: Increase in heart rate (HR) in response to moderate-and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of exercise intensity $p<0.001$).

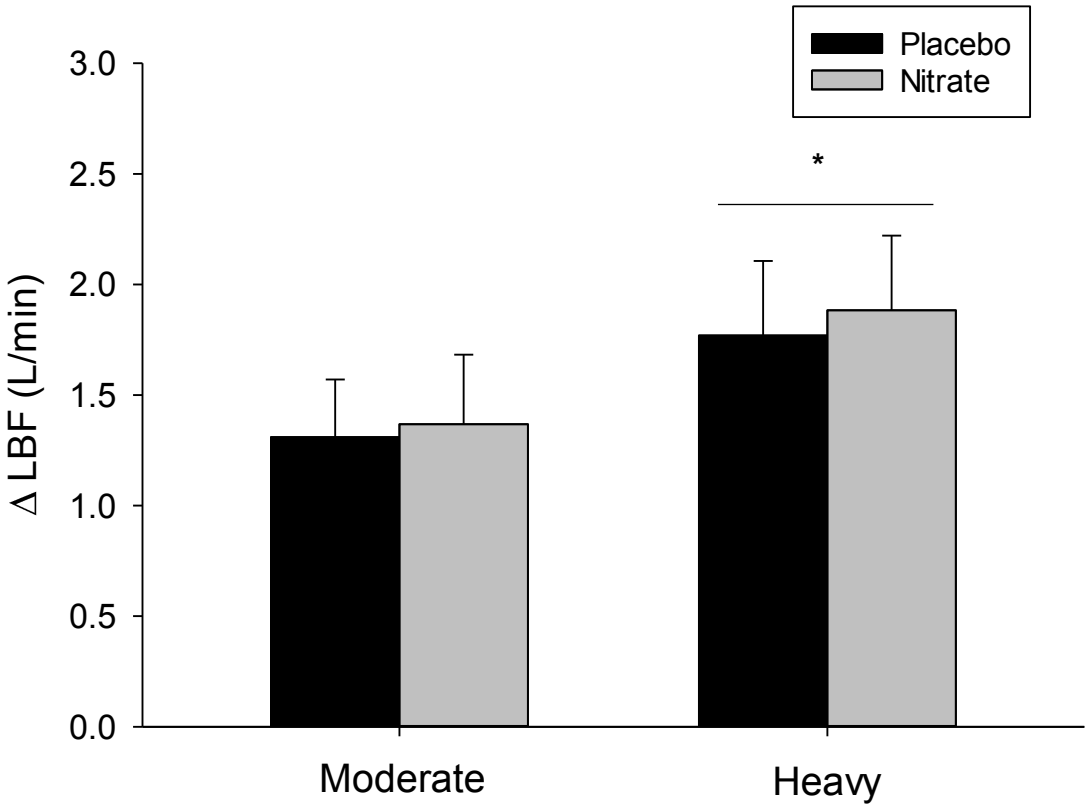

Figure 7: Increase in leg blood flow (LBF) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from heavy intensity exercise (main effect of intensity p=0.001).

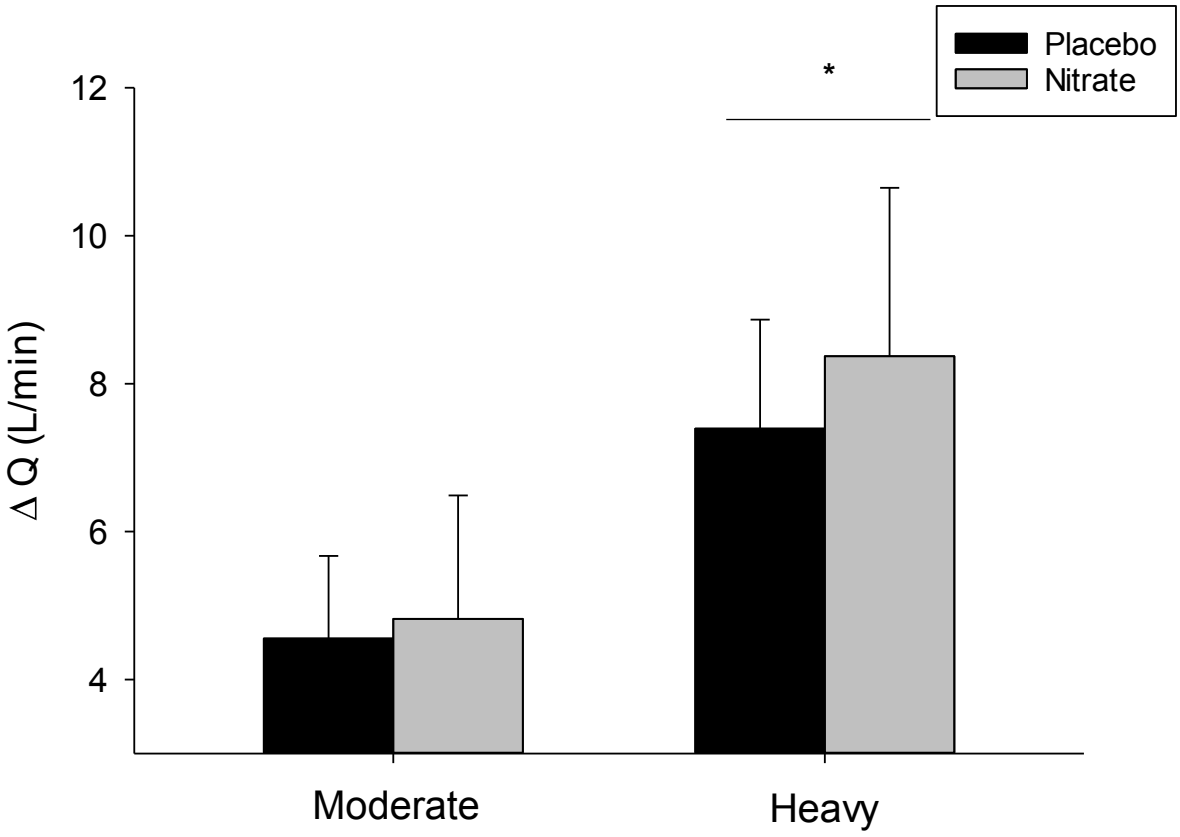

Figure 8: Increase in cardiac output (Q) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of intensity p=0.001).

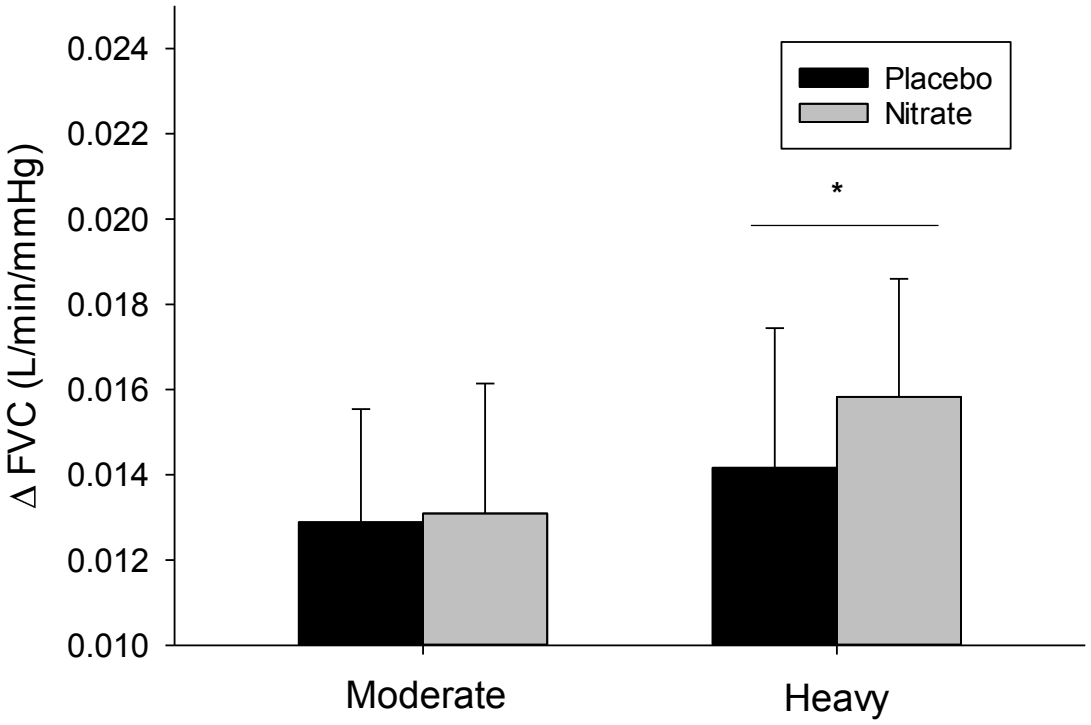

Figure 9: Increase in femoral vascular conductance (FVC) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of intensity p=0.001).

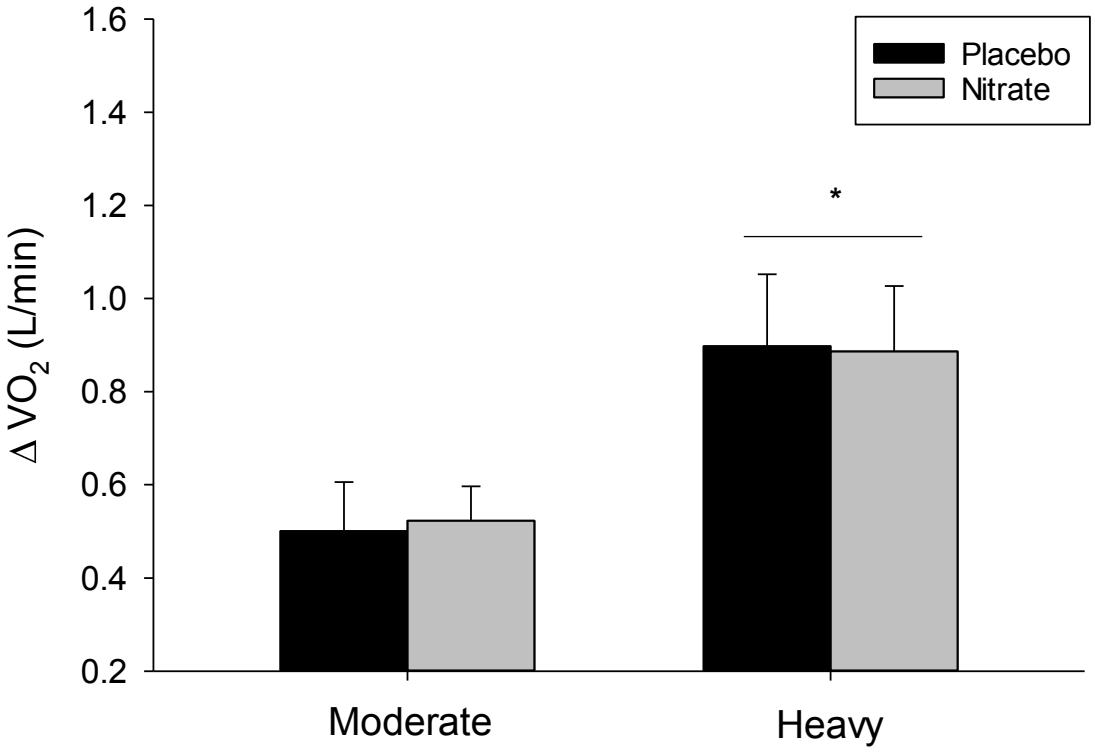

Figure 10: Increase in oxygen consumption (VO_2) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of exercise intensity p=0.001).

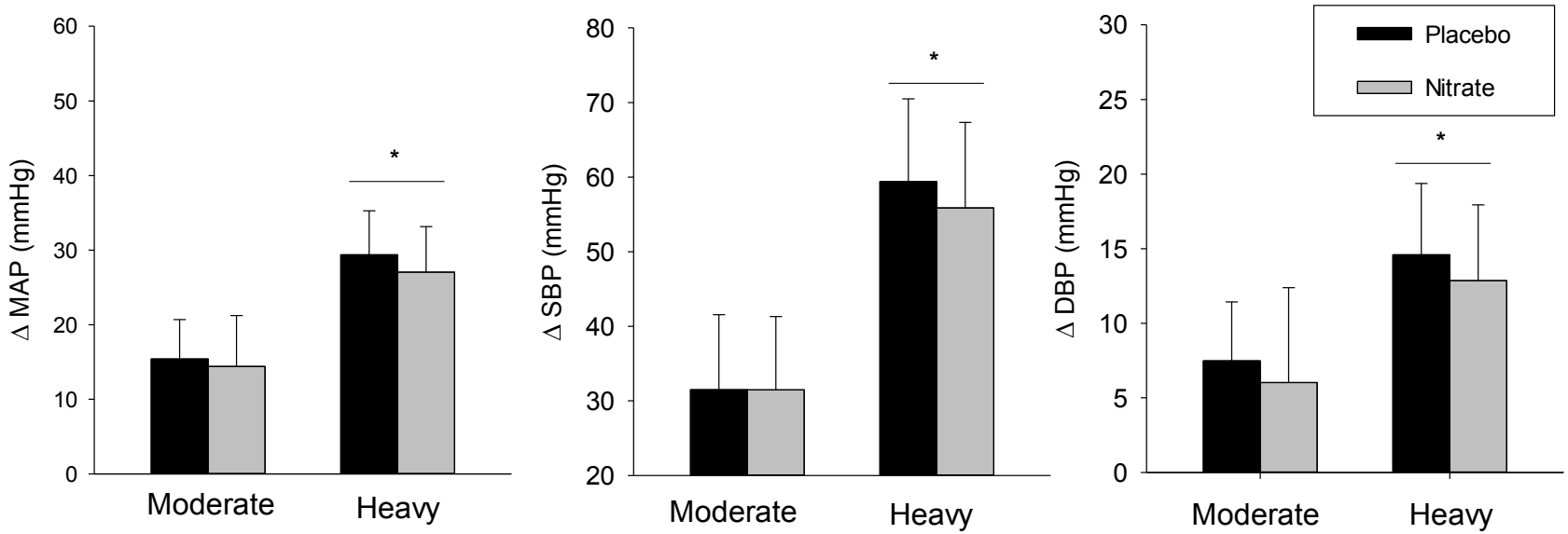

Figure 11: Increase in mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of intensity MAP: $p=0.001$; SBP: $p=0.001$; DBP: $p<0.001$).

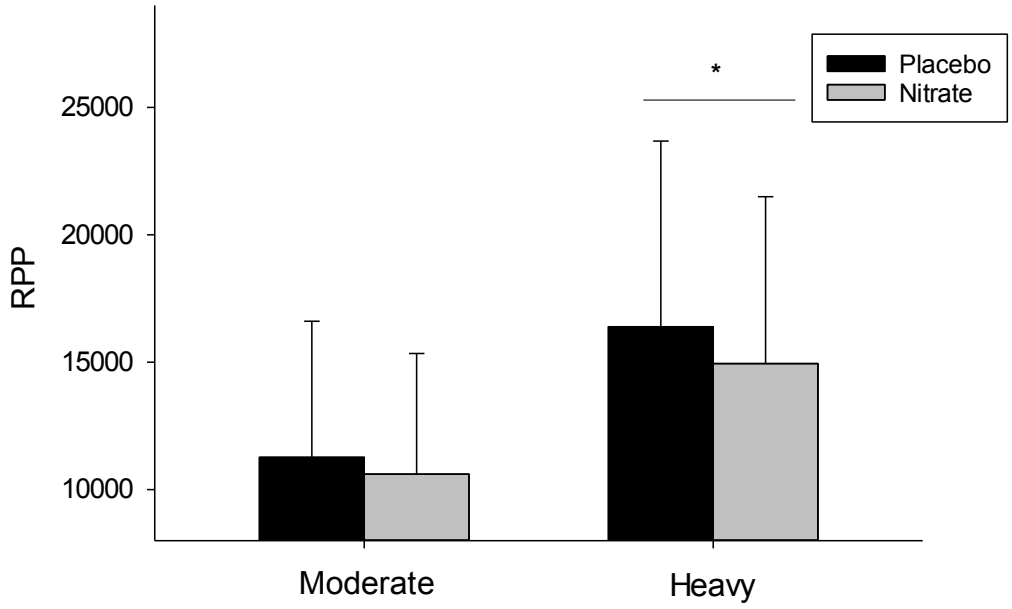

Figure 12: Increase in rate pressure product (RPP) in response to moderate- and heavy intensity-exercise with placebo or nitrate. Values are Mean±SD. * Indicates significant difference from moderate exercise (main effect of intensity p=0.001).

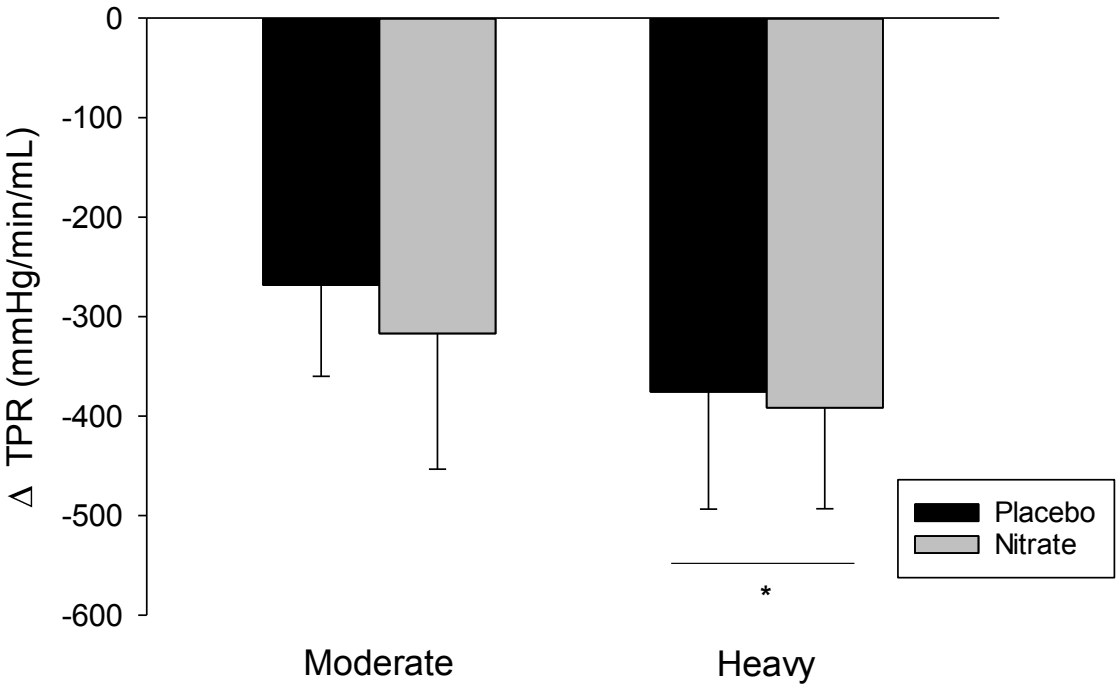

Figure 13: Decrease in total peripheral resistance (TPR) in response to moderate- and heavy-intensity exercise with placebo or nitrate. Values are Mean±SD. * indicates significant difference from moderate exercise (main effect of intensity p=0.006).

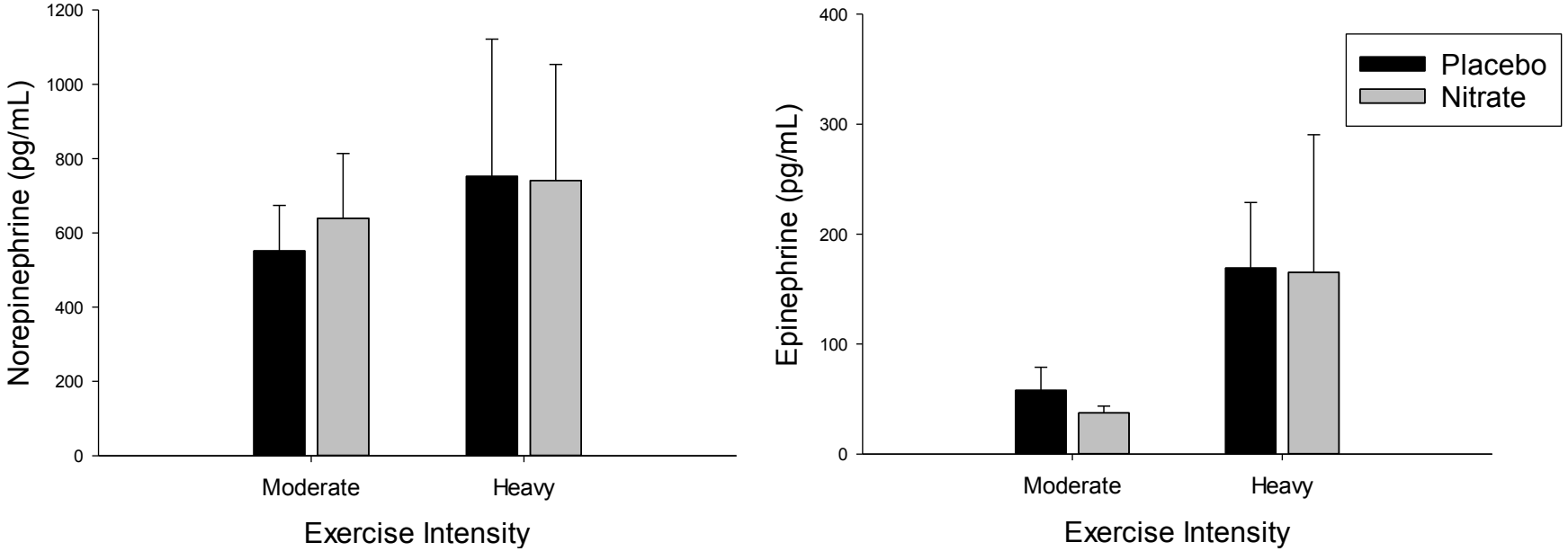

Figure 14. Circulating plasma [norepinephrine] and [epinephrine] during moderate- and heavy-intensity exercise in the placebo and nitrate conditions. Values are means ± SD.

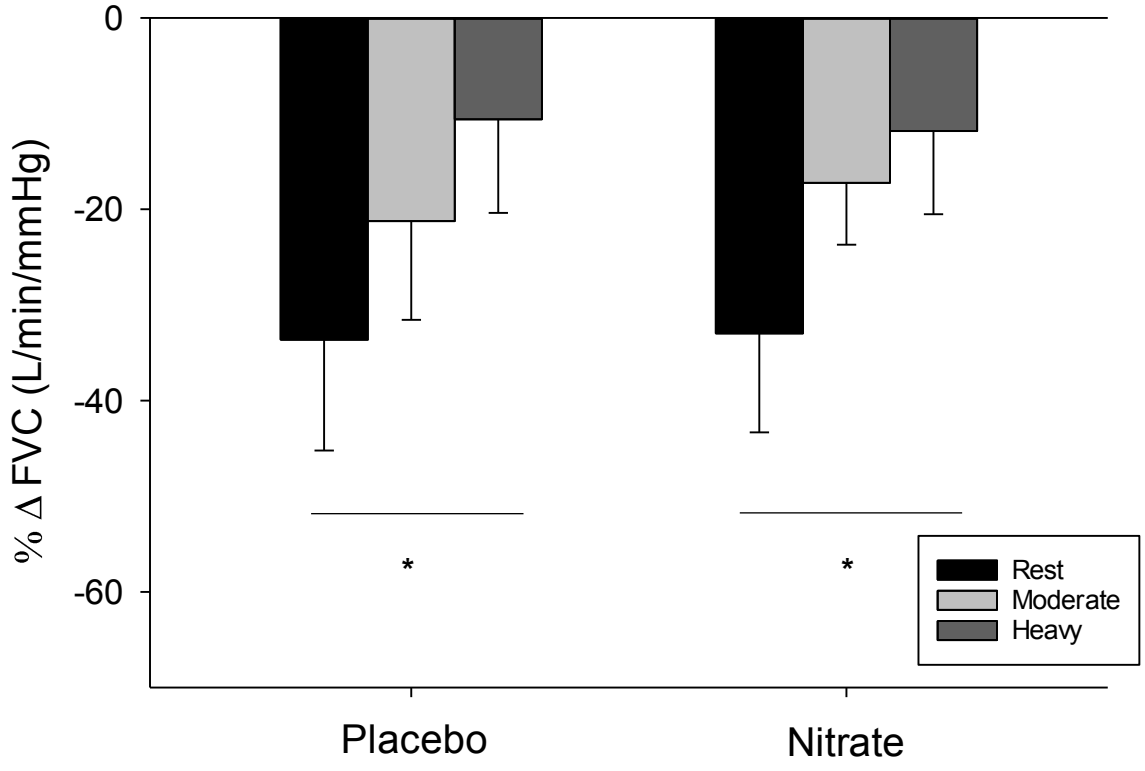

Figure 15: Percentage change in femoral vascular conductance (FVC) at rest and during moderate- and heavy-intensity exercise in the placebo and nitrate conditions in response to a cold pressor test. Values are Mean±SD. * indicates a significant difference across all exercise conditions (Main effect of exercise intensity $p<0.001$).

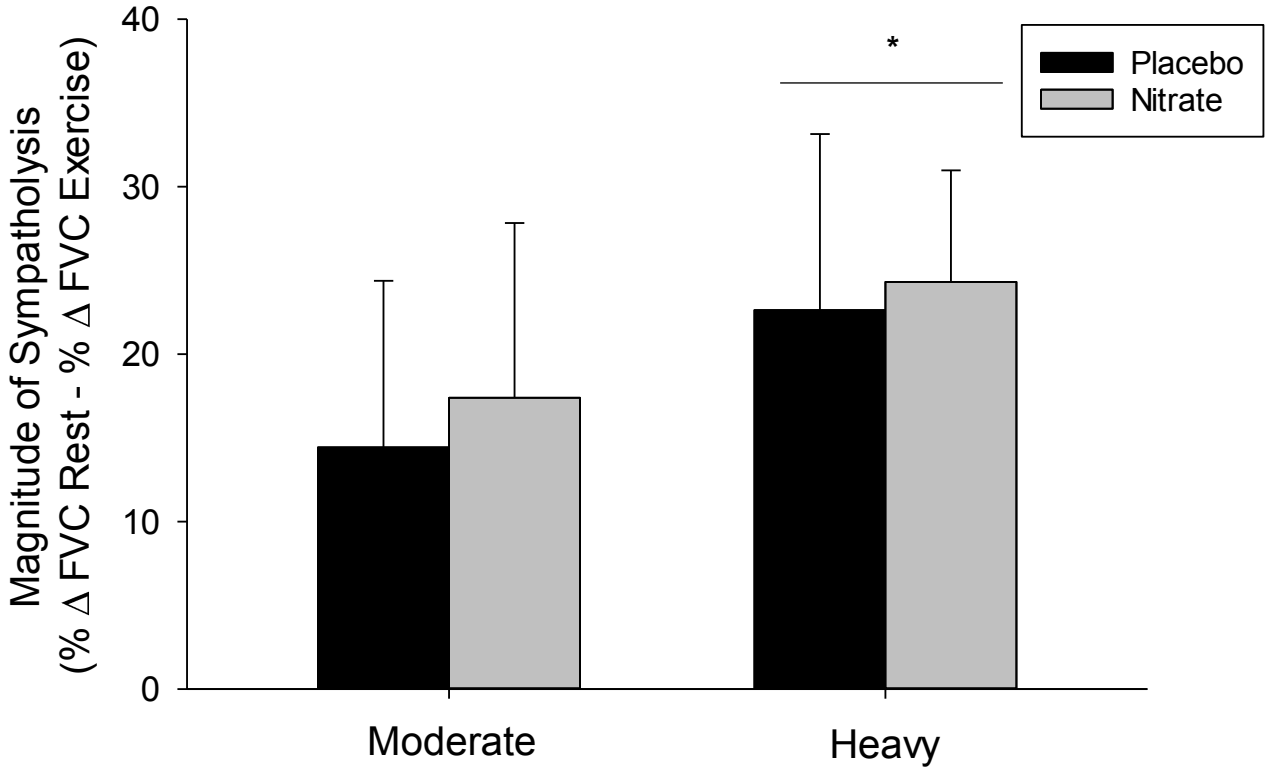

Figure 16: Magnitude of sympatholysis during moderate- and heavy-intensity exercise in the placebo and nitrate trial. Values are Mean±SD. * Indicates significant difference from moderate exercise (main effect of intensity p=0.002).

Discussion

The purpose of the present study was to investigate the effect of acute dietary NO_3^- supplementation on plasma catecholamines, blood pressure and sympathetic vasoconstrictor responsiveness at rest and during exercise, and in response to sympathoexcitation. It was hypothesized that acute dietary NO_3^- supplementation would: 1) have no effect on plasma catecholamines, at rest and during exercise and in response to a sympatho-excitatory stimulus; 2) attenuate sympathetic vasoconstrictor responsiveness at rest and during exercise and enhance sympatholysis. Acute supplementation with dietary NO_3^- increased plasma NO_2^- / NO_3^- suggesting that NO bioavailability was enhanced. However, blood pressure, sympathetic vasoconstrictor responsiveness and plasma catecholamines were not different between placebo and NO_3^- supplementation conditions at rest or during exercise. The results of the present study suggest that dietary NO_3^- does not alter sympathetic nervous system mediated vascular control at rest and during exercise in young healthy men.

Effect of Nitrate on Sympathetic Vasoconstrictor Responsiveness at Rest and During Exercise.

Consistent with previous studies, acute consumption of NO_3^- rich beetroot juice increased plasma NO_3^-/NO_2^- suggesting that NO bioavailability was enhanced ~2.5 hours after consumption. This time frame has been shown to reduce BP in young healthy adults (Hobbs et al., 2012; Kapil et al., 2010; Lee et al., 2015; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013a). Despite an increase in NO bioavailability, dietary NO_3^- supplementation did not alter sympathetic vasoconstrictor responsiveness at rest or during moderate- or heavy-intensity exercise in the present study which suggests enhanced NO bioavailability does not alter sympathetic vasoconstrictor responsiveness at rest. Previous research investigating NO-mediated

inhibition of sympathetic vasoconstriction have produced conflicting results with reports of NOS inhibition increasing the vascular response to sympathetic stimulation (Jendzjowsky et al., 2013c; Thomas et al., 1998b), or not altering the vascular response to sympathetic stimulation (Dinenno et al., 2003, 2004). Jendzjowsky et al. (2013b) and Thomas et al. (1998b) reported the inhibition of NOS via L-NAME augmented the vascular response to sympathetic stimulation, indicating NO contributes to the inhibition of sympathetic vasoconstriction in exercise trained rats. In contrast, two studies by Dinenno et al. (2003, 2004) reported that NOS inhibition did not alter tyramine-evoked vasoconstrictor responses in the forearm at rest and during handgrip exercise. The collective results from these studies are inconclusive, however they do suggest that NO contributes to the inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. However, the present findings suggest that enhanced NO bioavailability does not appear to alter sympathetic vasoconstrictor responses to sympathetic excitation at rest or during exercise. Consistent with this notion, Rosenmeier et al. (2003) have shown the infusion of the NO donor sodium nitroprusside does not alter vasoconstrictor responsiveness to tyramine or selective alpha adrenergic receptor agonists.

The increase in NO bioavailability following dietary NO_3^- supplementation may augment the scavenging of reactive O_2 species (ROS), such as the superoxide anion and alter sympathetic vasoconstrictor responsiveness by reducing oxidative stress. Several studies have shown superoxide scavenging anions improve the inhibition of sympathetic vasoconstriction in resting and contracting muscle (Fadel et al., 2012; Gao et al., 2006; Jendzjowsky et al., 2013a; Thomas et al., 2001). Although ROS were not measured in the present study, there was no difference in sympathetic vasoconstrictor responsiveness with dietary NO_3^-, which suggests the concentration of ROS was unaltered.

Plasma catecholamines were not different between the placebo and NO_3^- condition at rest, during exercise and during the CPT in the present study. Local inhibition of NOS in cardiovascular control centers of the brainstem has been shown to increase sympathetic nerve activity and BP indicating that NO may inhibit efferent sympathetic nerve activity (Bruno et al., 2012; Hirooka et al., 2011; Patel et al., 2001). Furthermore, injection of NO-donors in the brain stem decreased sympathetic nerve activity and BP further indicating that NO inhibits sympathetic outflow (Bruno et al., 2012; Zanzinger et al., 1995). To my knowledge, the effect of dietary NO_3^- on direct measures of efferent sympathetic nerve activity has not been investigated. Moreover, whether dietary NO_3^- treatment results in an increased NO bioavailability in the cardiovascular control centers of the brain stem has not been established. However, Bond et al. (2014) reported increased heart rate variability with an unchanged low to high frequency spectral power ratio (LF/HF) following acute dietary NO_3^- supplementation indicating that NO_3^- supplementation may alter autonomic control of heart rate. We would not expect dietary NO_3^- to alter the expression of post-synaptic α-adrenergic receptors and therefore similar levels of circulating catecholamines appears consistent with similar vasoconstrictor responsiveness at rest and during exercise in the present study. Further studies that include direct measures of sympathetic outflow are required to establish the effect of dietary NO_3^- on sympathetic activity.

Effect of Nitrate on Blood Pressure at Rest and During Exercise

In the present study, dietary NO_3^- did not alter resting MAP, SBP, or DBP. Consistent with the present findings, Kim et al. (2015), Casey et al. (2015) and Aucouturier et al. (2015) reported resting SBP or DBP was not altered by dietary NO_3^- supplementation in young normotensive adults. In contrast, several studies have reported a reduction of resting BP following acute dietary NO_3^- supplementation with beetroot juice (Bond et al., 2014; Hobbs et

al., 2012; Kapil et al., 2010; Lansley et al., 2011; Lee et al., 2015; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013a). The conflicting findings in the scientific literature are difficult to reconcile, however differences in subject populations and resting blood pressures may contribute to the divergent findings. For example, Bond et al. (2014) reported reduced blood pressure in young healthy adult African-American females, however this population has been known to have greater reactivity to stimuli that alter in laboratory situations and an increased for hypertension (Knox et al., 2002; Lloyd-Jones et al., 2010). Furthermore, Hobbs et al. (2012) reported that acute consumption of dietary NO_3^- reduced SBP and DBP, in subjects with SBP values outside of the normotensive range, suggesting that the effect of dietary NO_3^- may be a function of resting BP. However, Lansley et al. (2011) and Vanhatalo et al. (2010) reported reductions in SBP following NO_3^- supplementation in subjects with blood pressures similar to those in the present study. We utilized a similar dose of dietary NO_3^- to that used in the other studies (Hobbs et al., 2012; Wylie et al., 2013a), thus the lack of effect on BP in the present study does not appear to be related to the dose of dietary NO_3^- used. Indeed, Wylie et al. (2013b) reported that BP was reduced at dietary NO_3^- doses below and similar (4.2, 8.4, and 16.8 mMol) to the dose used in the present study (12.9 mMol). The reason for the divergent effects of beetroot juice on the control of resting BP in young normotensives are not readily apparent, however a reduction in BP in young normotensives who presumably have normal levels of NO bioavailability is counterintuitive as peripheral vascular resistance and sympathetic outflow would presumably be normal in this population. Furthermore, fitness does not appear to influence the effect of dietary NO_3^- on resting BP despite increased production of NO following exercise training (McConell et al., 2007), as subjects in the present study had similar or lower fitness scores (VO_2 = 46±13 mL/Kg/min) to other studies who reported reduced BP with dietary

NO_3^- (Lansley et al., 2011; Lee et al., 2015; Vanhatalo et al., 2010; Wylie et al., 2013a). Interestingly, dietary NO_3^- supplementation has been shown to have minimal effects on resting BP in older patients with diabetes that have elevated BP and possibly reduced NO bioavailability (Gilchrist et al., 2013). Further investigation is required to fully understand the effect of dietary NO_3^- on the regulation of BP.

Dietary NO_3^- did not alter the increase in RPP (an index of myocardial O_2 consumption) in response to moderate- and heavy-intensity exercise in the present study. In contrast, Bond et al. (2014) reported a lower RPP during cycling exercise at 40% and 80% of VO_2 peak following the dietary NO_3^- supplementation. Total peripheral resistance was reduced in the NO_3^- condition suggesting that dietary NO_3^- caused peripheral vasodilation and reduced afterload. Consistent with this notion, chronic treatment with dietary NO_3^- for 15 days has also been shown to reduce RPP and TPR during incremental cycling exercise to maximum and enhanced brachial artery flow mediated vasodilation (Lee et al., 2015).

Effect of Dietary NO_3^- on Leg Blood Flow at Rest and During Exercise

Consistent with previous research, dietary NO_3^- did not alter blood flow at rest in the present study (Aucouturier et al., 2015; Casey et al., 2015; Ferguson et al., 2013; Kim et al., 2015). Casey et al. (2015) and Kim et al. (2015) reported no difference in resting forearm blood flow between dietary NO_3^- and placebo conditions. Additionally, Aucouturier et al. (2015) reported no change in muscle oxygenation with dietary NO_3^-, suggesting that local muscle blood flow was not different between NO_3^- and placebo. Finally, Ferguson et al. (2013) reported no change in resting blood flow in rats following 5 d of dietary NO_3^- supplementation compared to controls. Collectively, the results of these studies suggest that enhanced NO bioavailability via dietary NO_3^- may not alter the regulation of resting blood flow. Previous research studies that

have utilized NOS inhibition to investigate the role of NO in the control of skeletal muscle blood have been contradictory, with reports of NOS inhibition reducing skeletal muscle blood flow (Schrage et al., 2004; Wray et al., 2011), or not altering skeletal muscle blood flow (Buckwalter et al., 2004). Further research is required to determine the role of NO in the control of resting skeletal muscle blood flow in humans, however the present data suggest that an increase in NO bioavailability does not alter resting blood flow in young healthy men.

Consistent with previous research, dietary NO_3^- did not alter blood flow during exercise in the present study (Casey et al., 2015; Kim et al., 2015). Casey et al. (2015) and Kim et al. (2015) reported no difference in blood flow during handgrip exercise in dietary NO_3^- and placebo conditions. In contrast Ferguson et al. (2013) reported increased total exercising hind limb blood flow in rats given 5 d of supplemental dietary NO_3^-. The findings of Ferguson et al. (2013) may be specific to rats and may also reflect a difference between chronic versus acute NO_3^- supplementation. Additionally, Ferguson et al. (2013) reported improved muscle blood flow and limb vascular conductance during exercise following dietary NO_3^- supplementation primarily in fast-twitch IIb + d/x muscles of the hind limb, which suggests the effect of dietary NO_3^- on vascular control may be fiber-type specific. Previous studies have shown that NOS inhibition reduces forearm blood flow during moderate- and heavy-intensity handgrip exercise, suggesting that NO may be required for exercise hyperemia (Dinenno et al., 2003, 2004; Wray et al., 2011). However, the present data suggest that increased NO bioavailability does not improve skeletal muscle blood flow during exercise.

In the present study, dietary NO_3^- did not alter VO_2 during moderate- or heavy-intensity exercise. Previous research has been contradictory with reports of an attenuation in exercise O_2 consumption (Bailey et al., 2010; Larsen et al., 2007, 2010), and reports of no effect with dietary

NO_3^- (Aucouturier et al., 2015; Martin et al., 2014). Furthermore, Bailey et al. (2010) reported reduced O_2 consumption during 15% and 30% of maximum alternate-leg knee-extension exercise. Further studies are needed to investigate whether changes in O_2 consumption during exercise with NO_3^- are dependent on the type and intensity of exercise being performed.

Conclusion

In conclusion, acute dietary NO_3^- supplementation increased plasma NO_2^- / NO_3^- suggesting that NO bioavailability was enhanced. However, blood pressure, sympathetic vasoconstrictor responsiveness and plasma catecholamines were not different between placebo and NO_3^- supplementation conditions at rest or during exercise. These results suggest that enhanced NO bioavailability following dietary NO_3^- supplementation did not alter sympathetic nervous system mediated vascular control at rest and during exercise in young healthy males.

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Chapter 3: General Discussion

Main Findings

The purpose of this study was to investigate the effect of acute dietary NO_3^- supplementation via beetroot juice on plasma catecholamines [NE] and [EPI] (an index sympathetic activity), blood pressure and sympathetic vasoconstrictor responsiveness at rest and during exercise, and in response to sympathoexcitation in humans. Consistent with experimental design, acute dietary NO_3^- supplementation increased plasma NO_2^- / NO_3^- suggesting that NO bioavailability was enhanced. However, in contrast to my hypotheses, dietary NO_3^- did not alter blood pressure, plasma catecholamines or sympathetic vasoconstrictor responsiveness at rest or during exercise. The results of the present study indicate that despite enhanced NO bioavailability sympathetic nervous system-mediated vascular control in resting and contracting muscle is not altered following acute dietary NO_3^- supplementation.

Experimental Considerations and Limitations

An alternate-leg knee-extension exercise model was utilized in the present study which allowed for the measurement of blood flow to a relatively large muscle mass at rest and during exercise. Previous studies of the effects of NO_3^- supplementation on the control of skeletal muscle blood flow have utilized forearm exercise models (Casey et al., 2015; Kim et al., 2015). Forearm exercise models are excellent for studies of local vascular control, but are also associated with relatively small changes in cardiac output and sympathetic outflow during exercise, making the knee-extension exercise model the preferred choice for studies of sympathetic vascular control in an isolated vascular bed. An additional strength of the present study was that we attempted to control pre-testing dietary NO_3^- intake in order to maximize the potential effects of acute NO_3^- supplementation on plasma NO_3^- / NO_2^- . Participants were

provided with a list of foods containing dietary NO_3^- and were asked to avoid consuming these foods 48 hours prior to testing. Participants were also asked to consume the same foods prior to testing and on each experimental day. In the present study, plasma catecholamines levels were measured to determine the effect of acute dietary NO_3^- supplementation on sympathetic nervous system activity. Muscle sympathetic nervous system activity can be measured directly via microneurographic techniques, however direct microneurographic recordings to active muscle during exercise require a very high level of technical expertise and can be complicated by movement artifact (L. B. Rowell, 1993). Direct microneurographic recordings in an inactive limb were also an option in the present study, however these measures would also have been technically difficult since the upper arms were used to measure BP and to perform the CPT in the present study. Plasma catecholamines measured in forearm venous circulation reflect a mix of catecholamines released from several vascular bed and represent a fraction of the total amount of catecholamines released from the sympathetic varicosities (Esler et al., 1988; Esler et al., 1990; Grassi et al., 1999; Kopin, 1985), because plasma [NE] and [EPI] depend on secretion, tissue clearance and re-uptake (Esler et al., 1990; Grassi et al., 1999; Kopin, 1985; Parati et al., 1985) of NE and EPI. Therefore, the primary limitation with the use of circulating plasma as an index of sympathetic outflow is that they may not reflect nerve activity directed toward the skeletal muscle vascular bed (L. B. Rowell, 1993). However, several studies have shown that forearm venous plasma catecholamines correlate with measures of muscle sympathetic nervous system nerve recordings during dynamic exercise (Grassi et al., 1999; Mancia et al., 1991; L. B. Rowell et al., 1987; Seals et al., 1988) and during a CPT (Victor et al., 1987), indicating that plasma catecholamines provide an accurate reflection of muscle sympathetic nerve activity at rest, during exercise and in response to sympathetic stress.

Future Directions

The present study demonstrated no effect of enhanced NO bioavailability on sympathetic vasoconstriction in young healthy men. It seems likely that NO bioavailability was not limited in these subjects and, therefore an acute increase in NO bioavailability might not be expected to improve the inhibition of sympathetic vasoconstriction in this population. However, previous studies have demonstrated reduced BP following acute dietary NO_3^- supplementation in young healthy males suggesting that vascular control may be altered after consumption of dietary NO_3^- (Hobbs et al., 2012; Kapil et al., 2010; Lee et al., 2015; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013). It could be argued that NO_3^- supplementation may have a larger effect on vascular function in populations with reduced NO bioavailability. One such condition, is sickle cell disease. Individuals with sickle cell disease have reduced NO bioavailability, as a result of reduced expression of L-arginine (Mack et al., 2006). Individuals with sickle cell disease also demonstrate increased sympathetic nerve activity and altered responses to hypoxia (L'Esperance et al., 2013). Therefore dietary NO_3^- may serve to reduce the response to sympathetic stress in individuals with sickle cell disease. The production of NO is also altered in post-menopausal women. It has been argued that the decline in estrogen production following menopause leads to a decrease in NO bioavailability and vascular dysfunction that contributes to the elevated cardiovascular disease risk in post-menopausal women (Wenger et al., 1993). Fadel et al. (2004) reported impaired inhibition of sympathetic vasoconstriction in post-menopausal women. However, Rosselli et al. (1995) reported an increase in plasma $[NO_2^-]$ and $[NO_3^-]$ with estrogen infusion to the point that plasma $[NO_2^-]$ and $[NO_3^-]$ levels were similar to pre-menopausal women. Furthermore, Fadel et al. (2004) reported short-term oestrogen replacement therapy restored the inhibition of sympathetic vasoconstriction in exercising post-menopausal

women. Therefore if augmented NO bioavailability as a result of enhanced enzymatic production of NO restored the inhibition of sympathetic vasoconstriction in post-menopausal women, it is plausible increased NO bioavailability as a result of dietary NO_3^- may also alter sympathetic vasoconstrictor responsiveness.

Young healthy females have not been shown to have reduced levels of NO bioavailability, however young women appear to have lower BP compared to men and the relationships between BP, cardiac output and sympathetic nerve activity also appear to different between men and women (Hart et al., 2014; Hart et al., 2009; Harvey et al., 2015; Joyner et al., 2015; Orshal et al., 2004; Vianna et al., 2012). Interestingly Kapil et al. (2010) have reported that the reduction in BP following dietary NO_3^- supplementation was smaller in females compared to males and speculated that the conversion of NO_3^- to NO is different in women, as the same dose of dietary NO_3^- resulted in differing plasma [NO_3^-] and [NO_2^-]. Therefore additional research is needed to understand the effect of dietary NO_3^- and enhanced NO bioavailability on the control of BP in young healthy women.

In addition, future studies should be completed in populations in which the ratio of NO to ROS has been altered such as in ageing. It has been reported that there are enhanced activity of ROS with ageing (Drew et al., 2003). Previously, sodium NO_3^- was shown to reduce superoxide production and oxidative stress in old mice (Sindler et al., 2011), however whether an increase in NO production via dietary NO_3^- has the same effects has not been investigated in humans. An alternative model that has not been investigated, is the role of dietary NO_3^- in the response to hypoxia. In response to reduced oxygen, or in conditions such as chronic intermittent hypoxia episodes that occur with sleep apnea, ROS are elevated (Prabhakar et al., 2007). Future studies

could be completed that investigate whether dietary NO_3^- has the ability to reduce oxidative stress in ageing, or in response to hypoxic conditions.

Finally, future studies should investigate the effect of dietary NO_3^- in disease conditions in which the sympathetic nervous system activity is elevated, such as chronic heart failure. Sympathetic over-activity leads to increased peripheral vasoconstriction, and to enhanced sympatho-excitation responses (Notarius et al., 2001). Dietary NO_3^- could provide enhanced NO-bioavailability, which may inhibit vasoconstriction in conditions in which the sympathetic nervous system is overactive.

Conclusion

In conclusion, acute dietary NO_3^- supplementation appeared to enhance NO bioavailability, however blood pressure, sympathetic vasoconstrictor responsiveness and plasma catecholamines were not different between placebo and NO_3^- supplementation conditions at rest or during exercise. Collectively, the results of this study suggest enhanced NO via dietary NO_3^- did not alter sympathetic vascular control at rest and during exercise in young healthy males.

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