J Physiol 593.9 (2015) pp 2213–2224

The Journal of Physiology

Hindlimb unweighting does not alter vasoconstrictor responsiveness and nitric oxide-mediated inhibition of sympathetic vasoconstriction

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Key points

- Physical inactivity increases the risk of cardiovascular disease and may alter sympathetic nervous system control of vascular resistance.
- Hindlimb unweighting (HU), a rodent model of physical inactivity, has been shown to diminish sympathetic vasoconstrictor responsiveness and reduce NO synthase expression in isolated skeletal muscle blood vessels.
- Our understanding of the effects of HU on sympathetic vascular regulation *in vivo* is very limited.
- The present findings demonstrate that HU did not alter sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle.
- This study suggests that short-term physical inactivity does not alter *in vivo* sympathetic vascular control in the skeletal muscle vascular bed at rest and during contraction.

Abstract We tested the hypothesis that physical inactivity would increase sympathetic vasoconstrictor responsiveness and diminish NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. Sprague–Dawley rats ($n = 33$) were randomly assigned to sedentary time control (S) or hindlimb unweighted (HU) groups for 21 days. Following the intervention, rats were anaesthetized and instrumented for measurement of arterial blood pressure and femoral artery blood flow and stimulation of the lumbar sympathetic chain. The percentage change of femoral vascular conductance (%FVC) in response to sympathetic chain stimulation delivered at 2 and 5 Hz was determined at rest and during triceps surae muscle contraction before (control) and after NO synthase blockade with L-NAME (5 mg kg I.V.). Sympathetic vasoconstrictor responsiveness was not different ($P > 0.05$) in S and HU rats at rest (S, 2 Hz, $-26 \pm 8\%$ and 5 Hz, $-46 \pm 12\%$; and HU, 2 Hz, $-29 \pm 9\%$ and 5 Hz, $-51 \pm 10\%$) and during contraction (S, 2 Hz, $-10 \pm 7\%$ and 5 Hz, $-23 \pm 11\%$; and HU, 2 Hz, $-9 \pm 5\%$ and 5 Hz, $-22 \pm 7\%$). Nitric oxide synthase blockade caused a similar increase ($P > 0.05$) in sympathetic vasoconstrictor responsiveness in HU and S rats at rest (S, 2 Hz, $-41 \pm 7\%$ and 5 Hz, $-58 \pm 8\%$; and HU, 2 Hz, $-43 \pm 6\%$ and 5 Hz, $-63 \pm 8\%$) and during muscle contraction (S, 2 Hz, $-15 \pm 6\%$ and 5 Hz, $-31 \pm 11\%$; and HU, 2 Hz, $-12 \pm 5\%$ and 5 Hz, $-29 \pm 8\%$). Skeletal muscle NO synthase expression and ACh-mediated vasodilatation were also not different between HU and S rats. These data suggest that HU does not alter sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle.

DOI: 10.1113/JP270279

(Received 3 November 2014; accepted after revision 22 February 2015; first published online 5 March 2015)
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Abbreviations eNOS, endothelial nitric oxide synthase; FBF, femoral blood flow; FVC, femoral vascular conductance; HR, heart rate; HU, hindlimb unweighted/unweighting; iNOS, inducible nitric oxide synthase; MAP, mean arterial pressure; MCF, maximal contractile force; MSNA, muscle sympathetic nerve activity; MT, motor threshold; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; S, sedentary time control.

Introduction

Physical inactivity increases the risk of cardiovascular disease and is recognized as a leading cause of death by the Centres for Disease Control and Prevention (Mokdad *et al.* 2004). Physical inactivity has also been shown to alter the basic physiological regulation of the cardiovascular system (Booth & Lees, 2007). Indeed, chronic physical inactivity has been shown to reduce exercise tolerance (Desplanches *et al.* 1987), cause peripheral vascular dysfunction (Thijssen *et al.* 2010) and alter autonomic reflex control (e.g. baroreflex function, regulation of efferent sympathetic nerve activity) of the cardiovascular system (Moffitt *et al.* 1998; Mueller, 2010).

The sympathetic nervous system is essential for the maintenance of arterial blood pressure and vascular resistance and the distribution of cardiac output (Malpas, 2010). In young humans, resting muscle sympathetic nerve activity (MSNA) was increased following 120 days of bed rest (Kamiya *et al.* 1999). Likewise, resting splanchnic sympathetic nerve activity was increased, and the increase in sympathetic outflow in response to direct stimulation of the rostral ventrolateral medulla and unloading of baroreceptors was greater in sedentary compared with physically active rats (Kamiya *et al.* 1999; Mueller, 2007; Mischel & Mueller, 2011; Mischel *et al.* 2014). Collectively, these studies indicate that efferent sympathetic nerve activity is increased after physical inactivity. If an increase in sympathetic outflow occurs in the absence of changes in the expression and responsiveness of postsynaptic sympathetic receptors, sympathetic vasoconstriction and vascular resistance may be elevated in sedentary/inactive individuals.

A reduced ability to shunt cardiac output away from inactive tissues to active muscles during exercise has been reported in hindlimb-unweighted (HU) rats, suggesting that the regulation of peripheral vascular resistance and muscle blood flow may be altered after physical inactivity (Overton & Tipton, 1990; McDonald *et al.* 1992). Furthermore, maximal constrictor responses to K^+ and α-adrenergic agonists in isolated aorta (Delp *et al.* 1993, 1995; Purdy *et al.* 1998; Papadopoulos & Delp, 2003) and isolated femoral and carotid arteries (Purdy *et al.* 1998; Hwang *et al.* 2007) are reduced following HU. In skeletal muscle resistance vessels, HU decreased vasoconstrictor responsiveness to potassium chloride and noradrenaline in second-order arterioles isolated from white gastrocnemius muscle (type IIB glycolytic, fast-twitch fibres), whereas vasoconstrictor responsiveness was preserved in arterioles from the soleus muscle (type I oxidative, slow-twitch fibres; Delp, 1999). Collectively, these studies suggest that the transduction of sympathetic nerve activity to vascular resistance may be altered by inactivity and that the effects of inactivity on vasoconstriction may be muscle and/or fibre type dependent. However, studies in isolated vascular segments may not be indicative of *in vivo* vascular control and do not permit assessment of vascular reactivity in contracting muscle. For example, 2 weeks of leg immobilization did not alter tyramine-evoked vasoconstriction in young healthy men at rest, suggesting that physical inactivity did not change sympathetic vasoconstrictor responsiveness. During exercise, tyramine infusion reduced leg vascular conductance in the immobilized, but not the control leg, suggesting that the ability to blunt constrictor responses during exercise was reduced after inactivity (Mortensen *et al.* 2012). Unfortunately, the mechanism responsible for the diminished inhibition of vasoconstriction during exercise was not determined (Mortensen *et al.* 2012).

Hindlimb unweighting has been shown to reduce endothelium-dependent vasodilatation and expression of endothelial NO synthase (eNOS) in isolated rat soleus muscle feed arteries (Jasperse *et al.* 1999; Woodman *et al.* 2001) and 1A arterioles (Jasperse *et al.* 1999; Schrage *et al.* 2000). Nitric oxide has been shown to inhibit sympathetic vasoconstriction in resting and contracting skeletal muscle (Thomas & Victor, 1998; Jendzjowsky & DeLorey, 2013*b*, *d*). Given the potential reduction in NOS expression following HU, it is conceivable that NO-mediated inhibition of sympathetic vasoconstriction may decline with inactivity and increase sympathetic vasoconstrictor responsiveness.

Thus, the purpose of the present study was to investigate the effect of HU on sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. It was hypothesized that HU would increase sympathetic vasoconstrictor responsiveness and reduce NO-mediated inhibition of sympathetic vasoconstriction.

Methods

Animal use

Male Sprague–Dawley rats were obtained from the institutional animal colony (~100–150 g body mass) and housed in an environmentally controlled room (22–24°C, 40–70% humidity) with a 12 h–12 h light–dark cycle. Food (Lab Diet 5001; PMI Nutrition, Brentwood, MO, USA) and water were provided *ad libitum*. All experiments were conducted with approval from the University of Alberta Animal Care and Use Committee: Health Sciences and in accordance with the Canadian Council on Animal Care Guidelines and Policies and conform to the principles of UK regulations, as described by Drummond (2009).

Hindlimb unweighting

Rats were randomly assigned to HU ($n = 20$) or sedentary time control groups (S; $n = 15$). Rats were unweighted by tail suspension and were housed individually in custom-designed cages. Benzoin tincture was applied to the proximal two-thirds of the rat's tail and allowed to dry and then traction tape with a wire loop in the centre was placed on each side of the tail and secured with filament tape, taking care to maintain tail circulation. The wire loop was then connected to a crossbar and the hindlimbs were suspended at an angle of approximately 30 deg to prevent the hindlimbs from contacting the surface of the cage, as previously described (Morey-Holton & Globus, 2002). Suspension height was confirmed by visual observation of the rat and by manually stretching the hindlimbs to ensure that the toes were unable to reach the floor of the cage. Hindlimb-unweighted rats were not permitted to contact surfaces with their hindlimbs except during weekly weighing and cage cleaning (~5 min). The suspension height was adjusted to maintain unweighting of the hindlimb as the rats grew. The S rats were housed individually in traditional shoebox cages and were handled daily.

Instrumentation

Following 21 days of HU or S caging, anaesthesia was induced by inhalation of isoflurane (3.5%; balance O_2; Sigma-Aldrich, Canada) and subsequently maintained by syringe-pump infusion of a mixture of α-chloralose (8–16 mg kg^{-1} h^{-1}; Pharmaceutical Partners of Canada, Richmond Hill, ON Canada) and urethane (50–100 mg kg^{-1} h^{-1}; Pharmaceutical Partners of Canada) through a cannula placed in the right jugular vein. The depth of anaesthesia was assessed by absence of a withdrawal reflex to a paw pinch and the stability of haemodynamic variables. A tracheotomy was performed and a cannula placed in the trachea to maintain a patent airway and facilitate spontaneous breathing of room air. Work in our laboratory has previously demonstrated that arterial blood gases and acid–base status are maintained at rest and during contraction in this preparation (arterial P_{O_2}, 88–95 mmHg; arterial P_{CO_2}, 39–41 mmHg; and pH: 7.39–7.42; Jendzjowsky & DeLorey, 2013*d*). The left brachial artery was cannulated and attached to a pressure transducer (Abbott, North Chicago, IL, USA) for the continuous measurement of arterial blood pressure. The left femoral artery and vein were cannulated for drug delivery. Femoral blood flow (FBF) was measured at the right femoral artery with a flow probe (0.7 V; Transonic Systems, Ithaca, NY, USA) connected to a flowmeter (T106; Transonic Systems). Heart rate (HR) was derived from the arterial blood pressure waveform, and femoral vascular conductance (FVC) was calculated. Core body temperature was monitored by rectal thermistor, and body temperature was maintained at 36–37°C by an external heating pad (TCAT-2; Physitemp, Clifton, NJ, USA).

The right triceps surae muscle group was dissected and attached to a force transducer (FT03; Grass Technologies, Warwick, RI, USA) at the calcaneal tendon. A cuff electrode was placed around the sciatic nerve, and muscle contraction was produced by electrical stimulation (Stimulator Panel, Chart software; AD Instruments, Colorado Springs, CO, USA). The motor threshold (MT) and the optimal muscle length for tension development were determined. Maximal contractile force (MCF) was determined by stimulation of the triceps surae muscle group with 25 impulses of 1 ms duration delivered at 100 Hz at 10 times the MT. Rhythmic muscle contractions at 60% of MCF were produced by stimulation of the sciatic nerve with 40 Hz, 0.1 ms pulses in 250 ms trains at a rate of 60 trains min^{-1} at approximately six times MT.

The lumbar sympathetic chain was exposed by making a mid-line abdominal incision and temporarily retracting the aorta and vena cava. A bipolar silver-wire stimulating electrode was attached to the lumbar sympathetic chain at the level of the L3 and L4 vertebrae and electrically isolated in rapidly curing, non-toxic silicone elastomer (Kwiksil; WPI, Sarasota, FL, USA). An isolated constant-current stimulator (DS3; Digitimer, Welwyn Garden City, UK) was used to stimulate the sympathetic chain and evoke the release of sympathetic neurotransmitters.

Following the surgical procedures and an approximately 20 min stabilization period, the following experimental protocol was completed.

Experimental protocol

The vasoconstrictor response to 1 min of lumbar sympathetic chain stimulation (1 ms, 1 mA pulses) delivered at 2 and 5 Hz in random order was measured at rest and during muscle contraction in control conditions and following NOS blockade (L-NAME, 5 mg kg^{-1}, I.V.). Rhythmic muscle contraction was produced for a total of 8 min, and lumbar sympathetic chain stimulation was

delivered 3 and 6 min after the onset of muscle contraction in random order. Control and L-NAME conditions were separated by ∼60 min of recovery.

To investigate the effect of HU on endothelium-dependent vasodilatation, the magnitude of vasodilatation in response to an intra-arterial bolus injection of ACh (0.1 μg) was measured in control and L-NAME conditions in a subset of rats (S, $n = 10$; and HU, $n = 10$). To minimize any flow-induced vasodilatation during intra-arterial injections, small volumes (100 μl) were injected over ∼5 s. Vehicle injections at this volume and rate did not increase hindlimb blood flow.

After the experimental protocol, rats were killed by anaesthetic overdose (α-chloralose and urethane). The heart, soleus and lateral and medial gastrocnemius muscles were dissected and weighed, and skeletal muscles were then flash frozen in liquid nitrogen and stored at −80°C. The tibia was also dissected, and the length of the tibia was measured.

Data analysis

Data were recorded using Chart software (AD Instruments). Arterial blood pressure and FBF were sampled at 100 Hz, and FVC (in millilitres per minute per millimetre of mercury) was calculated as FBF divided by the mean arterial pressure (MAP). Muscle force production was measured continuously and peak force development determined for each muscle contraction. To compare force production between groups and experimental conditions, a mean of peak contractile forces was calculated between minutes 3 and 7 (the time period encompassing the sympathetic stimulations) for each contractile bout.

The change in HR, MAP, FBF and FVC in response to sympathetic stimulation was calculated as an absolute change and as a percentage change from the value preceding the sympathetic stimulation in control and L-NAME conditions. The percentage change in FVC is the accepted metric to assess the magnitude of sympathetic vasoconstrictor responses because the percentage change in FVC accurately reflects percentage changes in resistance vessel radius even across conditions with different baseline levels of vascular conductance (Buckwalter & Clifford, 2001; Thomas & Segal, 2004). The response to ACh was calculated as the difference between the peak FVC response (∼3 s average) and the pre-infusion baseline (∼20 s average) and expressed as a percentage change from the FVC baseline. All data are expressed as means ± SD.

Western blot analysis

Snap frozen (100–200 mg) soleus, medial gastrocnemius and lateral gastrocnemius muscles were homogenized in lysis buffer [20 mM Tris (pH 7.4), 5 mM EDTA, 10 mM sodium pyrophosphate tetrabasic, 100 mM sodium fluoride and 1% Tergitol type NP-40] containing 1× Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). One hundred micrograms of total protein was separated on a 7.5% SDS-polyacrylamide gel and then transferred onto nitrocellulose membrane (0.2 μm; Bio-Rad, Hercules, CA, USA). Membranes were incubated with anti-mouse inducible NOS (iNOS), eNOS or neuronal NOS (nNOS; all at 1:1000 dilution; BD Transduction Laboratories, Franklin Lakes, NJ, USA) and rabbit α-tubulin (1:1000 dilution; Abcam, Cambridge, MA, USA). Membranes were subsequently probed with IRDye® 800 CW donkey anti-mouse IgG and IRDye® 680 RD donkey anti-rabbit IgG secondary antibodies (1:10,000 dilution; Li-Cor Biosciences, Lincoln, NE, USA). The protein bands were detected and quantified by a Li-Cor Odyssey imager system v3.0. Results were normalized to α-tubulin.

Statistics

Group differences in body, muscle and heart mass, tibial length, heart-to-body mass and heart mass-to-tibial length ratios were determined by Student's unpaired t test. The vasoconstrictor response to sympathetic stimulation was analysed by three-way repeated-measures ANOVA (group × drug condition × muscle contractile state). Each frequency of sympathetic stimulation was analysed separately. Differences in baseline haemodynamics, the haemodynamic response to exercise, muscle force production, ACh-mediated vasodilatation and NOS isoform expression were determined by two-way repeated-measures ANOVA (group × drug condition). When significant main effects and interactions were detected, a Student–Newman–Keuls *post hoc* test was performed. A P value of < 0.05 was considered statistically significant.

Results

Two rats were removed from the HU experimental group because tail suspension could not be maintained throughout the protocol. Thus, experiments were completed on 33 rats (S, $n = 15$; and HU, $n = 18$).

Body, heart, soleus, medial gastrocnemius and lateral gastrocnemius muscle mass was lower ($P < 0.05$) in HU than in S rats (Table 1). The heart-to-body mass ratio was similar ($P > 0.05$) in HU and S rats, but the heart mass-to-tibial length ratio was decreased ($P < 0.05$) in HU compared with S rats (Table 1).

Resting HR, MAP, FBF and FVC were not different ($P > 0.05$) between HU and S rats (Table 2).

Table 1. Animal characteristics

Group	Body mass (g)	Heart mass (g)	Soleus mass (g)	Lateral gastrocnemius mass (g)	Medial gastrocnemius mass (g)	Tibial length (mm)	Heart-to-body mass ratio	Heart mass-to-tibial length ratio
Sedentary	405 ± 50	1.37 ± 0.15	0.19 ± 0.03	0.77 ± 0.13	1.16 ± 0.28	40.1 ± 1.9	0.34 ± 0.03	3.4 ± 0.3
Hindlimb unweighted	339 ± 42*	1.18 ± 0.10*	0.09 ± 0.02*	0.65 ± 0.16*	0.89 ± 0.14*	39.7 ± 1.8	0.35 ± 0.03	3.0 ± 0.2*

All values are means ± SD. *Statistically significant ($P < 0.05$) group difference.

Table 2. Resting haemodynamics

Group	Drug condition	HR (beats min^{-1})	MAP (mmHg)	FBF (ml min^{-1})	FVC (ml min^{-1} mmHg^{-1})
Sedentary	Control	402 ± 42	92 ± 5	3.4 ± 0.7	0.037 ± 0.008
	L-NAME	382 ± 27†	128 ± 10*,†	4.0 ± 0.9*,†	0.031 ± 0.007†
Hindlimb unweighted	Control	410 ± 28	90 ± 12	2.9 ± 0.5	0.033 ± 0.007
	L-NAME	381 ± 30†	118 ± 11*,†	3.2 ± 1.1*	0.027 ± 0.009†

All values are means ± SD. Abbreviations: FBF, femoral blood flow; FVC, femoral vascular conductance; HR, heart rate; and MAP, mean arterial pressure. *Significant group difference ($P < 0.05$). †Significant drug effect ($P < 0.05$).

Effect of HU on sympathetic vasoconstrictor responsiveness

The response of HR, MAP, FBF and FVC to sympathetic stimulation at rest and during muscle contraction in a representative HU rat is shown in Fig. 1.

The vasoconstrictor response to sympathetic stimulation delivered at 2 and 5 Hz was not different ($P > 0.05$) in HU and S rats at rest (Fig. 2). Muscle contraction attenuated ($P < 0.05$) the constrictor response to sympathetic stimulation in both HU and S rats, but sympathetic vasoconstrictor responsiveness was not different ($P > 0.05$) between HU and S rats during muscle contraction (Fig. 2 and Table 3).

Effects of NOS inhibition

Treatment with L-NAME increased resting MAP ($P < 0.05$) in HU and S rats. The increase in MAP was larger in S rats, such that resting MAP was greater ($P < 0.05$) in S compared with HU rats during NOS inhibition. Treatment with L-NAME decreased ($P < 0.05$) resting HR by a similar magnitude in HU and S rats. Resting FBF was reduced ($P < 0.05$) by NOS inhibition in both HU and S rats, but FBF was lower ($P < 0.05$) in HU compared with S rats. Resting FVC was decreased ($P < 0.05$) by L-NAME in both HU and S rats, but was not different ($P > 0.05$) between HU and S rats.

Sympathetic vasoconstrictor responsiveness was increased ($P < 0.05$) during NOS inhibition at rest and during contraction in both HU and S rats (Fig. 2); however, sympathetic vasoconstrictor responsiveness was not different ($P > 0.05$) between HU and S rats in resting and contracting muscle (Fig. 2 and Table 3).

Hyperaemic response to contraction and muscle force production

The increase in FBF and FVC in response to muscle contraction at 60% MCF was lower ($P < 0.05$) in HU than in S rats (Table 4). Treatment with L-NAME augmented the increase in FBF and FVC in response to muscle contraction at 60% MCF in both groups ($P < 0.05$), and the increase in FBF and FVC remained lower ($P < 0.05$) in HU compared with S rats in the presence of L-NAME. The blood pressure response to exercise was not different ($P > 0.05$) between HU and S rats in control and L-NAME conditions, but L-NAME reduced ($P < 0.05$) the increase in MAP during exercise in both HU and S rats (Table 4). Muscle force production was lower ($P < 0.05$) in HU compared with S rats in both control (S, 418 ± 93 g; and HU, 263 ± 90 g) and L-NAME conditions (S, 401 ± 87 g; and HU, 260 ± 105 g).

Effects of HU on ACh-dependent vasodilatation

The increase in FVC in response to ACh was not different ($P > 0.05$) between HU and S rats in control and L-NAME conditions (Fig. 3). Treatment with L-NAME reduced ($P < 0.05$) the vasodilator response to ACh by a similar magnitude in both HU and S rats.

Effect of HU on NOS isoform expression

The expression of iNOS, eNOS and nNOS in soleus, lateral gastrocnemius and medial gastrocnemius muscles was not different ($P > 0.05$) between HU and S rats (Fig. 4). Endothelial NOS and iNOS were expressed to a similar extent ($P > 0.05$) in all muscles in S and HU rats, whereas nNOS expression was lower ($P < 0.05$) in the soleus muscle compared with lateral and medial gastrocnemius muscles in both S and HU rats.

Discussion

The purpose of this study was to determine the effects of HU on sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. The important novel findings from this study were that HU did not alter the following factors: (i) sympathetic vasoconstrictor responsiveness in resting or contracting muscle; (ii) NO-mediated inhibition of sympathetic vasoconstriction at rest and during exercise; and (iii) skeletal muscle NOS expression and NO-dependent vasodilatation. To our knowledge, this is the first study to investigate the effects of HU on sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of vasoconstriction in resting and contracting skeletal muscle. The data indicate that HU does not alter *in vivo* sympathetic vascular control at rest and during exercise.

Hindlimb unweighting via tail suspension has been shown to cause marked skeletal muscle atrophy and, consistent with previous studies (Musacchia *et al.* 1983; McDonald *et al.* 1992), soleus and medial and lateral gastrocnemius muscle mass was reduced in HU compared with S rats in the present study. Consistent with a decline in skeletal muscle cross-sectional area, muscle force production was reduced in HU rats. Heart mass and the heart mass-to-tibial length ratio were also reduced in HU compared with S rats, indicating that HU caused significant cardiovascular deconditioning in the present study.

In contrast to our hypothesis, HU did not alter sympathetic vasoconstrictor responsiveness in resting skeletal muscle in the present study. Consistent with the preserved resting sympathetic vasoconstrictor responsiveness in this study, HU did not alter

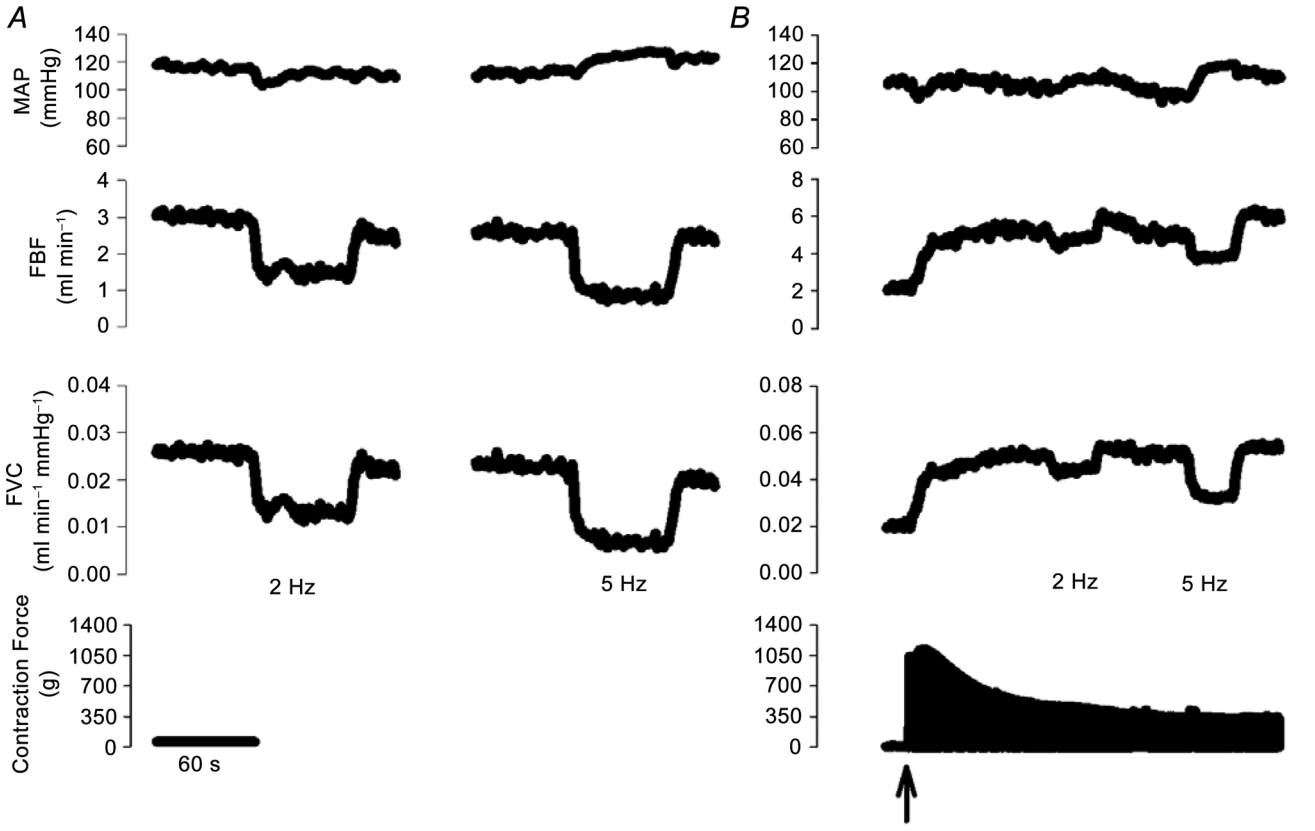

Figure 1. Original data from a representative hindlimb-unweighted rat illustrating the response of mean arterial blood pressure (MAP), femoral blood flow (FBF), femoral vascular conductance (FVC) and contractile force to lumbar sympathetic stimulation at 2 and 5 Hz in resting skeletal muscle (*A*) and during skeletal muscle contraction at 60% of maximal contractile force (MCF; *B*)
Arrow denotes the onset of contraction.

sympathetic vasoconstrictor responsiveness in soleus muscle second-order arterioles (Delp, 1999) and 2 weeks of limb immobilization did not change tyramine-evoked vasoconstriction in the resting leg of healthy, young men (Mortensen *et al.* 2012). In contrast to the present data, HU decreased vasoconstrictor responsiveness in second-order arterioles from white gastrocnemius muscle (Delp, 1999) and maximal constrictor responses in isolated vascular segments from the thoracic and abdominal aorta (Delp *et al.* 1993) and carotid and femoral arteries (Sangha *et al.* 2000). The reasons for the different effects of inactivity on vasoconstrictor responsiveness in these studies are not readily apparent. However, it is possible that the effects of inactivity on sympathetic vasoconstriction are vessel and muscle specific and that the limited data on the effects of HU in skeletal muscle resistance vessels are not reflective of the effects of inactivity on integrated sympathetic vascular control in a limb. Hindlimb unweighting has been shown to increase sensitivity to KCl in abdominal aortic rings (Delp et al. 1993); however, vasoconstrictor responses to sympathetic stimulation delivered at 2 and 5 Hz were similar in HU and S rats, suggesting that sensitivity to sympathetic neurotransmitters was not altered by HU in the present study. It is also possible that HU may alter postsynaptic receptor expression and/or the amount and composition of neurotransmitters released during sympathetic chain stimulation, and these changes may balance HU-induced alterations in vasoconstrictor properties. However, tyramine has been shown to produce a similar increase in circulating catecholamines before and after limb immobilization, suggesting that inactivity does not alter evoked neurotransmitter release (Mortensen *et al.* 2012). To our knowledge, the effect of inactivity on the expression of sympathetic receptors has not been investigated.

Compared with rest, the vasoconstrictor response to sympathetic stimulation was attenuated by muscle contraction in both HU and S rats in the present study. Sympathetic vasoconstriction was decreased by a similar magnitude in HU and S rats, and sympathetic vasoconstrictor responsiveness was not different during muscle contraction in HU and S rats (Fig. 2). In humans,

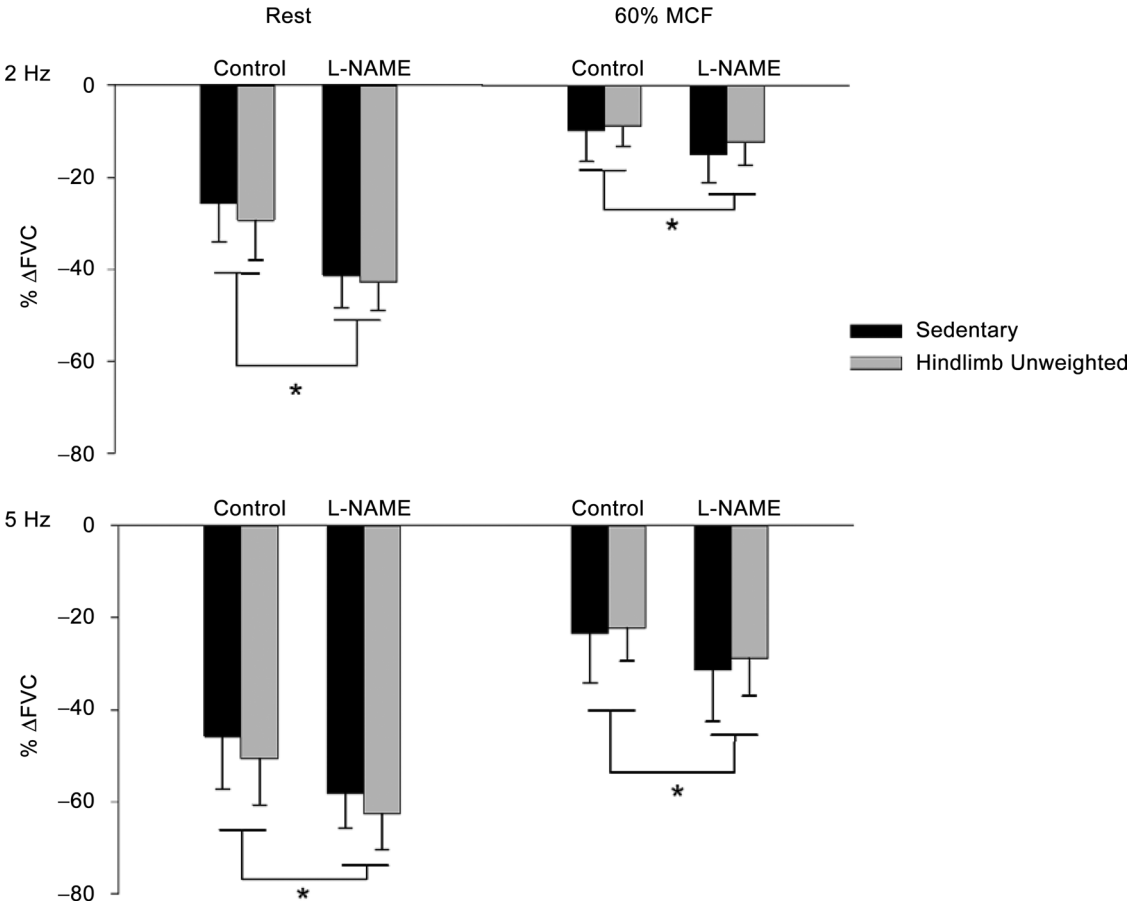

Figure 2. The percentage change of FVC in response to 2 and 5 Hz sympathetic stimulation at rest and during contraction at 60% MCF in sedentary (filled columns) and hindlimb-unweighted rats (grey columns) during control conditions and after administration of L-NAME (5 mg kg^{-1} I.V.)
Values are means ± SD. *Significant difference between control and drug conditions ($P < 0.05$).

Table 3. Haemodynamic response to sympathetic stimulation at rest and during contraction

Muscle contractile state	Group	Drug condition	Stimulation frequency (Hz)	ΔMAP (mmHg)	ΔFBF (ml min^{-1})	ΔFVC (ml min^{-1} mmHg^{-1})
Rest	Sedentary	Control	2	0.5 ± 5.4	−0.8 ± 0.4	−0.009 ± 0.004
			5	10.7 ± 5.2	−1.3 ± 0.5	−0.017 ± 0.006
		L-NAME	2	10.1 ± 5.5*	−1.4 ± 0.4*	−0.012 ± 0.003*
			5	19.8 ± 7.1*	−2.0 ± 0.6*	−0.018 ± 0.004
	Hindlimb unweighted	Control	2	0.9 ± 6.9	−0.8 ± 0.3	−0.010 ± 0.003
			5	11.8 ± 7.4	−1.2 ± 0.3	−0.016 ± 0.004
		L-NAME	2	11.0 ± 4.9*	−1.2 ± 0.5*	−0.011 ± 0.004*
			5	20.3 ± 6.8*	−1.7 ± 0.7*	−0.017 ± 0.007
60% MCF	Sedentary	Control	2	2.6 ± 2.6	−0.7 ± 0.7	−0.009 ± 0.007
			5	9.2 ± 6.1	−1.5 ± 1.0	−0.021 ± 0.012†
		L-NAME	2	8.4 ± 4.3*	−0.9 ± 0.6†	−0.012 ± 0.005*
			5	18.1 ± 7.5*	−2.0 ± 1.1*	−0.025 ± 0.009*,†
	Hindlimb unweighted	Control	2	2.2 ± 2.6	−0.4 ± 0.4†	−0.006 ± 0.003†
			5	8.0 ± 4.7†	−1.0 ± 0.6	−0.015 ± 0.006
		L-NAME	2	6.7 ± 3.3*,†	−0.5 ± 0.5†	−0.008 ± 0.004*,†
			5	16.8 ± 6.9*,†	−1.3 ± 0.8	−0.018 ± 0.007

All values are means ± SD. Abbreviations are as in Table 2. *Significant ($P < 0.05$) effect of L-NAME within a group and stimulation frequency. †Significant ($P < 0.05$) effect of muscle contraction within a group and stimulation frequency.

Table 4. Hyperaemic and blood pressure response to muscle contraction

Group	Drug condition	ΔMAP (mmHg)	ΔFBF (ml min^{-1})	ΔFVC (ml min^{-1} mmHg^{-1})
Sedentary	Control	11 ± 9	5.4 ± 1.2	0.050 ± 0.010
	L-NAME	−2 ± 5†	6.5 ± 1.8†	0.053 ± 0.010
Hindlimb unweighted	Control	10 ± 7	3.6 ± 1.0*	0.034 ± 0.011*
	L-NAME	0 ± 7†	4.6 ± 0.9*,†	0.040 ± 0.008*,†

All values are means ± SD. Abbreviations are as in Table 2. *Significant group difference ($P < 0.05$). †Significant drug effect ($P < 0.05$).

tyramine infusion during exercise produced a similar percentage decrease in leg vascular conductance in a leg that had been immobilized (casting for 2 weeks) and a control leg, consistent with preserved sympathetic vasoconstrictor responsiveness during contraction following inactivity (Mortensen *et al.* 2012). In the immobilized leg, however, tyramine infusion during exercise reduced vascular conductance in comparison to exercise alone, whereas in the control leg the vascular conductance was not different between exercise alone and exercise with tyramine; this suggests that in humans the ability to inhibit vasoconstriction during exercise was diminished by inactivity (Mortensen *et al.* 2012).

Work in our laboratory and others has shown that NO inhibits sympathetic vasoconstriction in resting and contracting muscle (Thomas & Victor, 1998; Jendzjowsky & DeLorey, 2013*b*). Consistent with previous studies, NOS blockade augmented the constrictor response to sympathetic stimulation at rest and during muscular contractions in both HU and S rats. However, during NOS inhibition the sympathetic vasoconstrictor responsiveness was similar at rest and during contraction in HU and S rats, suggesting that HU did not reduce NO-mediated inhibition of sympathetic vasoconstriction in the present study. Consistent with the preserved NO-mediated inhibition of vasoconstriction, expression of skeletal muscle eNOS, nNOS and iNOS was not different in HU compared with S rats in the present study (Fig. 4). Similar eNOS expression has been reported in gastrocnemius muscle feed arteries and 1A arterioles of HU and sedentary rats (Woodman *et al.* 2001), whereas eNOS expression was reduced in soleus feed (Jasperse *et al.* 1999; Woodman *et al.* 2001) and 1A arterioles (Schrage *et al.* 2000; Woodman *et al.* 2001) following 14 days of HU. Ma *et al.* (2003) reported that femoral artery eNOS and iNOS expression was unchanged following HU, whereas NOS expression was reduced in mesenteric arteries and increased in carotid arteries and thoracic aorta in HU compared with sedentary-control rats. Another study reported that iNOS expression was upregulated in thoracic aorta, heart and kidney and nNOS expression was increased in brain and kidney tissue following HU, whereas eNOS expression

was not different in HU and sedentary-control rats (Vaziri *et al.* 2000). Collectively, these data indicate that the effects of HU on NOS expression are highly isoform and tissue specific. Work in our laboratory has demonstrated that NO derived from both nNOS and eNOS contributes to NO-mediated inhibition of sympathetic vasoconstriction (Jendzjowsky & DeLorey, 2013*b*) in resting and contracting skeletal muscle. The similar skeletal muscle eNOS and nNOS expression in the present study is consistent with preserved NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. Skeletal muscle homogenates are unable to distinguish tissue-specific (muscle, endothelium and nerve) changes in NOS expression, and whether HU altered NOS expression in other tissues and vascular beds was not determined in the present study. However, the blood pressure response to L-NAME was blunted in HU compared with S rats, suggesting that systemic NO bioavailability may have been decreased in HU rats.

Nonetheless, skeletal muscle NO-dependent vascular function did not appear to be altered by HU in the present study, because ACh-mediated vasodilatation was not different between HU and S rats (Fig. 3). Treatment with L-NAME also caused a similar decrease in ACh-mediated vasodilatation in HU and S rats (Fig. 3). Maintained ACh-mediated vasodilatation following HU has also been reported in gastrocnemius muscle feed arteries and 1A

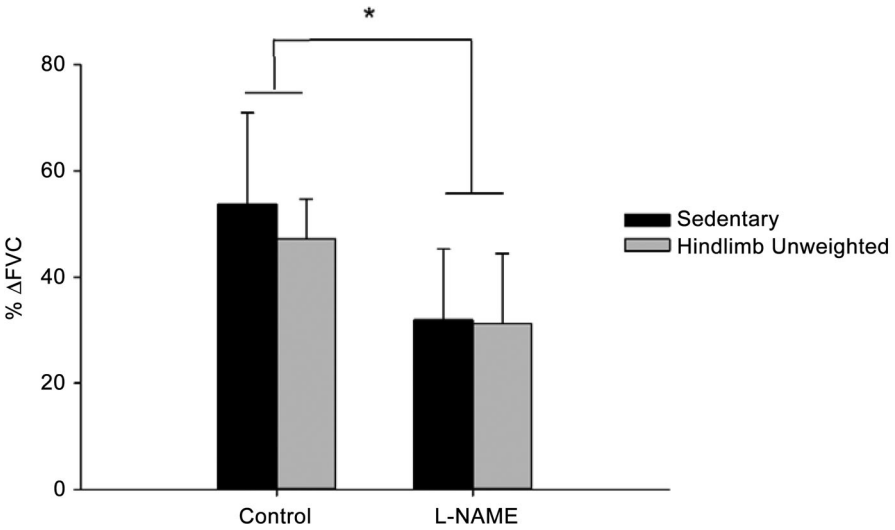

Figure 3. The percentage change of FVC in response to a single bolus dose of ACh (0.1 μg) in sedentary (filled columns) and hindlimb-unweighted rats (grey columns) during control conditions and after administration of L-NAME (5 mg kg^{-1} I.V.)
Values are means ± SD. *Significant difference between control and drug conditions ($P < 0.05$).

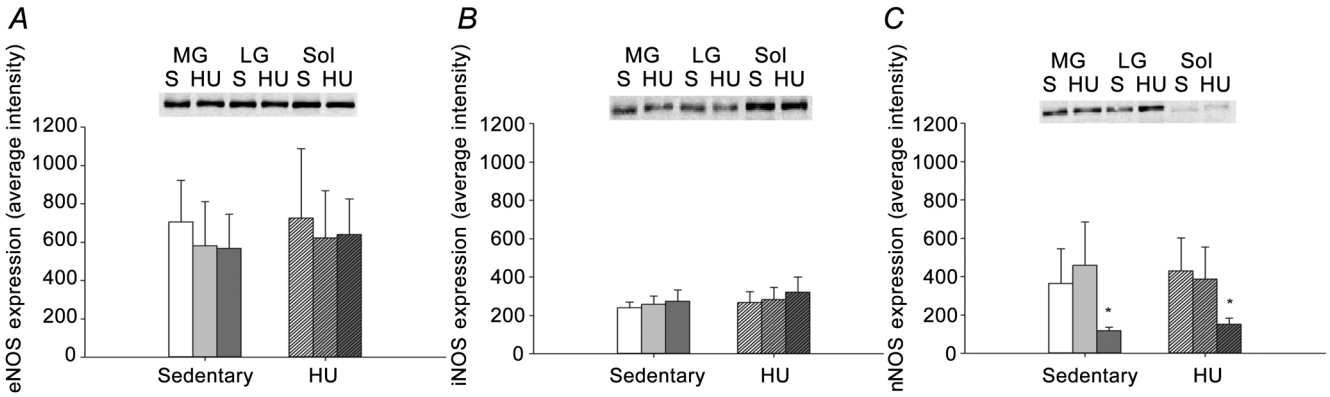

Figure 4. Normalized expression of endothelial (eNOS; *A*), inducible (iNOS; *B*) and neuronal NO synthase (nNOS; *C*) from medial gastrocnemius (MG; open columns), lateral gastrocnemius (LG; light grey columns) and soleus muscles (Sol; dark grey columns) in sedentary (S; solid columns) and hindlimb-unweighted groups (HU; hatched columns) with representative Western blots
All values are means ± SD. *Significant difference between muscles within a group. A value of $P < 0.05$ was considered statistically significant.

arterioles (Woodman *et al.* 2001), whereas ACh-mediated vasodilatation was reduced in soleus feed (Jasperse *et al.* 1999; Woodman *et al.* 2001) and 1A arterioles (Schrage *et al.* 2000; Woodman *et al.* 2001) in HU rats. In humans, the increase in leg vascular resistance following NOS blockade with N(G) monomethyl-L-arginine and the vasodilatory response to the NO donor sodium nitroprusside were not altered by 28 days of unilateral lower limb suspension (Bleeker *et al.* 2005*c*). Flow-mediated dilatation of the femoral artery was also preserved after 28 days of unilateral lower limb suspension (Bleeker *et al.* 2005*a*) and 52 days of bed rest (Bleeker *et al.* 2005*b*). In summary, the available scientific evidence regarding the effects of inactivity on NO-mediated vascular function is conflicting. Further investigation will be required to provide full characterization of the tissue-specific effects of inactivity on NO-mediated vascular function at rest and during exercise.

Experimental considerations and limitations

Consistent with previous studies that have investigated the effect of HU on vascular function, sedentary caged rats were used as the control group in the present study (Overton & Tipton, 1990; McDonald *et al.* 1992; Delp *et al.* 1993,1995; Purdy *et al.* 1998; Delp, 1999; Papadopoulos & Delp, 2003). It could be argued that sedentary caged animals and HU rats experienced a similar level of inactivity and that physically active rats may be a more suitable control group. However, the decrement in skeletal and cardiac muscle mass as well as reduced skeletal muscle force production in HU compared with S rats are all indicative of marked deconditioning in the HU rats and suggest that it is unlikely that similar levels of inactivity were experienced by S and HU rats. Furthermore, we (Jendzjowsky & DeLorey, 2012, 2013*a*,*c*,*d*) and others (Mizuno *et al.* 2014) have shown that a modest amount of physical activity alters sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of sympathetic vasoconstriction. Therefore, if a physically active group were to have been used as the control group in the present study, it would have been difficult to determine whether group differences were attributable to an increase or a decline in physical activity.

The present study indicates that vasoconstrictor responsiveness was not altered by HU, suggesting that sympathetic vascular transduction was unchanged following physical inactivity. However, further investigation is required to elucidate the effect of HU on each component of vascular transduction. For example, it is conceivable that changes in the responsiveness and/or density/distribution of postsynaptic sympathetic receptors may balance alterations in evoked neurotransmitter release and maintain constrictor responses following HU.

Additional limitations include the use of electrically evoked sympathetic activity and muscle contractions in anaesthetized rats that do not reflect naturally occurring nerve activity in conscious animals/humans. Finally, NOS protein levels were measured in muscle homogenates, and specific effects of HU on NOS expression in skeletal muscle, endothelium, nerve and smooth muscle could not be determined.

Perspectives and conclusions

Previous studies have shown that reflex control of the sympathetic nervous system is altered by inactivity (Moffitt *et al.* 1998; Foley *et al.* 2005; Jung *et al.* 2005) and that sympathetic outflow may be increased in sedentary people/animals (Kamiya *et al.* 1999; Mischel & Mueller, 2011). In the present study, evoked sympathetic vasoconstrictor responses were not different in resting and contracting muscle in control and HU rats, suggesting that sympathetic vascular regulation was unaltered by hindlimb unweighting. Interestingly, a previous *in vitro* study reported that following HU the vasoconstrictor responses to noradrenaline were decreased and maintained in isolated second-order arterioles from the gastrocnemius and soleus muscles, respectively (Delp, 1999). Collectively, these data suggest that isolated vessels may not reflect integrated vascular control *in vivo* or that integrative vascular control mechanisms that regulate limb vascular conductance may be able to compensate for inactivity-induced alterations in vascular function in specific muscles and/or vascular segments.

Recent studies from our laboratory have shown that there is considerable plasticity in sympathetic vascular regulation and that a relatively modest amount of exercise training alters sympathetic vasoconstrictor responsiveness and NO-mediated inhibition of vasoconstriction (Jendzjowsky & DeLorey, 2012, 2013*a*,*c*,*d*). Similar plasticity does not appear to exist in response to physical inactivity. Thus, the accumulated evidence from our recent studies suggests that adaptions in sympathetic vascular control in response to changes in physical activity may not occur along a continuum and do not appear to be a simple linear function of physical activity level. Indeed, the present findings suggest that compensatory, protective mechanisms may exist that maintain sympathetic vascular control and the control of skeletal muscle blood flow in response to a short (21 days) but severe decline in physical activity. Further studies will be required to determine whether prolonged physical inactivity modifies sympathetic vascular control in the skeletal muscle vasculature.

In conclusion, the present study demonstrated that HU did not increase sympathetic vasoconstrictor responsiveness or reduce NO-mediated inhibition of sympathetic vasoconstriction in resting and contracting skeletal muscle. Skeletal muscle NOS expression and NO-dependent vasodilatation were also not altered by HU. This study advances our understanding of the effects of chronic physical inactivity on sympathetic nervous system control of vascular conductance in resting and contracting skeletal muscle.

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Additional information

Competing interests

None declared.

Author contributions

T.P.J. contributed to conception of the project, design of experiments, data collection, analysis and interpretation, and manuscript preparation and revision. N.G.J. contributed to data collection, analysis and interpretation, and manuscript preparation and revision. D.S.D. contributed to the conception of the project, design of experiments, data analysis and interpretation, and manuscript preparation and revision. All authors have read and approved the final submission. All experiments were completed at the University of Alberta.

Funding

This project was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), Canadian Foundation for Innovation and Alberta Advanced Education and Technology. T.P.J. was supported by an NSERC Canada Graduate Doctoral Scholarship and a Queen Elizabeth II Doctoral Scholarship from the University of Alberta. N.G.J. was supported by an NSERC Canada Graduate Doctoral Scholarship, University of Alberta Presidents' Scholarship and Izaak Walton Killam Memorial Scholarship.

Acknowledgements

The authors would like to thank Dr Sandra Davidge and Ms Anita Quon for assistance with the measurement of nitric oxide synthase.