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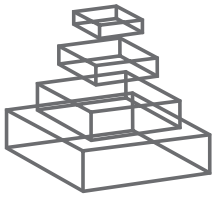

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NEURAL CONTROL OF THE CIRCULATION DURING EXERCISE IN HEALTH AND DISEASE

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The neural cardiovascular and hemodynamic adjustments to exercise are necessary to meet the metabolic demands of working skeletal muscle. These demands are met, in part, by precise alterations in sympathetic and parasympathetic nerve activity of the autonomic nervous system. Several neural mechanisms working in concert are responsible for these reflex adjustments and through complex interactions control the cardiovascular and hemodynamic changes in an intensity-dependent manner. It is well accepted that central command (a feed-forward mechanism originating from higher brain centers), the exercise pressor reflex (a feed-back mechanism originating from skeletal muscle), the arterial baroreflex (a negative feed-back mechanism originating from the carotid sinus and aortic arch), and the cardiopulmonary baroreflex (a negative feed-back mechanism originating from the heart, and blood vessels of the lungs) are all involved in mediating the characteristic cardiovascular adjustments to physical activity. Although certain fundamental mechanisms about each of these reflex mechanisms are continuing to be examined, there is a growing interest in regard to alterations in the activity of each of these neural inputs after the development of cardiovascular disease. This call for papers is for any aspects of neural cardiovascular control during exercise both in health and disease. Appropriate topics include but are not limited to, central and peripheral factors involved in these reflex control mechanisms, alterations in reflex control and the neural cardiovascular responses to exercise with disease, and potential integrative relationships between these neural control mechanisms. Studies performed in a variety of experimental models including humans, animals, cell culture, etc are all welcome.

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EDITORIAL
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Neural control of the circulation during exercise in health and disease

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Keywords: exercise pressor reflex, arterial baroreflex, blood pressure, sympathetic nerve activity, vascular responses to exercise

During exercise, appropriate cardiovascular, and hemodynamic adjustments are necessary to meet the metabolic demands of active skeletal muscle. Autonomic alterations in sympathetic and parasympathetic nerve activity play a major role in ensuring these adjustments are adequately made. Several neural mechanisms working in concert are responsible for regulating this autonomic activity and through complex interactions, control the cardiovascular and hemodynamic changes in an intensity-dependent manner. Central command (a feed-forward mechanism originating from higher brain centers), the exercise pressor reflex (EPR; a feed-back mechanism originating in skeletal muscle), the arterial baroreflex (a negative feed-back mechanism originating from the carotid sinus and aortic arch), and the cardiopulmonary baroreflex (a negative feed-back mechanism originating from the heart, and blood vessels of the lungs) are all known to contribute to the neural cardiovascular adjustments to physical activity. This research topic was designed to provide an update in the area of neural control of the circulation and present some of the newest work and current ideas in this continually progressing field. Indeed, fundamental mechanisms driving each of these neural inputs continue to be elucidated. Moreover, there is a growing interest in regard to alterations in the activity of each input after the development of cardiovascular disease. The papers compiled in this e-book highlight some of the most recent work regarding neural cardiovascular control during exercise in health and disease using a variety of experimental models and methodologies.

Given its importance in mediating the cardiovascular responses to exercise, the EPR (activated via excitation of group III and IV afferent fibers within skeletal muscle), has been a major focus of past and current research. This is highlighted by 4 contributions in this research topic. An important advancement is the growing understanding of impairments in EPR function accompanying the development of cardiovascular disease. Indeed, sympathetic activation during exercise is exaggerated in a number of disease states increasing the risk of a cardio- and/or cerebral-vascular event while also contributing to exercise intolerance. For example, EPR dysfunction has been shown to contribute to the augmented exercise-induced sympatho-excitation in heart failure and hypertension. In this research topic, for the first time, alterations in muscle temperature are considered as a possible contributing factor to the exaggerated EPR activity in heart failure (Li et al., 2012). More importantly, Li and Xing (2012) extend the list of diseases with impairments in EPR function to include peripheral arterial disease. These authors summarize recent mechanistic work demonstrating a role for the EPR in contributing to the augmented sympathetic and pressor responses to exercise in peripheral arterial disease. With the goal of treating EPR dysfunction, Wang et al. (2012) provide a thorough review of the mechanisms that contribute to the exaggerated EPR in heart failure, while demonstrating the effectiveness of chronic exercise training as a non-pharmacological therapy to ameliorate heightened EPR-induced sympathetic activation. This is accompanied by a paper by Mueller and Mischel (2012) in which regular physical activity is shown to modulate excitatory/inhibitory neurotransmission within the rostral ventrolateral medulla, a brainstem region integral in the central control of sympathetic outflow. Thus, exercise training can have important beneficial central as well as peripheral effects that modulate sympathetic control of the circulation. Lastly, Murphy et al. (2013) provide novel insight into the timing at which EPR function may be altered following injury to the spinal cord; information critical to the implementation of exercise prescription in patients with spinal cord injury (a population known to be at an increased risk for the development of cardiovascular disease).

Another reflex critical for appropriate neural cardiovascular adjustments to exercise is the arterial baroreflex. It is well established that the arterial baroreflex resets to remain functional during exercise and plays an important role in ensuring appropriate neural cardiovascular responses are elicited. Notably, the majority of work contributing to our knowledge of the arterial baroreflex in humans during exercise has been performed in young Caucasian men. In this research topic, Holwerda et al. (2013) have begun to extend these findings to young African Americans clearly showing for the first time a similar magnitude of resetting in this group compared to young Caucasian Americans. However, impairments in the ability of African Americans to defend against a hypertensive challenge were observed during steady-state exercise, providing novel information that may begin to explain the greater cardiovascular responsiveness to physiological stressors in African Americans compared to Caucasian Americans. Another interesting concept put forth in this research topic is provided by Schwartz and Stewart (2012). As noted above, although arterial baroreflex resetting is well known to occur during exercise, less information is available regarding other forms of stress. These authors provide data suggesting the presence of resetting during upright tilt with the sympathetic baroreflex being augmented and potentially contributing to an increase in peripheral resistance that may improve an individual's ability to defend against hypotension. This is important information that highlights the need for

additional human studies to further characterize resetting during orthostasis.

While reflex control of sympathetic outflow is critical, vascular changes ultimately determine the pressor responses evoked. Indeed, augmented central sympathetic activation would be minimized by insufficient transduction into a vascular response. Thus, vascular responses are critical to fully elucidating the neural control of the circulation during exercise. Importantly, sympathetic responses are only one facet of vascular control competing with metabolic, hormonal, mechanical, shear-induced, conducted, myogenic, and autoregulatory mechanisms. In this research topic, Matsukawa et al. (2013) provide a mini-review on vasodilator responses at the onset of exercise and the potential contribution of central command in mediating a cholinergic and/or beta-adrenergic vasodilator signal to skeletal muscle. Casey and Joyner (2012) examine the interaction between sympathetically-mediated vasoconstriction and vasodilation in the partial restoration of flow following hypoperfusion evoked by intra-arterial balloon inflation. They demonstrate that alpha-adrenergic mediated vasoconstriction restricts compensatory vasodilation during forearm exercise with hypoperfusion, but is not responsible for the initial increase in vascular resistance at the onset of hypoperfusion. These findings further underscore the interactions and complexities of vascular control during exercise. Interestingly, this is likely extended to post exercise as well. Indeed, Moynes et al. (2013) demonstrate a persistent attenuation of vascular responses to sympathetic activation acutely after the cessation of exercise that may have important implications for blood pressure regulation and immediate post exercise hypotension. Lastly, it is important to remember that not all vascular beds respond alike. For the most part, skeletal muscle blood flow has been discussed; however, increases in skin blood flow as well as cerebral blood flow are also important in the adjustments to exercise. Miyazawa et al. (2012) demonstrate that the regulation of these vascular beds are likewise quite complex.

Overall, this research topic highlights recent advances in the neural control of the circulation in health and disease and at the same time, continues to demonstrate the diversity and complexities involved. Indeed, the more we learn, the more we appreciate the integration needed to coordinate appropriate neural cardiovascular and hemodynamic adjustments to exercise as well as the plasticity of the neural cardiovascular system in both health and disease. We now await further advances in this important field of research.

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Exaggerated pressor response in relation to attenuated muscle temperature response during contraction in ischemic heart failure

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It is known that muscle temperature (T_m) increases with exercise. The purpose of this study was to examine if contraction-induced increase in T_m was altered in rats with heart failure (HF) induced by chronic myocardial infraction (MI) as compared with healthy control animals. A temperature probe was inserted in the triceps surae muscle to continuously measure T_m throughout experiments. Static muscle contraction was induced by electrical stimulation of the sciatic nerve for 1 min. As baseline T_m was 34˚C, contraction increased temperature by 1.6 ± 0.18˚C in nine health control rats and by 1.0 ± 0.15˚C in 10 MI rats ($P < 0.05$ vs. control). Note that there were no differences in developed muscle tension and muscle weight between the two groups. In addition, muscle contraction increased mean arterial pressure by 23 ± 3 mmHg in control rats and by 31 ± 3 mmHg in MI rats ($P < 0.05$ vs. control). A regression analysis further shows that there is an inverse liner relationship between the pressor response and static contraction-induced increase in T_m. Our data suggest that T_m increase evoked by contraction is impaired in MI rats. The abnormal alteration in T_m likely modifies the reflex cardiovascular responses in MI via mechanisms of temperature-sensitive receptors on muscle afferent nerves.

Keywords: heart failure, exercise, muscle temperature, blood pressure, sympathetic nervous system

INTRODUCTION

The sympathetic nervous system is activated during exercise (Mark et al., 1985; Victor et al., 1988; Sinoway et al., 1989; Matsukawa et al., 1990). This contributes to increases in blood pressure, heart rate (HR), myocardial contractility, and peripheral vasoconstriction (Coote et al., 1971; McCloskey and Mitchell, 1972; Mitchell et al., 1977; Lind, 1983). Two mechanisms of neural control contribute to these exercise responses: "central command" and "the exercise pressor reflex" (Goodwin et al., 1972; Mitchell et al., 1983; Waldrop et al., 1996). Central command is a mechanism whereby signals from a central site responsible for recruiting motor units activate cardiovascular control areas in the brain stem (Goodwin et al., 1972; Waldrop et al., 1996). The exercise pressor reflex is a mechanism whereby signals from thin-fiber skeletal muscle group III (predominately mechanically sensitive) and group IV (predominately metabolically sensitive) afferents likewise evoke increases in blood pressure and HR via coordinated changes in autonomic outflow (Kaufman et al., 1983; Mitchell et al., 1983; Kaufman and Forster, 1996). This system responds to mechanical deformation of the muscle afferent receptive field (i.e., "mechanoreceptor" stimulation) as well as to metabolic stimulation (i.e., "metaboreceptor" stimulation). When these receptors are stimulated, thin-fiber muscle afferent nerves are engaged, cardiovascular circuits in the brain stem are activated, sympathetic activity increases, and blood pressure rises (Mitchell et al., 1983).

The sympathetic nerve and blood pressure responses to exercise are exaggerated in heart failure (HF; Middlekauff et al., 2000, 2001; Li et al., 2004b; Momen et al., 2004; Gao et al., 2007). However, the underlying mechanisms to cause the abnormal autonomic responses are poorly understood.

In general, purinergic P2X receptors and transient receptor potential vanilloid type 1 (TRPV1 or VR1) are widely found on thin-fiber afferent nerves (Guo et al., 1999; Ma, 2002) and mediates numerous sensory afferent activations (Caterina et al., 1997; Nault et al., 1999; Smith and McQueen, 2001; Zahner et al., 2003). Specifically, it has been reported that activation of P2X receptors on the nerve endings of muscle afferents plays a role in mediating the autonomic adjustments to active muscle (Hanna et al., 2002; Li and Sinoway, 2002; Hanna and Kaufman, 2003, 2004). Although TRPV1 has been reported to play little role in mediating the cardiovascular responses to activation of muscle afferent (Kindig et al., 2005), capsaicin, a TRPV1 agonist injected into the arterial supply of the hindlimb muscles evokes increases in blood pressure and HR (Li et al., 2004a,b; Smith et al., 2005).

Furthermore, abnormal responses of P2X and TRPV1 have been observed in rats with HF. For instance, previous studies have demonstrated that muscle afferent-mediated pressor response of P2X activation is exaggerated in HF animals and the responsiveness is related to the degree of left ventricular dysfunction (Li et al., 2004b; Gao et al., 2007). The augmented reflex response is likely linked to upregulated P2X receptors in the sensory neurons of thin-fiber afferent nerves in HF (Gao et al., 2007; Wang et al., 2010). In addition, less TRPV1 expression is found in the sensory neurons of HF rats compared with control animals, and the

pressor response of TRPV1 activation is attenuated in HF (Li et al., 2004b; Smith et al., 2005; Wang et al., 2010).

Muscle temperature (T_m) rises in exercising muscles (Shellock et al., 1985; Kenny et al., 2003), and P2X and TRPV1 are sensitive to change of temperature (Garcia-Villalon et al., 1997; Wang et al., 2003; Kluess et al., 2005). Thus, it was necessary to study contraction-induced increase in T_m in order to better understand heightened sympathetic activity in HF, likely due to P2X and TRPV1. Moreover, the published data have demonstrated that blood pressure response to stimulation of P2X receptors on muscle afferents is attenuated with increasing T_m (Gao et al., 2006). This result suggests that higher T_m blunts effects of P2X on the reflex pressor response. Likewise, lower T_m augments effects of P2X. In contrast, higher temperature increases its response as TRPV1 is activated (Caterina et al., 1997). Given that P2X is increased and TRPV1 is decreased after induction of myocardial infraction (MI; Smith et al., 2005; Gao et al., 2007), we hypothesized that contraction-induced increase in T_m is attenuated in HF rats and then abnormal T_m response in HF may affect the muscle pressor reflex via temperature-sensitive P2X and TRPV1 (Garcia-Villalon et al., 1997; Wang et al., 2003; Kluess et al., 2005). Moreover, a relationship between the reflex pressor response and T_m response during contraction was further determined in this report.

MATERIALS AND METHODS

CORONARY ARTERY LIGATION

All procedures outlined in this study were approved by the Animal Care Committee of this institution. Sprague Dawley male rats (150–180 g) were anesthetized by inhalation of isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen), intubated, and artificially ventilated. A left thoracotomy between the fourth and fifth ribs was performed, exposing the left ventricular wall. The left coronary artery was ligated. Experiments were performed 6–10 weeks after coronary ligation. Age- and body weight-matched rats served as controls.

Transthoracic echocardiography was performed 1–2 weeks before the experiments. The rats were anesthetized by inhalation of isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen). The transducer was positioned on the left anterior chest, and left ventricular dimensions were measured. The fractional shortening (FS) was determined by echocardiographic measurements. FS is >40% in controls ($n = 9$) and <30% in HF ($n = 10$), respectively.

EXPERIMENTAL PREPARATION

The rats were anesthetized by inhalation of isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen). An endotracheal tube was inserted into the trachea and attached to a ventilator. Polyethylene catheters (PE-50) were inserted into the common carotid artery and external jugular vein for measurement of arterial blood pressure and for fluid administration, respectively. The skin covering the triceps surae muscle and femoral region was surgically separated from the muscle below in order to eliminate inputs from cutaneous afferents in the hindlimb. The sciatic nerve of each leg was isolated and then placed on a stimulating electrode. A needle microprobe connected to a thermometer (Model BAT-12, World Precision Instrument, Sarasota, FL, USA) was directly inserted into the gastrocnemius muscle of the hindlimb to continuously monitor baseline T_m throughout experiments and measure T_m responses to muscle contraction. The animals were ventilated, and tidal CO_2 was monitored by a respiratory gas monitor (Datex-Ohmeda, Madison, WI, USA) and maintained within normal ranges, as previously described (Li et al., 2004a,b; Gao et al., 2007). Body temperature was maintained between 36.5 and 38.5°C by a heating pad and external heat lamps, and fluid balance was stabilized by a continuous infusion of saline.

Decerebration was performed as previously described (Li et al., 2004a,b; Smith et al., 2005; Gao et al., 2007). A transverse section was made anterior to the superior colliculus. Once this procedure was completed, anesthesia was removed from the inhaled mixture. A recovery period of 60 min after decerebration was employed to allow sufficient time for elimination of the effects of anesthesia gas from the preparation.

Arterial blood pressure was measured by connecting the carotid arterial catheter to a pressure transducer (model P23ID, Statham). Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was determined from the arterial pressure pulse. All measured variables were continuously recorded on an eight-channel chart recorder (model TA 4000, Gould, Valley View, OH, USA) and stored on a PC computer that used the PowerLab system (ADInstruments, Castle Hill, Australia). The triceps surae muscle was isolated and the calcaneal bone of the hindlimb was cut. The Achilles tendon was connected to a force transducer for the measurement of muscle tension during electrically induced muscle contraction. The pelvis was stabilized in a spinal unit and the knee joints were secured by clamping the patellar tendon to a spinal unit.

On completion of each experiment, a 2-Fr microMillar pressure transducer catheter (Millar Instruments) was inserted into the right carotid artery and threaded into the left ventricle for measurement of left ventricular end-diastolic pressure (LVEDP). The heart was exercised after intravenous injection of an overdose of sodium pentobarbital (120 mg/kg body weight) followed by 2 ml of a saturated solution of potassium chloride. Wet heart and triceps surae muscle weight were measured. The data collected from rats whose left ventricle FS was <30% were included in MI group of this report.

EXPERIMENTAL PROTOCOLS

A 60 min equilibration period was allowed after completion of experimental procedure. Contractions induced by electrical stimulation of the sciatic nerve were then performed in nine healthy control rats and 10 MI rats. Static contraction of the triceps surae muscle was conducted at frequencies of 30 Hz (2.5 times motor threshold and 0.1 ms duration; Gao et al., 2006). Stimulation was sustained for 1 min. Baseline T_m was controlled at 30, 34, and 38°C at a random way, respectively, by using a water-perfused heating pad and an ice bag around the hindlimb muscle. Contraction was performed at different baselines of T_m. There was a 60 min rest period between each bout of contraction. The T_m was measured before, during, and after each of stimulations. Body core temperature

and T_m on the contralateral leg were also measured during stimulation.

DATA ACQUISITION AND ANALYSES

Arterial blood pressure and developed muscle tension during muscle contraction were recorded on a PC computer that used Power Lab software. T_m was recorded on a temperature monitor. Control values were determined by averaging at least 1 min of the data immediately before the interventions. The peak change of each variable was determined by the peak response from control.

Peak change data for each variable were analyzed with a two-way ANOVA. Changes in temperature during 1 min-stimulation in control rats and MI rats were also analyzed with a two-way ANOVA. Tukey *post hoc* analyses were utilized to determine differences between groups, as appropriate. All values were expressed mean ± SE. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for windows version 13.0.

RESULTS

Rats with the left ventricular FS < 30% showed increases in heart weight, LVEDP, and left ventricular diastolic dimension (**Table 1**). These rats were used as a MI group. In addition, there were no significant differences in resting T_m and core body temperature as well as body and muscle weight between control and MI groups (**Table 2**).

T_M RESPONSE IN CONTROL AND MI RATS

Following a start of electrical stimulation of the sciatic nerve, T_m increased. The response gradually returned to a steady level after the end of stimulation. At baseline T_m of 30, 34, and 38°C, time courses of contraction-induced increase in T_m during 1 min of contraction are shown in **Figures 1A–C**. Peak temperature responses to contraction in control rats and MI rats are shown in **Figure 1D**. It is noted that an increase in T_m was significantly attenuated in MI rats compared with control rats, as resting T_m was 30 and 34°C. However, this effect wasn't observed, as resting

Table 1 | Echocardiographic and cardiac characteristics.

	LVAW (cm)	LVDD (cm)	LVPW (cm)	LVSD (cm)	FS (%)	Heart weight (g)	LVEDP (mmHg)
Control ($n = 9$)	0.14 ± 0.01	0.89 ± 0.01	0.15 ± 0.01	0.40 ± 0.02	55.32 ± 2.14	1.42 ± 0.02	0.4 ± 0.18
HF ($n = 10$)	0.08 ± 0.00*	1.09 ± 0.02*	0.16 ± 0.01	0.87 ± 0.02*	19.74 ± 1.33*	1.79 ± 0.04*	15 ± 3.00*

*LVAW, the thickness of left ventricular anterior wall; LVDD, left ventricular end-diastolic dimension; LVPW, left ventricular posterior wall; LVSD, left ventricular end-systolic dimension; FS, shortening fraction of LV; LVEDP, LV end-diastolic pressure. Values are mean ± SE. *indicates, P < 0.05, vs. control.*

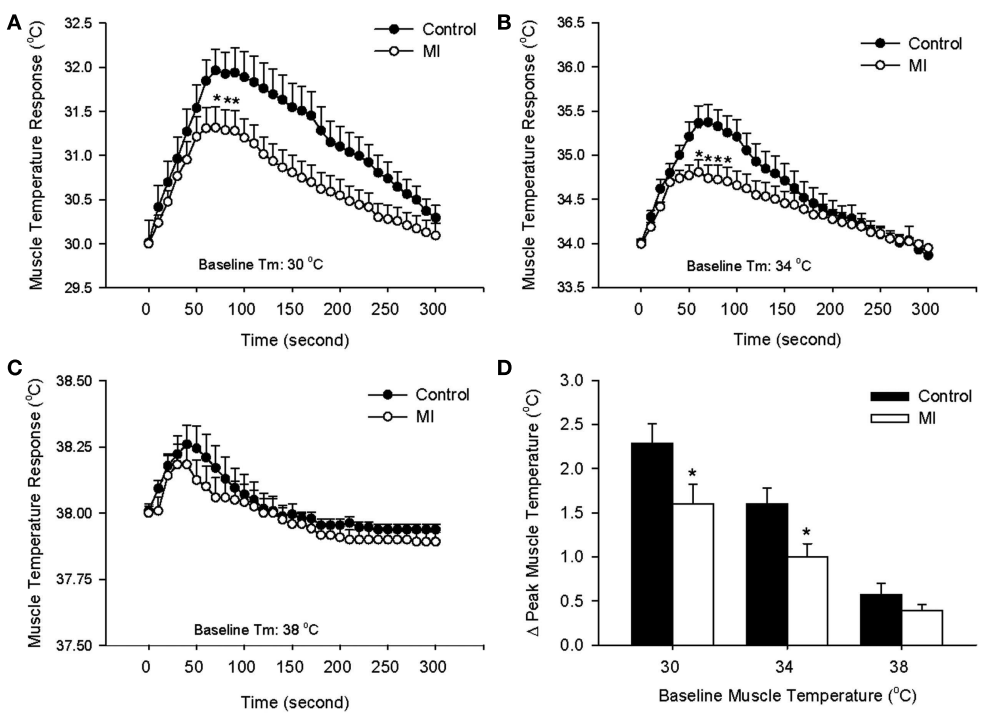

FIGURE 1 | Contraction-induced increase in muscle temperature (T_m) in control rats and MI rats during 1 min electrical stimulation of sciatic nerve and 4 min follow-up at different basal T_m: 30°C (A), 34°C (B), and 38°C (C). The peak temperature changes in the muscle during static contraction at three levels of basal T_m are shown in **(D)**. Values are means ± SE (Control: 9 rats; MI: 10 rats). The increases in T_m during contraction were significantly lower in MI at baseline T_m of 30, 34°C. *$P < 0.05$, compared with healthy control group.

Table 2 | Muscle characteristics.

	Number of rats	Body weight (BW, g)	Muscle weight (MW, g)	MW/BW (mg/g)	Basal body temperature (˚C)	Basal muscle temperature (˚C)
Control	9	535 ± 19.4	3.69 ± 0.081	6.9 ± 0.1	36.83 ± 0.20	34.50 ± 0.16
HF	10	515 ± 9.28	3.67 ± 0.078	7.1 ± 0.1	36.99 ± 0.14	34.43 ± 0.11

Muscle indicates the triceps surae muscle. Values are mean ± SE. There are no differences in all parameters between the two groups.

Table 3 | Baseline MAP (mm Hg) and HR (beats/min), and peak responses.

Baseline T_m	Groups	Baseline MAP	Peak MAP	Baseline HR	Peak HR
30˚C	Control	80 ± 3	99 ± 4*	390 ± 13	410 ± 13
	HF	84 ± 5	119 ± 6*	399 ± 16	423 ± 17
34˚C	Control	94 ± 6	117 ± 7*	390 ± 23	414 ± 25
	HF	93 ± 6	124 ± 7*	418 ± 20	443 ± 20
38˚C	Control	88 ± 7	109 ± 11*	400 ± 16	406 ± 17
	HF	94 ± 6	114 ± 8*	422 ± 20	432 ± 24

*Values are means ± SE. The number of animals = 9 in control; and 10 in HF. MAP, mean arterial pressure; HR, heart rate; T_m, muscle temperature. There are no significant differences among basal values. *$P < 0.05$, versus baseline.*

T_m was 38˚C. Body core temperature and T_m on the contralateral leg were not significantly changed during stimulation.

CARDIOVASCULAR RESPONSES IN CONTROL AND MI RATS

Baseline MAP values before contraction were not different in the healthy control animals and the MI rats (**Table 3**). Electrical stimulation of the sciatic nerve significantly increased MAP in both groups. A greater MAP increase was observed in MI rats, as baseline T_m was 30 and 34˚C but not 38˚C (**Figure 2A**). There was no significant difference in HR response to the stimulation (**Table 3**).

It is noted that there were no significant differences in the muscle tension indicated by time-tension index in the two groups (**Figure 2B**). This suggests that attenuated T_m and enhanced pressor responses to contraction in the MI rats were not due to development of muscle tension. In addition, absolute muscle tensions appeared to be smaller in MI rats; however, no significant differences were observed in both groups. When baseline T_m was 30, 34, and 38˚C, peak tensions were 0.55 ± 0.04, 0.57 ± 0.06, and 0.46 ± 0.04 kg in control; and 0.52 ± 0.03, 0.51 ± 0.02, and 0.44 ± 0.03 kg in MI ($P > 0.05$, vs. control at all conditions).

A RELATIONSHIP BETWEEN PRESSOR AND T_M RESPONSE

Changes in MAP response to static muscle contraction were plotted against contraction-induced increase in T_m. A significant inverse correlation was seen between the pressor response and increased T_m during contraction (**Figure 3**). Thus, the greater pressor response is likely linked to the lower T_m response in rats with MI.

DISCUSSION

Our data demonstrate that contraction-induced increase in T_m is attenuated in MI rats compared with control rats. A regression analysis further shows that the lower T_m response is closely related to the greater pressor response during contraction. Notably, there are no differences in the muscle tension development and muscle mass in the control rats and rats with HF. This suggests that altered T_m and pressor responses in HF are unlikely due to muscle tension and/or muscle mass. Additionally, body core temperature is unchanged during contraction, indicating that body temperature is unlikely to affect the T_m and pressor responses. Thus,

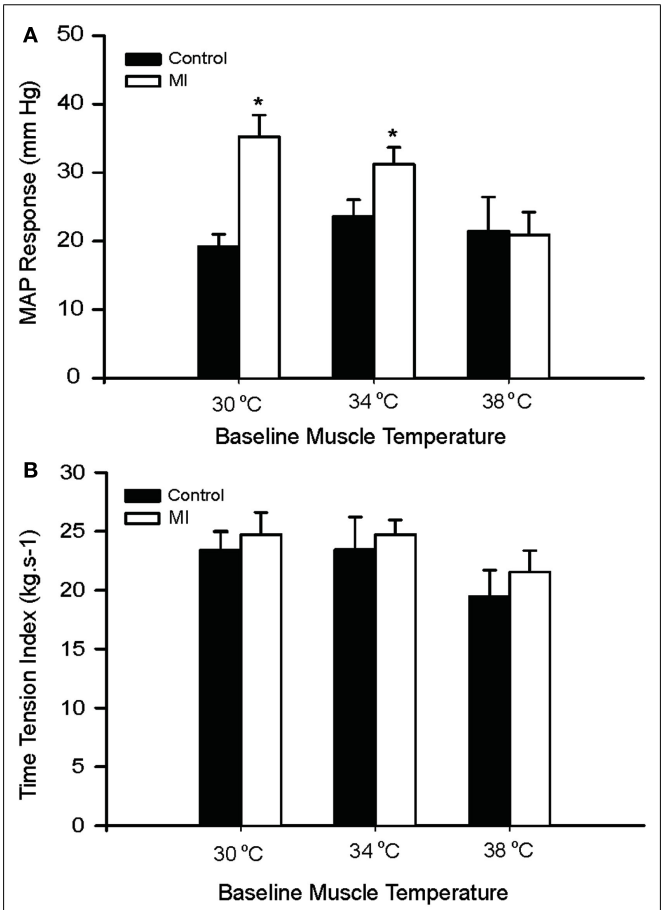

FIGURE 2 | Peak pressor response and developed muscle tension evoked by sciatic nerve stimulation at three different basal T_m: 30, 34, and 38˚C in control and MI rats. (A) MAP responses were significantly augmented during static muscle contraction in MI group at baseline T_m of 30 and 34˚C. **(B)** Developed tensions are indicated by time-tension index (TTI), and the TTI induced by static contraction were similar in two groups. *$P < 0.05$, compared with healthy control group. The number of rats = 9 in control; and 10 in MI.

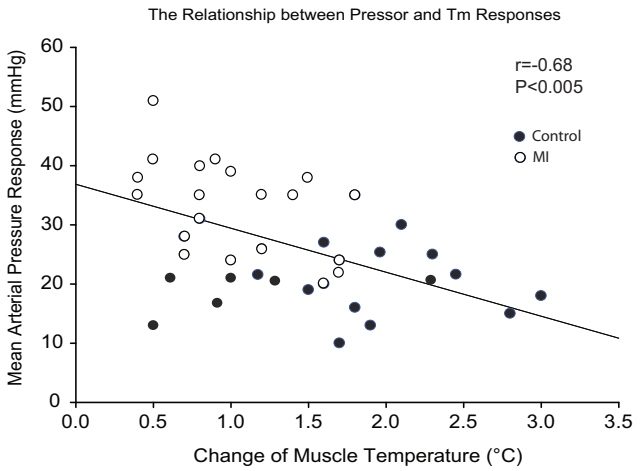

FIGURE 3 | Regression analyses between MAP response and muscle temperature change during static contraction at baseline T_m of 30 and 34°C. Note that a higher MAP response was seen as a lower T_m was induced by muscle contraction.

findings of this study suggest that impaired T_m response is likely responsible for the exaggerated muscle pressor reflex in HF.

EFFECTS OF SENSORY NERVES' P2X AND TRPV1 ON MUSCLE PRESSOR REFLEX

Evidence supports that ATP sensitive P2X receptors play a role in evoking mechano- and metaboreceptors mediated stimulation of the exercise pressor reflex (Hanna et al., 2002; Li and Sinoway, 2002; Hanna and Kaufman, 2003, 2004). In HF, P2X plays a role in sympathetic abnormalities by demonstrating that the muscle pressor response induced by α, β-me ATP injected into rat hindlimb muscles is exaggerated in HF animals (Gao et al., 2007). Also, P2X receptor-mediated muscle mechanoreceptor contribution to the sympathetic nerve response to exercise is augmented in HF (Li et al., 2004b; Wang et al., 2010). Moreover, the greater response to activation of PX receptor is related to the severity of left ventricular dysfunction (Gao et al., 2007). This may be due to that HF induces upregulation of P2X receptors in the sensory neurons (Gao et al., 2007). However, the intrinsic mechanism responsible for the P2X receptor alternations in rats with HF is unclear.

It has been reported that P2X receptors are temperature-sensitive and P2X activity increases as temperature falls (Garcia-Villalon et al., 1997; Ziganshin et al., 2002; Kluess et al., 2005). The effect of P2X receptor on reflex muscle response has recently been reported to be sensitive to alternations of T_m (Gao et al., 2006). This prior study further suggests that elevated T_m attenuates the pressor response to static muscle contraction.

Result of the present study shows that T_m and pressor responses during contraction tended to be smaller in both healthy and MI rats as baseline T_m was set at 38°C. Moreover, the greater pressor response was linearly related to the lower T_m response. This is consistent with the concept that the effect of P2X receptor on reflex muscle response is sensitive to alternations of T_m and that elevated temperature attenuates the response (Gao et al., 2006). In addition, our data also show that an increase in T_m during contraction was attenuated in HF rats compared with control rats. Thus, the enhanced P2X activity and exaggerated muscle reflex in HF is likely attributed to a lower T_m response.

Metabolite sensitive-TRPV1 receptors have been reported to respond to heating (Wang et al., 2003). In this report, a rise in T_m in contracting muscle was smaller in the MI rats than in the controls. Thus, we postulate that the muscle metaboreflex is blunted by less stimulation of TRPV1 due to lower temperature response in HF. A lower T_m in HF is likely to contribute to the attenuated TRPV1 activities. It has previously been reported that TRPV1 of sensory neurons is downregulated and TRPV1 response is attenuated in HF rats (Li et al., 2004b; Smith et al., 2005; Wang et al., 2010). Data of the present report provide further evidence that suggest that abnormal autonomic adjustments to exercise may be due to abnormalities in T_m response in HF. We believe that in HF mechanoreceptor contribution to muscle sympathetic nerve activity is augmented, whereas metaboreceptor engagement is attenuated. Overall, mechanosensitive afferents may contribute to a greater degree and muscle sympathetic response is exaggerated in HF (Sterns et al., 1991; Middlekauff et al., 2000, 2001; Li et al., 2004b; Momen et al., 2004; Smith et al., 2005).

MUSCLE TEMPERATURE AND MUSCLE PRESSOR REFLEX

It is known that T_m rises with exercise (Shellock et al., 1985; Kenny et al., 2003). During exercise, T_m observed in HF patients is abnormal as compared with healthy subjects (Shellock et al., 1985). Cold temperature increases cardiac demand in response to exercise and significantly reduces maximal exercise capacity in patients with HF (Juneau et al., 2002). A further study suggests that warming of exercising legs improves exercise capacity in patients with cardiac disease and low exercise tolerance (Yamanouchi et al., 1996).

An elevation of T_m by heating muscles increases reflex blood pressure and sympathetic nerve responses to exercise and cooling muscle delays those reflex activities (Ray and Gracey, 1997; Ray et al., 1997). It is noted that T_m is increased >4°C and decreased >7°C. When T_m is altered largely, this could directly change sympathetic nervous activity. Also, afferent inputs from skin could be activated in these human studies. This may lead to different patterns of sympathetic responses than pure muscle afferent activation. In contrast, in our current report the skin covering the triceps surae muscle and femoral region was surgically separated from the muscle below, thus we have eliminated inputs from cutaneous afferents in the limb.

It should be noted that in the present report the effects of muscle blood flow on changes of T_m are not determined as muscle contraction is evoked at different temperature conditions. However, previous studies have demonstrated that blood flow directed to exercising muscles is decreased in HF (Lejemtel et al., 1986; Shoemaker et al., 1999), suggesting that blood flow is likely to contribute to attenuated T_m increase in HF as contraction is induced.

Finally, static contraction was induced by the sciatic nerve stimulation in the current study to optimize T_m and pressor responses. A study limitation is that the pressor response induced by the stimulation was possibly due, in part, to direct electrical activation of afferent nerves. However, contraction-induced increase in T_m was unlikely due to the direct stimulation of afferent nerves

because core temperature and T_m on the contralateral leg were not altered. Also, the pressor response was closely related to change of T_m during contraction. This is important to address the issue that impaired T_m response can modify the muscle reflex in HF.

In summary, the data of present study demonstrate that contraction-induced increase in T_m is attenuated in HF animals compared with control animals, and the magnitude of the pressor response to contraction is greater as the temperature increase is lower. The impaired temperature response may alter muscle afferent-mediated pressor response via temperature-sensitive P2X and TRPV1 receptors. This investigation provides evidence for the role played by T_m in the exaggerated sympathetic responses to exercise in HF.

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Muscle afferent receptors engaged in augmented sympathetic responsiveness in peripheral artery disease

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The exercise pressor reflex (EPR) is a neural control mechanism responsible for the cardiovascular responses to exercise. As exercise is initiated, thin fiber muscle afferent nerves are activated by mechanical and metabolic stimuli arising in the contracting muscles. This leads to reflex increases in arterial blood pressure (BP) and heart rate primarily through activation of sympathetic nerve activity (SNA). Studies of humans and animals have indicated that the EPR is exaggerated in a number of cardiovascular diseases. For the last several years, studies have specifically employed a rodent model to examine the mechanisms at receptor and cellular levels by which responses of SNA and BP to static exercise are heightened in peripheral artery disease (PAD), one of the most common cardiovascular disorders. A rat model of this disease has well been established. Specifically, femoral artery occlusion is used to study intermittent claudication that is observed in human PAD. The receptors on thin fiber muscle afferents that are engaged in this disease include transient receptor potential vanilloid type 1 (TRPV1), purinergic P2X, and acid sensing ion channel (ASIC). The role played by nerve growth factor in regulating those sensory receptors in the processing of amplified EPR was also investigated. The purpose of this review is to focus on a theme namely that PAD accentuates autonomic reflex responses to exercise and further address regulatory mechanisms leading to abnormal sympathetic responsiveness. This review will present some of recent results in regard with several receptors in muscle sensory neurons in contribution to augmented autonomic reflex responses in PAD. Review of the findings from recent studies would lead to a better understanding in integrated processing of sympathetic nervous system in PAD.

Keywords: static exercise, muscle afferents, sympathetic nerve activity, PAD, ASIC, P2X, TRPV1, NGF

INTRODUCTION

During exercise, sympathetic nervous activity (SNA) increases and this leads to rises in blood pressure (BP) and heart rate (HR), myocardial contractility, and peripheral vasoconstriction (Victor et al., 1988; Sinoway et al., 1989). A basic mechanism termed the "Exercise Pressor Reflex" (EPR; Coote et al., 1971; McCloskey and Mitchell, 1972; Mitchell et al., 1977, 1983) is thought to contribute to sympathetic engagement during exercise (**Figure 1**). This autonomic reflex is initiated as thin fiber afferents arising from contracting skeletal muscle are engaged (McCloskey and Mitchell, 1972; Mitchell et al., 1983; Kaufman and Forster, 1996a). This system responds to mechanical deformation of the muscle afferents receptive field as well as to muscle by-products (Kaufman and Forster, 1996a). Group III afferents are predominantly mechanically sensitive (mechanoreceptor) and Group IV afferents are predominantly metabosensitive (metaboreceptor; Kaufman et al., 1984). When these receptors are stimulated, thin fiber muscle afferent nerves are engaged, cardiovascular nuclei in the brainstem are activated, SNA increases, and BP and HR rise (Mitchell et al., 1983). In addition, the sympathetic and cardiovascular responses to exercise are modulated by the "Central Command" (Goodwin et al., 1972; Waldrop et al., 1996), and the arterial baroreflex (Potts and Li, 1998; Fadel et al., 2001).

These reflex mechanisms found in healthy individuals are altered in cardiovascular diseases in the processing of muscle afferent signals via afferent nerves' receptors (Li et al., 2004b; Sinoway and Li, 2005; Smith et al., 2006; Gao et al., 2007; Xing et al., 2008a; Liu et al., 2010, 2011; Tsuchimochi et al., 2010a). For example, as the EPR is activated in patients with peripheral arterial disease (PAD), increases in SNA, BP, and HR are exaggerated (Baccelli et al., 1999; Bakke et al., 2007). As noted, PAD caused by a restriction of the blood vessels in the lower limbs is typically popular in the older adults (Ouriel, 2001; Critchley and Capewell, 2003; Muir, 2009). The most common symptom of this disease is intermittent claudication, which frequently occurs during physical activity but is relieved promptly by rest (Rejeski et al., 2008). Thus, a rat model of femoral artery ligation that displays impaired reserve capacity of limb blood flow with exercise, has been employed to study PAD in humans (Waters et al., 2004). Using this rat model, a number of prior studies have further demonstrated that the SNA and pressor responses to static muscle contraction and stimulation of muscle metabolite receptors, i.e., capsaicin sensitive transient receptor potential vanilloid type 1 (TRPV1), purinergic P2X, and acid sensing ion channels (ASICs) are amplified in occluded rats as compared with control rats (Xing et al., 2008a; Liu et al., 2010, 2011; Tsuchimochi et al., 2010a; **Figure 1**). Nevertheless,

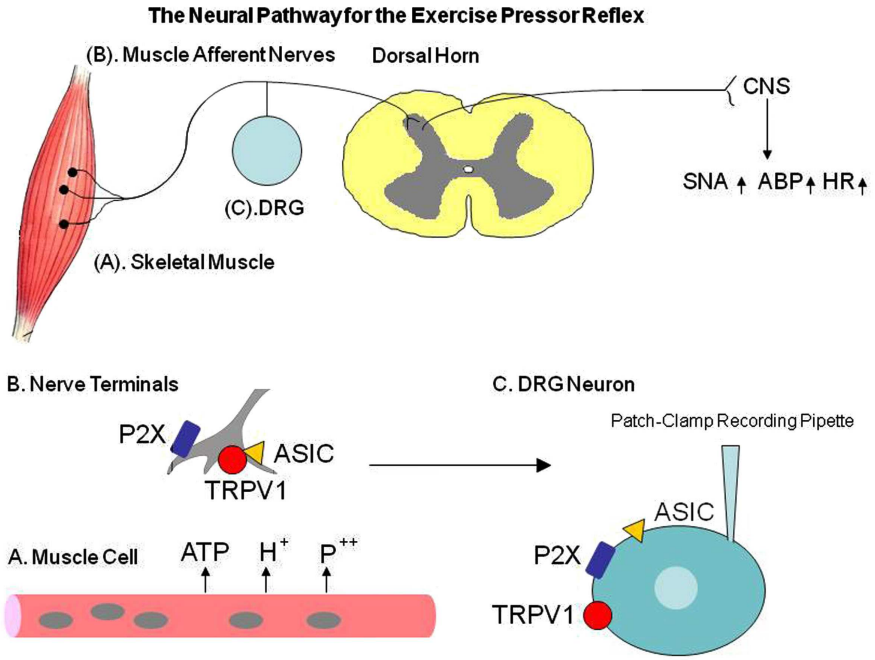

FIGURE 1 | The diagram describes the neural pathway for the exercise pressor reflex. Interstitial ATP, H^+, and phosphate are increased in exercising muscle. P2X, TRPV1, and ASIC mediate the muscle metabolite-induced reflex responses. In peripheral artery disease (PAD), those receptors in dorsal root ganglion (DRG) are altered, which likely leads to abnormal cardiovascular responses during exercise. Note that the whole cell patch clamp methods are used to characterize receptor responsiveness in the primary afferent neuron of control rats and PAD rats. Those receptors appear at the peripheral terminals and cell body of the DRG (sensory afferent neurons). The receptor activity in the DRG cell body can be used to reflect its effect in the nerve endings. TRPV1, transient receptor potential vanilloid type 1; ASIC, acid sensing ion channel; SNA, sympathetic nerve activity; ABP, arterial blood pressure; HR, heart rate.

the underlying mechanisms by which femoral occlusion augments responsiveness of SNA and BP to activation of muscle mechano- and metabo-sensitive afferents remain to be determined.

Prior studies demonstrated that femoral artery occlusion elevates the levels of nerve growth factor (NGF) in the hindlimb muscles and dorsal root ganglion (DRG) neurons of rats (Emanueli et al., 2002; Xing et al., 2009). NGF can induce expression of TRPV1, P2X3, and ASIC3 receptors in the DRG neurons (Ramer et al., 2001; Mamet et al., 2003). Therefore, NGF was infused into the hindlimb muscles using the osmotic minipump, and the role for NGF in regulating expression and response TRPV1, P2X3, and ASIC3 receptors was examined. In addition, NGF can change the neuronal phenotype such as capsaicin-insensitive sensory neurons (Hunter et al., 2000), which possibly alters afferent mediated response in the processing of sensory signals. Accordingly, the dual immunofluorescence techniques were employed to examine if femoral ligation and NGF can alter distribution of DRG neurons with the two thin fiber phenotypes: C-fiber and A-fiber. Also, whether femoral occlusion and NGF can selectively increase expression of ASIC3 in DRG neurons that project C-fiber/A-fiber afferents was examined. Moreover, NGF-antibody (NGF-Ab) was previously administered into the hindlimb muscles of occluded rats to neutralize effects of NGF, and then SNA and BP responses to static muscle contraction and passive tendon stretch were examined. Muscle contraction was performed to evoke both mechano- and metabo-components of the EPR, and muscle stretch was employed to activate muscle mechanoreceptor. Also, to examine effects of NGF on the reflex responses to activation of muscle metaboreceptors, lactic acid was injected into the arterial blood supply of the hindlimb muscles after infusion of NGF-Ab in the hindlimb muscles of occluded rats.

The general hypotheses were that (1) protein expression of TRPV1, P2X3, and ASIC3 receptors in DRG and their responses with stimulation of those receptors are increased after femoral artery ligation and (2) NGF contributes to augmented reflex SNA and BP responses evoked by stimulation of metabolically sensitive muscle afferent nerves via enhancement of metabolic receptors expression such as ASIC3 in thin C-fiber afferent neurons.

ALTERED METABOLIC RECEPTORS ON MUSCLE SENSORY NERVES AFTER FEMORAL ARTERY OCCLUSION

A number of studies have been performed to examine how the muscle reflex mediated-SNA is engaged via sensory receptors mechanism. This review will focus on the roles of TRPV1, P2X, and ASICs receptors on muscle sensory nerves in mediating the exaggerated sympathetic response in hindlimb muscle ischemia seen in PAD patients. Note that those receptors to be studied appear at the peripheral terminals and the cell body of the sensory afferent neurons-DRG (**Figure 1**). Receptor activity of DRG cell bodies has been used frequently as a surrogate nerve ending receptor activity and physiology (Tsuzuki et al., 2003; Puntambekar et al., 2004). Especially, the whole cell patch clamp methods (**Figure 1**) are used

to characterize the precise mechanisms by which those receptors mediate responses (Tsuzuki et al., 2003; Puntambekar et al., 2004).

TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1

It is known that TRPV1 receptor appears preferentially on metabolite sensitive Group III and IV sensory neurons (Ma, 2001). These receptors are located on afferents in a variety of tissues and mediate the effect of the vanilloid compound capsaicin (Caterina et al., 1997). When capsaicin is injected into the pulmonary circulation it activates C-fibers and evokes a pulmonary chemoreflex (Coleridge et al., 1989). The epicardial application of capsaicin stimulates cardiac TRPV1 receptors evoking a sympathoexcitatory reflex (Zahner et al., 2003). The competitive capsaicin antagonist capsazepine can reduce capsaicin-induced activation of the cloned non-selective cation channel TRPV1 (Caterina et al., 1997). Capsazepine also abolishes capsaicin-induced C-fiber activity both *in vitro* and *in vivo* (Fox et al., 1995; Lee et al., 1996). Although the endogenous TRPV1 ligand has not been determined, both the metabolic by-products accompanying the inflammatory process (lactic acid, H^+) and inflammatory mediators themselves (histamine, serotonin, prostaglandin E2) have been identified as potential endogenous ligands for the C-fiber "capsaicin" receptor. Hydrogen ions (H^+) in general and lactic acid in particular have been shown to activate C-fiber afferents similar to the effect seen with capsaicin (Stahl and Longhurst, 1992; Bevan and Geppetti, 1994; Hong et al., 1997). *In vitro* studies have demonstrated that H^+ inhibits the binding of the capsaicin analog resiniferatoxin (RTX) to vanilloid receptors, a finding that was attributed to competition for the same binding site (Szallasi et al., 1995).

Activation of thin fiber muscle afferent nerves causes increases in BP and HR via a reflex muscle response (Kaufman et al., 1983, 1984; Kaufman and Forster, 1996b). When capsaicin is injected into the arterial supply of the dog hindlimb, BP rises by 20%, an effect is abolished by sectioning afferent nerves (Crayton et al., 1981). The muscle pressor response is likely to be due to the stimulation of both Group III and IV fibers since capsaicin stimulates 71% of Group IV and 26% of Group III dog hindlimb muscle afferent fibers (Kaufman et al., 1982). In a prior study, it has been observed that when capsaicin injected into the arterial supply of the hindlimb muscles of rats, BP increases and the effect is mediated via the TRPV1 receptors engagements on sensory afferents (Li et al., 2004a).

Consistent with those previous findings (Li et al., 2004a,b; Gao et al., 2006), it has been reported that TRPV1 can mediate SNA and pressor responses via a reflex mechanism (Xing et al., 2008a), and the responses are exaggerated by the femoral artery occlusion, suggesting that ischemia sensitizes TRPV1 receptors (Xing et al., 2008a). In addition, evidence from this prior study shows that: (1) arterial occlusion leads to upregulation of TRPV1 expression in the DRG neurons; and (2) the magnitude of capsaicin-evoked currents of the DRG neuron is greater in rats with the arterial occlusion. Thus, it is well reasoned that alterations in TRPV1 can contribute to enhanced sympathetically mediated vasoconstriction leading to reduced muscle blood flow in PAD.

It should be noted that a prior study in the cat model indicates that blockade of TRPV1 does not attenuate the EPR (Kindig et al., 2005). In a rat model of femoral artery ligation, augmented BP response to static contraction of the hindlimb muscles was not seen to be significantly attenuated after blocking TRPV1 (Tsuchimochi et al., 2010a). It is speculated that TRPV1-induced reflex responses require H^+ (lower pH) in the muscle interstitium. It has been reported that TRPV1 and ASIC play a coordinated role in the processing of muscle sensory signals (Gao et al., 2006). Likewise, ASIC responds to the accumulation of muscle metabolites (such as lactic acid/lowered pH, ATP, and inorganic phosphates) that are liberated by exercising muscles (Light et al., 2008). In situations without acidosis, the TRPV1 may not be effectively active. This hypothesis is supported by another work showing that receptors mediating protons and capsaicin responses coexist in the DRG neurons innervating muscle (Xing et al., 2008b). The responsiveness of acidosis and capsaicin is sensitized by each other, and amplitudes of inward currents responsive to protons and capsaicin are greater in the neurons innervating muscle comprised predominately of glycolytic fibers than that in those innervating muscle comprised predominately of oxidative fibers (**Figure 2**). This study also provides evidence at a cellular level that responsiveness of sensory neurons with nerve endings in different fiber types respond differently to a given level of metabolic stimulation. Note that the effect of fiber type composition on the cardiovascular responses evoked by static muscle contraction was studied previously (Wilson et al., 1995). Data of this study suggest that the reflex responses evoked by static contraction of oxidative muscle (red portion) are less, compared with the changes elicited by contraction of glycolytic muscle (white portion).

P2X RECEPTORS

It has been reported that ATP and analogs of ATP stimulate and excite sensory afferent nerves *via* P2X purinoceptors on sensory nerves (Burnstock and Wood, 1996; Burnstock, 1999). Specifically, it has been shown that increased ATP in the hindlimb muscles elevates BP (Hanna et al., 2002; Li and Sinoway, 2002). In these studies, stimulation of ATP-sensitive P2X receptors in the hindlimb muscle increased BP. It has been confirmed that Group III and IV afferents are responsible for the increase in BP after arterial infusions of α, β-me ATP (Hanna and Kaufman, 2004). Also, it has been demonstrated that ATP enhances cardiovascular responses induced by stimulation of muscle mechanoreceptors *via* P2X receptors (Li and Sinoway, 2002).

Data have been published demonstrating that interstitial ATP levels are elevated in active muscle of human subjects, dogs, and cats (Hellsten et al., 1998; Mo and Ballard, 2001; Li et al., 2003). It is anticipated that ischemic insult of the hindlimb muscles is likely to accumulate ATP to a larger degree, and thereby greater ATP levels can upregulate P2X receptors on thin fiber afferent nerves (Xu and Huang, 2002) and augment the P2X mediated-SNA response. On the basis of these data, it was hypothesized that femoral artery occlusion increases P2X3 receptors in DRG neurons which thereby leads to the enhanced reflex responses to stimulation of P2X3. Western blotting and immunohistochemistry were employed to examine P2X3 in DRG neurons of control rats and those with femoral artery occlusion. In order to determine P2X responsiveness, sympathetic, and cardiovascular responses to injections of α, β-me ATP into the arterial blood supply of the hindlimb muscles were further examined in both groups.

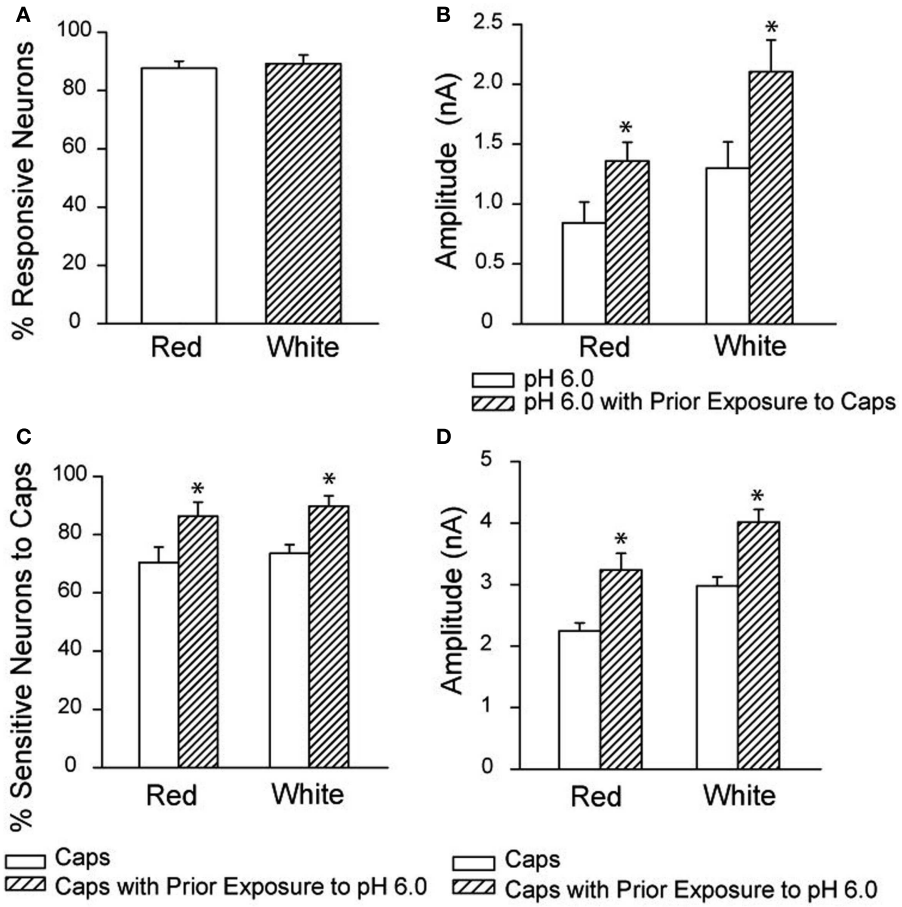

FIGURE 2 | Overlapping response to low pH and capsaicin in DRG neurons. (A) The neurons that responded to pH 6.0 with a sustained inward current also responded to capsaicin. The overlap was similar for DRG neurons innervating the red and white muscle. **(B)** Prior exposure to capsaicin increased the amplitude of the proton-evoked currents in DRG neurons innervating the red and white muscles. $*P < 0.05$ compared with pH 6.0 without exposure to capsaicin. **(C,D)** Show that prior exposure to protons increased the proportion of DRG neurons responding to capsaicin, and increased the amplitude of the capsaicin-induced currents in the neurons. $*P < 0.05$ compared with capsaicin without exposure to pH 6.0. Reprinted from Xing et al. (2008b).

Results of this study demonstrated that 24 and 72 h of femoral artery occlusion significantly elevated the protein levels of P2X3 in lumbar DRGs (Liu et al., 2011). Twenty hours following the ligation surgery, the level of P2X3 was 1.47-fold greater in occluded rats than in control animals. Fluorescence immunohistochemistry further confirmed that femoral occlusion increased P2X3 immunoreactivity in small- to medium-diameter of DRG neurons. These results are summarized in **Figure 3**. In addition, injection of α, β-me ATP into the arterial blood supply of the hindlimb muscles evoked greater increases in RSNA and MAP in occluded rats than in control rats (Liu et al., 2011). The findings of this study suggest that there is a close linkage in increased P2X3 receptors on afferent nerves and augmented sympathetic responsiveness to stimulation of muscle afferent nerves under conditions of femoral occlusion.

Notably, recent studies suggest that reactive oxidative stress (ROS) contributes to regulation of discharges of vagal lung thin afferent fiber nerves (Ruan et al., 2005, 2006). Also, it has been reported that an increase in muscle NADPH oxidase-derived ROS sensitizes the EPR in a decerebrate rat model (Wang et al., 2009). Likewise, a decrease in ROS can attenuate the reflex (Wang et al., 2009). Thus, it is speculated that ROS is engaged in augmented SNA and BP response during activation of the EPR in rats with femoral occlusion. Superoxide dismutases (SOD), a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide as considered an important antioxidant. In a published work, tempol, a mimic of SOD, was arterially injected into the hindlimb muscles of rats and results demonstrated that tempol attenuates BP response evoked by contraction of occluded hindlimb muscles, but the attenuation was not seen when contraction was induced in freely perfused control legs (McCord et al., 2011). A following study suggests that effects of tempol on the BP response during contraction are via ATP dependent potassium channels. However, a prior study suggests that ROS plays an important role in regulating discharges of vagal lung thin afferent fiber nerves via engagement of TRPV1 and P2X receptors (Ruan et al.,

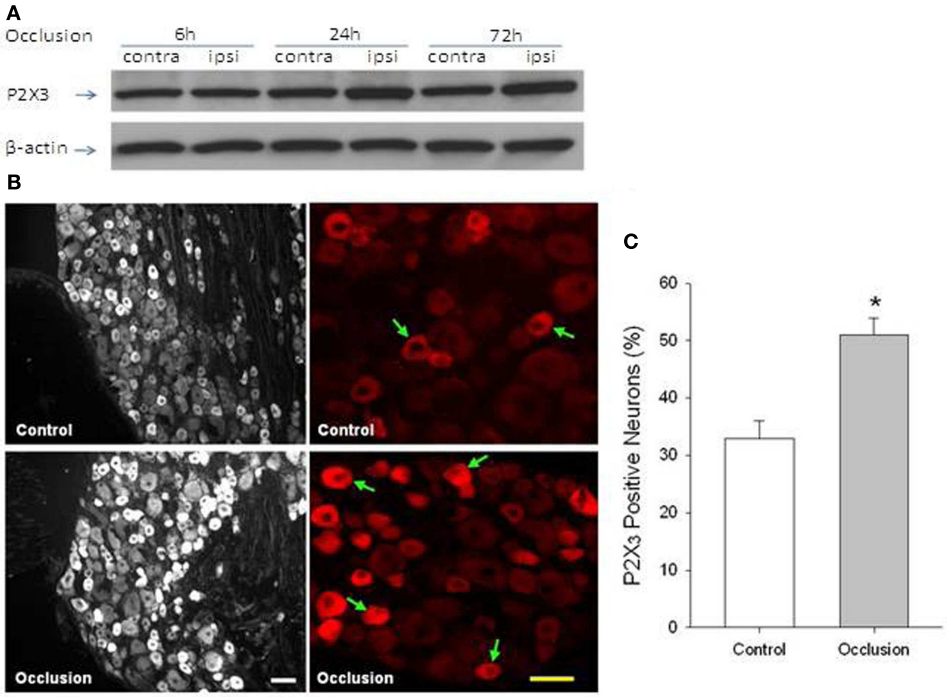

FIGURE 3 | (A) Western blot assay was employed to examine P2X3 proteins in L4–L6 DRG at time of 6, 24, and 72 h following femoral artery occlusion. Representative bands of P2X3 expression show effects of femoral artery occlusion on P2X3 expression in dorsal root ganglion (DRG) neurons at different time courses. The ligation was performed on the one hindlimb [ipsilateral (ipsi)]. The sham-operated procedure was performed on the contralateral limb [contralateral (contra)] of the same rats, and this served as control. Bands of β-actin are used as control for an equal protein loading. **(B,C)** Fluorescence immunohistochemistry was employed to examine expression of P2X3 in DRG neurons of control limb and occluded limb. Representative photomicrographs and average data show that P2X3 appears within lumbar DRG neurons and that percentage of P2X3 positive neurons is greater in DRG neurons of 24 h of femoral artery occlusion ($n = 6$) than that in control ($n = 6$). Arrows indicate P2X3 positive cells. Scale bar = 50 μM. $^*P < 0.05$, compared with control. Reprinted from Liu et al. (2011).

2005, 2006). In those experiments, the reflex pulmonary chemical response induced by a ROS stimulant hydrogen peroxide is attenuated by the prior application of i-RTX (TRPV1 antagonist) and PPADS (P2X antagonist; Ruan et al., 2005, 2006). Thus, it is likely that ROS can alter response of sensory nerves with activation of TRPV1 and P2X. Nevertheless, the augmented EPR is significantly attenuated after tempol is given to compensate SOD in occluded muscles of rats (McCord et al., 2011). If the levels of SOD in the hindlimb muscles are altered after femoral artery occlusion may be necessary to be examined given that anti-oxidation is likely to be beneficial to the augmented cardiovascular responses during exercise after femoral occlusion.

ACID SENSING ION CHANNEL

Lactic acid infused into the arterial supply of the hindlimb increases BP (Rotto et al., 1989; Sinoway et al., 1993; MacLean et al., 2000; Li et al., 2004a). A prior report demonstrates that H^+ evokes reflex muscle responses via the stimulation of ASIC but not TRPV1 (Li et al., 2004a). Specifically, H^+ evokes a pressor response that is not blocked by capsazepine but is attenuated by amiloride, an ASIC blocker. Of note, with pretreatment of RTX to destroy muscle afferents containing TRPV1 receptors, both capsaicin and H^+ responses are blunted (Li et al., 2004a). This suggests that ASIC are likely to be frequently found on afferents containing TRPV1 receptors. Another report suggests that TRPV1 and ASIC play a coordinated and interactive role in the processing of muscle afferent response to acid phosphate (Gao et al., 2006). In this report, it has been observed that simultaneous attenuation of TRPV1 and ASIC blunts acid phosphate-induced pressor response to a larger degree than when the respective blockers are given separately (Gao et al., 2006).

Furthermore, a study has used a rat model of femoral artery ligation to demonstrate that the cardiovascular responses to static contraction are amplified in occluded rats compared with control rats (Liu et al., 2010). A recent study has further shown that arterial injection of a specific ASIC3 blocker markedly attenuates the reflex pressor response to muscle contraction in the rats with a ligated femoral artery, but has only modest effects in the rats with freely perfused hindlimbs (Tsuchimochi et al., 2011). Notably, ASIC3 expression is upregulated in DRG neurons innervating the hindlimb muscles with the occluded femoral artery (Liu et al., 2010). Additionally, injecting lactic acid into the arterial blood supply of hindlimb muscles to stimulate ASIC3 of muscle afferent nerves increases SNA and BP to a greater degree in occluded rats (Liu et al., 2010).

Among ASICs, ASIC3 is found predominantly on sensory neurons and maintains functional channels in response to proton concentration fluctuation (Waldmann et al., 1997a,b, 1999; Light

et al., 2008). The pH range required to activate ASIC3 is ∼6.5–7.0 (Deval et al., 2008, 2011), which is close to what is observed in exercising muscle and/or moderately ischemic tissues (Rotto et al., 1989; MacLean et al., 1998, 2000; Yagi et al., 2006). Thus, in a prior study whole cell patch clamp methods were employed and acid-induced current with ASIC3 activation in DRG neurons of control rats and rats with 24 h of femoral artery occlusion were characterized to examine the mechanisms by which ASIC3 is engaged in femoral occlusion-augmented responses (Xing et al., 2012). The data of this study indicate that in DRG neurons with nerve endings in the hindlimb muscles, ASIC3-containing channels represent the majority of acid-induced currents elicited by moderate external acidosis in a range that is relevant to exercising muscle and/or hindlimb ischemia. Additionally, a greater current response with activation of ASIC3 is observed as the arterial blood supply to the hindlimb is deficient under ischemic conditions (**Figure 4**). Note that the size of DRG neurons that have ASIC3-like currents is typically small to large, and the size distribution is similar in control and occluded animals. Also, the percentage of DRG neurons with pH 6.7-triggered action potential is greater in occluded rats than in control rats, suggesting that femoral occlusion increases the probability of sensory neurons to evoke neuronal activities (Xing et al., 2012). The results of immunohistochemical experiments further suggest that ASIC3 appears in both C- and A-fibers of DRG neurons, and that femoral artery occlusion largely increases expression of ASIC3 in DRG neurons that project C-fiber afferents (Xing et al., 2012).

OTHER RECEPTORS

In addition to TRPV1, P2X3, and ASIC3, it needs to point out that other muscle afferents' receptors including μ-opioid and thromboxane (TP) receptors are engaged in processing chronic ischemia

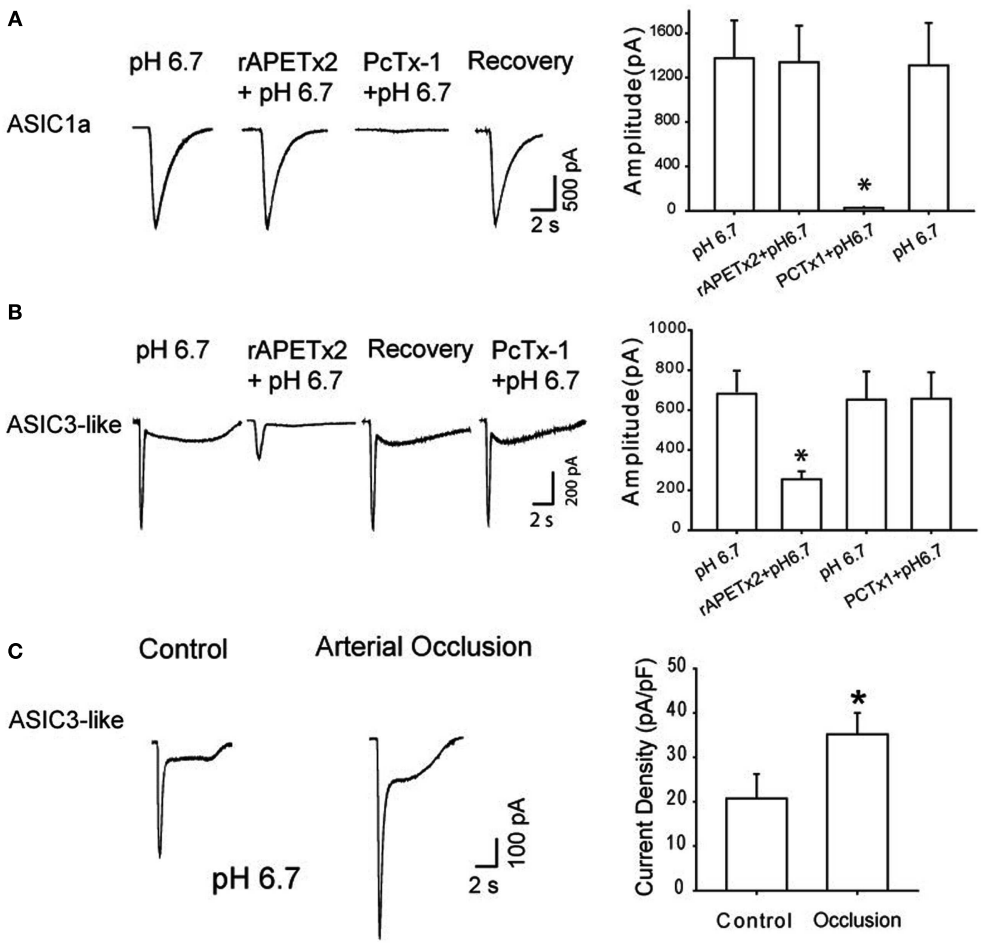

FIGURE 4 | (A,B) Toxins PcTx1 and rAPETx2 were used as antagonists to specifically block ASIC1a and to ASIC3-like currents evoked by pH 6.7 in DRG neurons innervating the hindlimb muscles. Toxin PcTx1 (20 nM) significantly inhibited current responses induced by pH 6.7 in DRG neurons exhibiting ASIC1a currents. However, rAPETx2 had no significant effects on this type of current in DRG neurons. Similarly, a prior application of rAPETx2 (1 μM) significantly attenuated peak amplitudes of currents evoked by pH 6.7 in DRG neurons that displayed ASIC3-like currents. PcTx1 had negligible effects on this type of currents. $*P < 0.05$ vs. pH 6.7 alone. Note that the inhibitory effects of PcTx1 on ASIC1a currents and rAPETx2 on ASIC3-like currents were both reversible. **(C)** Original traces and averaged data show effects of femoral arterial occlusion on ASIC3-like currents response to pH 6.7. Current density was used to analyze response of ASIC3-like currents. Twenty-four hours of arterial occlusion induced a larger current density compared with control. $*P < 0.05$ vs. control. Reprinted from Xing et al. (2012).

of the hindlimb muscles (Tsuchimochi et al., 2010b; Leal et al., 2011). Stimulating peripheral μ-opioid receptors using DAMGO significantly attenuates the pressor responses to static contraction in rats with 72 h of femoral artery ligation compared with freely perfused rats (Tsuchimochi et al., 2010b). Also, this study demonstrates that the inhibitory effect of DAMGO can be prevented by the injection of naloxone, an opioid blocker. In another published work, arterial injection of daltroban, a TP receptor antagonist, into the hindlimb muscles has been shown to attenuate the cardiovascular responses to static contraction and tendon stretch in 72 h ligated rats, suggesting that TP receptor contributes to the EPR in PAD (Leal et al., 2011).

ROLE FOR NGF IN REGULATING RESPONSES OF AFFERENT METABOLIC RECEPTORS AND EXERCISE PRESSOR REFLEX IN ISCHEMIC MUSCLE

Prior studies demonstrated that femoral artery occlusion elevates the levels of NGF in the hindlimb muscles and DRG neurons of rats (Emanueli et al., 2002; Xing et al., 2009). NGF can induce expression of TRPV1, P2X3, and ASIC3 receptors in the DRG neurons (Ramer et al., 2001; Mamet et al., 2003). In addition, NGF can change the neuronal phenotype such as capsaicin-insensitive sensory neurons (Hunter et al., 2000), which possibly alters afferent mediated response in the processing of sensory signals. Therefore, in a series of experiments, the role for NGF in regulating those metabolically sensitive receptors in muscle afferent nerves was examined. Also, NGF-Ab was previously administered into the hindlimb muscles of occluded rats to neutralize effects of NGF, and then SNA and BP responses to static muscle contraction and passive tendon stretch were examined. Muscle contraction is performed to evoke both mechano- and metabo-components of the EPR, and muscle stretch is employed to activate muscle mechanoreceptor. Also, to determine effects of NGF on the reflex responses to activation of muscle metaboreceptors, SNA and BP responses to lactic acid injected into the arterial blood supply of the hindlimb muscles were examined after infusion of NGF-Ab in the hindlimb muscles of occluded rats.

Thin fiber afferent nerves (neurons) are distinct as IB_4-negative and IB_4-positive because of their distinct neurochemical characteristics and neurotrophic factor responsiveness. The IB_4-negative neurons express trkA receptors for NGF, depend on NGF for survival during postnatal development, and contain neuropeptides such as calcitonin gene-related peptide and substance P (Averill et al., 1995; Bennett et al., 1996, 1998; Molliver et al., 1997). The IB_4-positive neurons express receptors for glial cell line-derived neurotrophic factor (GDNF), depend on GDNF for survival during postnatal development, and are relatively "peptide poor" but express a surface carbohydrate group that binds IB_4 (Averill et al., 1995; Bennett et al., 1996, 1998; Molliver et al., 1997).

The data show that arterial occlusion augments responses with activation of metabolite sensitive TRPV1 receptors in IB_4-positive, and -negative DRG neurons (Xing et al., 2009). Additional experiment showed that increased NGF in the muscles and in the culture dish containing DRG neurons amplifies the magnitude of TRPV1 response to capsaicin in IB_4-negative DRG neurons but not in IB_4-positive DRG neurons. These findings suggest that NGF plays a role in augmented TRPV1 responses in the processing of muscle ischemia or vascular insufficiency induced by the femoral artery occlusion (Xing et al., 2009). The data further suggest that a selective subpopulation of the afferent neurons is engaged in NGF augmented TRPV1 response. Thus, evidence of this study provides strong support for the proposition that NGF regulation in muscle metabolic changes associated TRPV1 receptors contributes to augmented sympathetic activity and may lead to a reduction in exercise capacity seen in PAD.

Furthermore, infusion of NGF into the hindlimb of healthy rats through a micro-osmotic pump induces 1.39-fold increases in P2X3 protein of the DRGs of the infused leg as compared to the control leg (Liu et al., 2011). Also, NGF infused into the hindlimb significantly enhanced the pressor response to arterial injection of α, β-me ATP. On the other hand, blocking NGF attenuated exaggeration of the reflex response induced by α, β-methylene ATP in occluded rats (Liu et al., 2011). These findings suggest that NGF is closely related to upregulation of P2X3 expression in DRG neurons and to augmentation in the SAN and BP responses to activation of P2X3 as the hindlimb vascular insufficiency occurs.

Finally, the role of NGF in regulating the enhanced sympathetic responsiveness during stimulation of primary muscle afferent nerves was examined as the blood flow directed to hindlimb muscles is insufficient following femoral artery ligation. In the first set of experiments, NGF-Ab was previously injected into the hindlimb muscles to block effects of NGF that were induced by femoral occlusion. Then, mechano- and/or metabo-sensitive afferents nerves were activated by three interventions, namely muscle contraction, tendon stretch, and stimulation of ASICs using lactic acid. The data demonstrate that NGF neutralization significantly attenuates femoral occlusion-augmented reflex sympathetic and BP responses evoked by static contraction and lactic acid, but not by muscle stretch. Given that administration of NGF-Ab into the hindlimb muscles significantly attenuates occlusion-enhanced protein levels of ASIC3 in DRG tissues, these results suggest that NGF that is increased in sensory nerves of occluded limbs contributes to augmented reflex SNA and BP responses to stimulation of chemically, but not mechanically sensitive muscle afferent nerves. Also, this study has examined whether NGF is engaged in the role of sensory nerves' ASIC3 in augmented responses evoked by the hindlimb vascular insufficiency. In the second set of experiments, effects of NGF on expression of ASIC3 in DRG neurons that project thin C-fiber and A-fiber were examined. NGF infused into the hindlimb muscles significantly increases the protein levels of ASIC3 in DRG and selectively increase expression of ASIC3 in DRG neurons that project C-fiber afferents. Thus, these data support the hypothesis that NGF plays a role in exaggeration of the muscle metaboreflex via enhancement of ASIC3 expression in thin C-fiber afferent neurons.

SUMMARY

Peripheral artery disease affects life styles in 20% of adults who are older than 65 years. The narrowing of blood vessels of the lower limbs, mainly due to atherosclerotic vascular disease, is a main cause of PAD. Intermittent claudication is the most common symptom of this disease and it regularly occurs during exercise/physical activity but is relieved promptly by rest. It is well

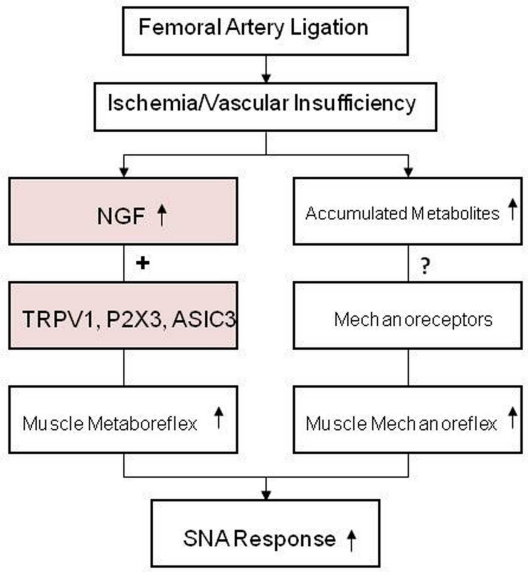

FIGURE 5 | The schematic diagram summarizes the regulatory mechanisms of the exaggerated sympathetic response to activation of the muscle metabo- and mechano-sensitive afferent nerves after femoral artery ligation.

known that the sympathetic nerve system plays an important role in regulating blood flow directed to skeletal muscle tissues during exercise. Thus, the goal of those studies was to examine the role of metabolite sensitive receptors on muscle afferent neurons in the responses of SNA after limiting blood flow to hindlimb muscle, as seen in PAD. Currently, several studies using a rat model of femoral artery ligation show that sympathetic responses of the EPR engagement are exaggerated as seen in PAD patients. Findings of the completed studies also suggest that enhanced protein levels of TRPV1, P2X3, and ASIC3 in muscle afferent nerves and amplified responses of those receptors contributes to the exaggerated reflex sympathetic and pressor responses to their individual receptor stimulus (**Figure 5**). The findings further suggest that NGF is likely responsible for enhanced TRPV1, P2X3, and ASIC3 and plays a role in modulating the metaboreceptor component of the EPR in the hindlimb muscle ischemia (**Figure 5**). Lactic acid and ATP are the major muscle by-products in exercising muscles and TRPV1, P2X3, and ASIC3 receptors are sensitive to those individual metabolites or combined metabolites. Current data presented here provide evidence that alteration in chemically sensitive receptors TRPV1, P2X3, and ASIC3 in primary afferent neurons innervating ischemic muscles plays an important role in the development of the exaggerated reflex sympathetic response, likely leading to worsening exercise capacity in patients with PAD. Also, NGF that increased in sensory nerves is engaged in abnormal responses of those metabolic receptors. A mechanism responsible for the augmented sympathetic response to the muscle mechanoreflex needs to be determined in the future. It is speculated that muscle metabolites are accumulated to a greater degree in ischemic muscles of PAD, which can sensitize mechanically sensitive muscle afferent nerves and enhance sympathetic response during activation of muscle mechanoreflex (**Figure 5**).

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Muscle reflex in heart failure: the role of exercise training

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Exercise evokes sympathetic activation and increases blood pressure and heart rate (HR). Two neural mechanisms that cause the exercise-induced increase in sympathetic discharge are central command and the exercise pressor reflex (EPR). The former suggests that a volitional signal emanating from central motor areas leads to increased sympathetic activation during exercise. The latter is a reflex originating in skeletal muscle which contributes significantly to the regulation of the cardiovascular and respiratory systems during exercise. The afferent arm of this reflex is composed of metabolically sensitive (predominantly group IV, C-fibers) and mechanically sensitive (predominately group III, A-delta fibers) afferent fibers. Activation of these receptors and their associated afferent fibers reflexively adjusts sympathetic and parasympathetic nerve activity during exercise. In heart failure, the sympathetic activation during exercise is exaggerated, which potentially increases cardiovascular risk and contributes to exercise intolerance during physical activity in chronic heart failure (CHF) patients. A therapeutic strategy for preventing or slowing the progression of the exaggerated EPR may be of benefit in CHF patients. Long-term exercise training (ExT), as a non-pharmacological treatment for CHF increases exercise capacity, reduces sympatho-excitation and improves cardiovascular function in CHF animals and patients. In this review, we will discuss the effects of ExT and the mechanisms that contribute to the exaggerated EPR in the CHF state.

Keywords: physical training, myocardial infarction, muscle afferents, exercise, sympathetic nerve activity

INTRODUCTION

A hallmark of patients suffering from chronic heart failure (CHF) is exercise intolerance characterized by fatigue and shortness of breath during exercise (Francis, 1985; Cohn, 1990; Sullivan et al., 1990; Wilson, 1995). The degree of exercise intolerance is important to characterize in patients with CHF, since it has implications for morbidity, disability, and prognosis, and is often the reason a patient seeks medical attention. Originally, the explanation for exercise intolerance in CHF appears to be mainly due to inadequate delivery of oxygen from the failing heart to the working muscle. However, evidence suggests that the degree of exercise intolerance is not directly related to the degree of cardiac dysfunction (Franciosa et al., 1981; Sullivan and Hawthorne, 1995). Rather, it is generally thought that the factors in the peripheral musculature may play a critical role in mediating exercise intolerance. These peripheral factors include abnormalities in endothelial function, vasodilatory capacity, changes in skeletal muscle structure, oxidative enzyme activity and a reflex originating in skeletal muscle (termed "exercise pressor reflex," EPR) that contributes significantly to the regulation of the cardiovascular and respiratory function during exercise (Myers and Froelicher, 1991; Sullivan and Hawthorne, 1995; Myers et al., 1999; Piepoli et al., 1999). In CHF patients, this reflex is exaggerated and causes extreme activation of the sympathetic nervous system even during moderate exercise. Exaggerated sympathetic activation by the EPR during exercise likely restrains muscle blood flow, arteriolar dilatation, and capillary recruitment, leading to under perfused areas of working muscle. In addition to vasoconstriction in skeletal muscle, hyperventilation is another consequence of the exaggerated EPR during exercise, both of which accentuates the symptoms of exercise intolerance. It is important to understand how the exaggerated EPR contributes to the exercise intolerance in CHF patients. Furthermore, the exaggerated sympatho-excitation that occurs during exercise also increases the risk of experiencing myocardial ischemia, myocardial infarction, cardiac arrest, and/or stroke during or immediately after exercise in these patients.

As exercise intolerance and exaggerated sympatho-excitation are important clinical features in these patients, therapeutic interventions are largely aimed at improving these symptoms. A particular interest has recently been directed toward the exaggerated EPR in CHF (Piepoli et al., 1996, 1999; Khan and Sinoway, 2000; Piepoli, 2006; Wang et al., 2010b, 2012). Once thought to be contraindicated in patients with CHF, long-term regular exercise training (ExT for at least 8 weeks) as a non-pharmacological treatment for CHF is now commonly employed in these patients, and has been shown to increase the quality of life as well as survival (Belardinelli et al., 1999; Piepoli et al., 2004; Smart and Marwick, 2004; Jankowska et al., 2007; Wisloff et al., 2007; Flynn et al., 2009; O'Connor et al., 2009). The beneficial effects of ExT include improved autonomic balance, reduced neurohumoral activation, increase in exercise capacity and ameliorated myopathy in CHF patients and animals (Pliquett et al., 2003; Roveda et al., 2003; Rondon et al., 2006; Jankowska et al., 2007; Mueller, 2007b; Negrao and Middlekauff, 2008). Adequate discussion of the beneficial effects of ExT in CHF is a large endeavor and beyond the

scope of the current review. Therefore, this review will be narrowed and focus on the role of ExT in improving the exaggerated EPR in CHF. Furthermore, we will also discuss the mechanisms underlying the beneficial effect of ExT on the exaggerated EPR in CHF.

SYMPATHETIC ACTIVATION DURING EXERCISE

During exercise the sympathetic nervous system is activated, which results in an increase in arterial pressure (AP), heart rate (HR), and peripheral vasoconstriction, especially to non-exercising tissues. Two theories have been postulated to explain the increases in cardiovascular and ventilatory function during exercise: central command and the EPR. Central command is a mechanism whereby neural motor and sympathetic activation occur in parallel, i.e., a volitional signal from the motor cortex or subcortical nuclei, responsible for recruiting motor units, activate cardiovascular control areas in the brainstem to modulate sympathetic and parasympathetic activity during exercise (Goodwin et al., 1972; Eldridge et al., 1985). It has been suggested that this system is linked to skeletal muscle metabolic needs via parallel rostral brain activation of motor and autonomic centers. These autonomic adjustments elicit changes in ventilation, HR and AP proportional to the intensity of exercise. The EPR is a peripheral neural reflex originating in skeletal muscle which contributes to the regulation of the cardiovascular and respiratory systems during physical activity. This reflex is essential for the maintenance of adequate blood perfusion to the exercising muscle, thereby matching the metabolic demands that exercise creates (McCloskey and Mitchell, 1972).

THE EXERCISE PRESSOR REFLEX

Although several reviews have been published describing the EPR (Sinoway and Li, 2005; Smith et al., 2006a; Murphy et al., 2011), a brief synopsis is warranted here. Alam and Smirk (1937) were the first to offer evidence suggesting that chemical byproducts of muscle contraction could evoke a pressor reflex. These authors demonstrated that dynamic calf exercise evoked increases in BP and HR that were maintained by circulatory arrest at the end of exercise. These findings provided the earliest evidence that the accumulation of metabolites in the contracting muscle elicited the EPR. A study by McCloskey and Mitchell (1972) demonstrated that anodal blockade of the L7–S1 dorsal roots of the cat blocked thickly myelinated group I and II afferents but did not affect the cardiovascular responses to contraction whereas topical application of a local anesthetic to the dorsal roots did not block group I and II afferents but did abolish the cardiovascular responses to contraction, indicating that activation of this reflex is mediated by stimulation of thinly myelinated group III and IV but not thickly myelinated group I and II afferents. Studies by Kaufman et al. (1983, 1984) demonstrated that group III fibers in the triceps surae muscle of the cat are predominantly mechanically sensitive, whereas unmyelinated group IV muscle afferents are chemically sensitive.

Anatomically, group III nerve endings terminate in the collagenous connective tissue, the endoneurium of the triceps surae and calcaneal tendon of the cat, which are rapidly excited by mechanical deformation of their receptive field and then quickly adapted during the steady state period of muscle contraction (Kniffki et al., 1978; Kaufman et al., 1983, 1984; Andres et al., 1985; Mense and Meyer, 1985; Hayward et al., 1991; Adreani et al., 1997; Adreani and Kaufman, 1998). As such, receptors associated with these afferent fibers are termed "mechanoreceptors," although a few are responsive to chemical stimuli. On the other hand, sensory receptors associated with group IV afferent neurons are located on unencapsulated nerve endings that terminate within the walls of capillaries, venules, and lymphatic vessels of skeletal muscle, which are predominately excited by the accumulation of metabolites produced by contracting muscle (Kaufman et al., 1983; Andres et al., 1985). With regard to the time needed for accumulation of metabolites, activation of group IV afferents are always delayed (5–20 s) following muscle contraction (Kaufman et al., 1983; Mense and Meyer, 1985). Sensory receptors associated with group IV afferents are termed "metaboreceptors" although a few are also responsive to mechanical stimuli.

The first site of synapse for most muscle group III and IV afferents is the dorsal horn of the spinal cord, specifically Rexed's laminae I, II, V, and X (Kalia et al., 1981; Mense and Craig, 1988; Li and Mitchell, 2002; Wilson et al., 2002). Although the specific pathway remains unknown, muscle afferents project from the dorsal horn to the brain stem along a path that includes the dorsolateral sulcus and the ventral spinal cord (Iwamoto et al., 1984; Kozelka and Wurster, 1985; Dykes and Craig, 1998). From the dorsal horn, ascending projections activate cells in the medulla. Above the medulla, the central integration of the pressor reflex is complex, involving multiple regions. However, evidence suggests that full expression of the EPR at least requires an intact pontomedullary region of the brainstem (Iwamoto et al., 1985). Those nuclei responsive to EPR stimulation have been described in the nucleus tractus solitarius (NTS), rostral ventral medulla, caudal ventrolateral medulla, lateral tegmental field, nucleus ambiguus, and the ventromedial region of the rostral periaqueductal grey (Iwamoto et al., 1982; Iwamoto and Kaufman, 1987; Li et al., 1997; Li and Mitchell, 2000). From the medulla, descending projections synapse on sympathetic pre-ganglionic neurons in the intermediolateral cell columns (IML) of the spinal cord and then project to the synapse at the paravertebral sympathetic chain ganglia, and finally innervate the heart and vasculature. The EPR-mediated adjustments in parasympathetic and SNA result in increases in cardiac contractility, SV, HR, and BP (Longhurst and Mitchell, 1979; Murata and Matsukawa, 2001; Koba et al., 2006; Wang et al., 2010b). It is through the pathways, outlined in **Figure 1**, that skeletal muscle reflexes contribute to cardiovascular and respiratory regulation during physical activity.

ABNORMALITIES OF EXERCISE PRESSOR REFLEX IN CHF

There is general agreement that the EPR is exaggerated in humans with CHF and that these exaggerations correlate with morbidity and mortality as well as with decreased left ventricular (LV) function (McClain et al., 1993; Middlekauff et al., 2000, 2001; Piepoli and Coats, 2007; Piepoli et al., 2008). The emerging evidence describing dysfunction of the reflex with the advent of CHF has been highlighted in several recent reviews (Sinoway and Li, 2005; Smith et al., 2006a; Garry, 2011; Murphy et al., 2011). Despite this, defining the mechanisms that mediate the abnormal EPR

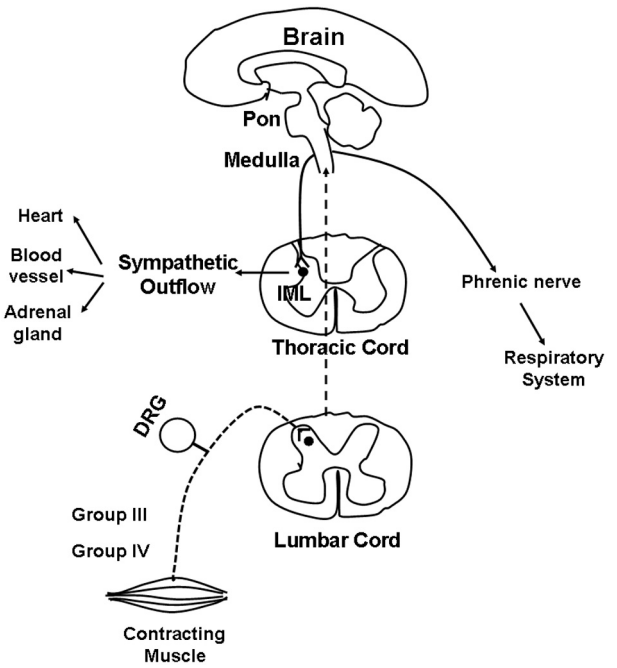

FIGURE 1 | Schematic illustrating the proposed pathway for the reflex cardiovascular and respiratory changes evoked by skeletal muscle contraction. Dashed lines indicate the afferent limb of this reflex pathway whereas solid lines points out the efferent limb. See text for additional discussion.

in CHF patients has proven to be difficult. For example, studies in human subjects have not been able to clearly discern whether peripheral primary afferent neurons or central areas that process the EPR are responsible for the exaggerated EPR that is observed in CHF. In addition, the literature surrounding the issue defining the contribution of metabo- or mechano-reflex to the exaggerated EPR in CHF patients is conflicted. A great deal of controversy exists regarding the contribution of the metabolic component of the EPR (metaboreflex) because its activity has been reported to be both enhanced (Piepoli et al., 1996, 2008; Piepoli and Coats, 2007) and reduced (Sterns et al., 1991; Middlekauff et al., 2000) in response to exercise in CHF patients. Based on measurements of ventilation, the studies of Piepoli et al. (Piepoli et al., 1996, 2008; Piepoli and Coats, 2007) showed that CHF patients had an overactive metaboreflex compared with control subjects. However, based on measurements of blood pressure or sympatho-excitatory responses to post-contraction circulatory arrest (PCCA, an isolated activator of the muscle metaboreflex), the studies of Sterns et al. (1991) and Middlekauff et al. (2000) showed that metaboreflex function is blunted rather than exaggerated in this disease state. The discrepant conclusions that the metaboreflex is blunted or exaggerated in CHF appear to be due to different measurements of physiologic parameters such as ventilation, blood pressure and sympathetic nerve activity. In addition, a central command mechanism, which cannot absolutely be excluded in human studies, may also contribute to this discrepant conclusion. Compared to studies concerning the metaboreflex in CHF patients, the studies focusing on the role of the mechanoreflex in mediating the exaggerated EPR is generally consistent, suggesting that an overactive mechanoreflex contributes to the exaggerated EPR in CHF patients. McClain et al. (1993) reported that limb congestion, a common feature of congestive CHF, increases the sympathetic nerve response to handgrip exercise. Moreover, subsequent studies from the same laboratory demonstrated that limb congestion could sensitize muscle mechanoreceptors and in the process increase synchrony between contraction and sympathetic discharge (Mostoufi-Moab et al., 2000). Middlekauff et al. (2001) suggesting that reflex renal vasoconstriction is exaggerated in both magnitude and duration during dynamic exercise in HF patients. Moreover, subsequent studies from this laboratory have shown that the mechanoreflex is exaggerated in humans suffering from CHF which is most likely due to sensitization of the mechanoreceptor afferents by cyclooxygenase products (Middlekauff and Chiu, 2004; Middlekauff et al., 2004).

In animal studies, using a decerebrate rat model, Smith et al. (2003, 2005a,b) conducted a series of convincing experiments designed specifically to examine EPR function in CHF and to determine the contribution of the muscle mechanoreflex and metaboreflex to the EPR in this disease. Their findings suggest (1) that the overactive cardiovascular response to exercise in CHF is mediated, in part, by an exaggerated EPR; (2) that the muscle metaboreflex is blunted and that the muscle mechanoreflex is enhanced in CHF; (3) that the mechanoreflex mediates the exaggerated EPR activity observed in CHF; (4) that the decreased sensitivity of group IV afferent neurons is important to the development of EPR hyperactivity. In parallel studies, Li et al. (2004) also reported a similar finding as that of Smith et al. (2003, 2005a,b), showing that the muscle metaboreflex control of cardiovascular activity is blunted and that the muscle mechanoreflex is enhanced in rats with large myocardial infarcts. Subsequent studies from the same laboratory (Koba et al., 2008) showed that renal and lumbar sympathetic nerve responses to muscle contraction were larger in CHF rats than in healthy control rats, indicating that the EPR contributes to the exaggerated sympathoexcitation during exercise. Recently, using the technique of single fiber recording, we (Wang et al., 2010a) demonstrated that the responses of group III afferents to contraction and stretch were enhanced in rats with dilated cardiomyopathy (induced by ligation of the left anterior descending coronary artery) whereas the responses of group IV afferents to contraction and capsaicin were reduced in these rats compared to sham-operated rats (**Figures 2** and **3**), which provide direct evidence that the exaggerated EPR in CHF is, at least in part, due to the peripheral sensitization of muscle mechanically sensitive afferents. However, the EPR is a multisynaptic reflex involving the following: (1) the receptors activating the afferent fibers; (2) primary afferent neurons, (3) second-order spinal neurons, (4) neurons in medullary centers, (5) sympathetic and parasympathetic efferent neurons, and (6) the end organs that the efferent fibers innervate. Whether other parts of this reflex arc are also involved in the genesis of the exaggerated EPR in the CHF state remains unclear. Further studies are worthy of being carried out to address this issue.

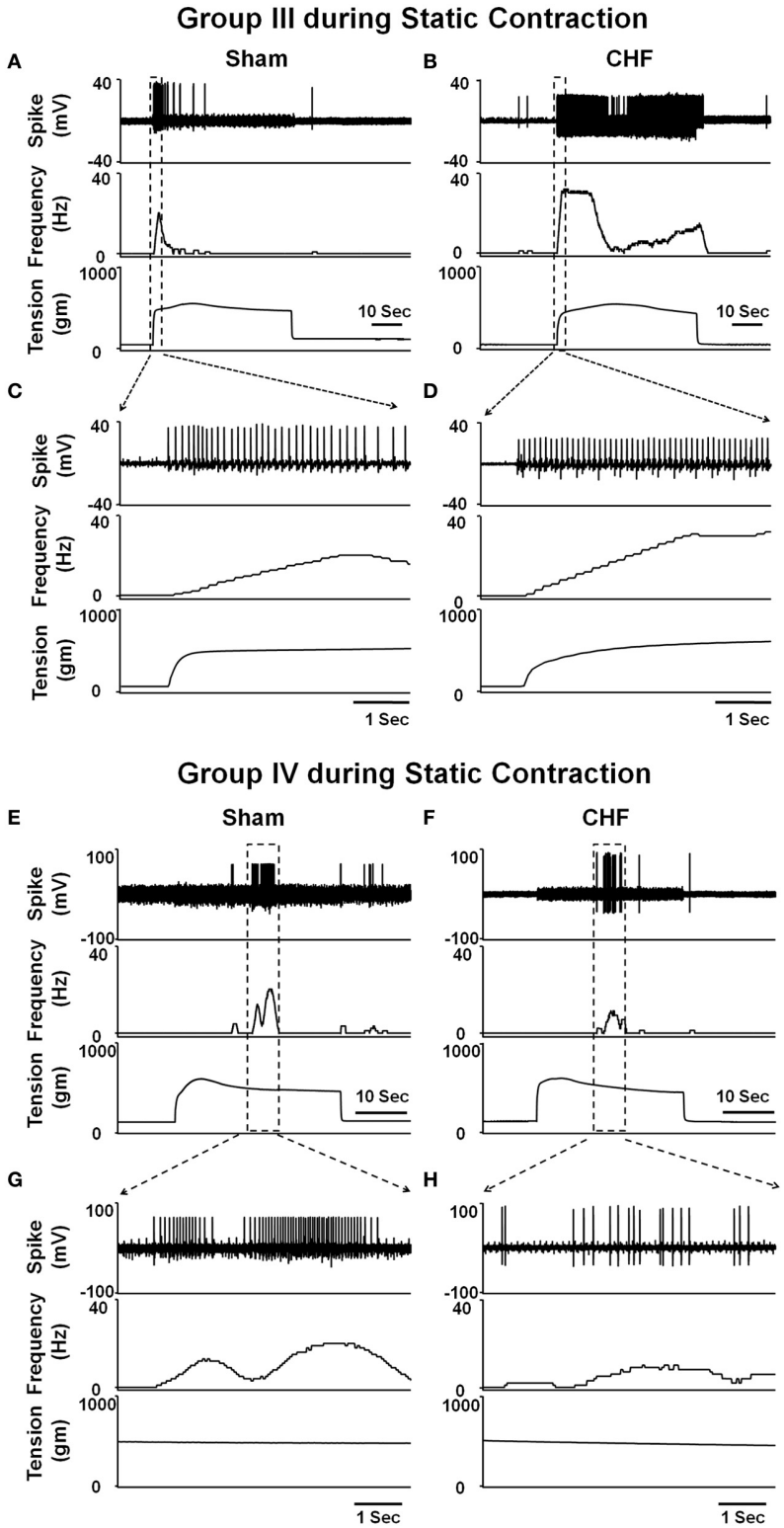

FIGURE 2 | Representative recordings showing the discharge of group III (A–D) and IV (E–H) afferents in response to static contraction induced by electrical stimulation of L5 ventral root in sham (Group III in panel (A): CV, 5.2 m/s; Group IV in panel (E): CV, 0.8 m/s) and CHF rats (Group III in panel (B): CV, 7.0 m/s; Group IV in panel (F): CV, 0.5 m/s). (C) and **(D)**, 5-s recording of two group III fibers discharge derived from the broken-lined box in **(A)** and **(B)**, respectively. **(G)** and **(H)**, 6-s recording of two group IV fibers discharge derived from the broken-lined box in **(E)** and **(F)**, respectively. [Reprinted from Wang et al. (2010a). Copyright @ 2010 The Physiological Society. Used with permission.]

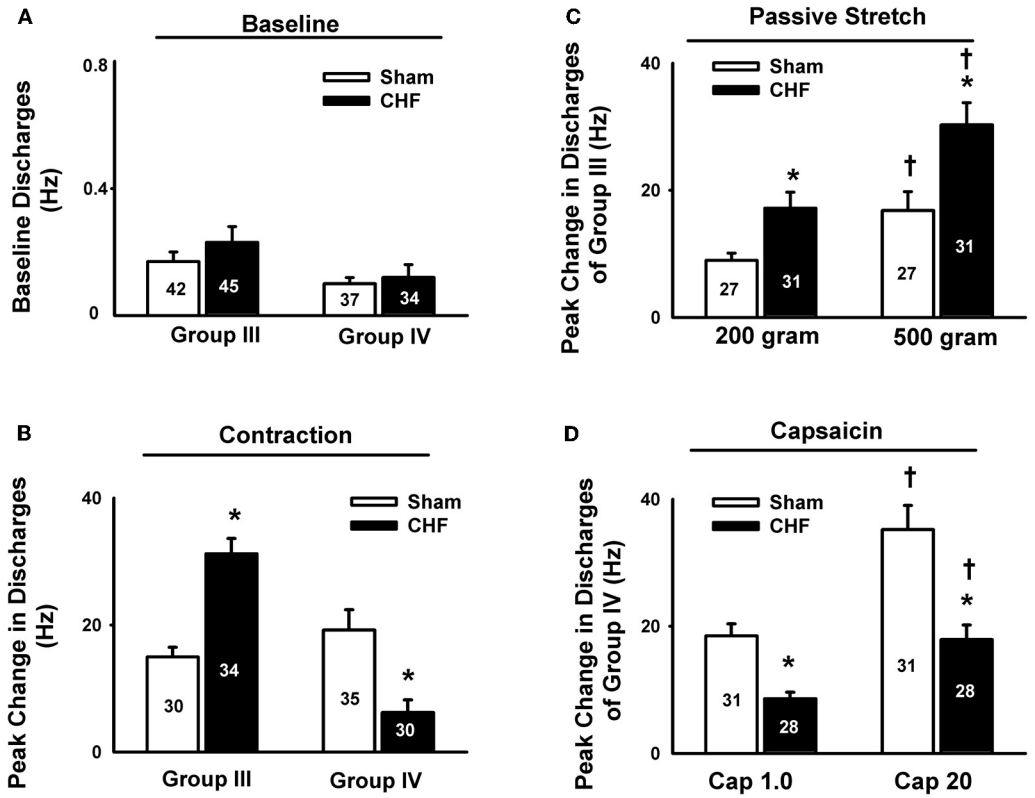

FIGURE 3 | Mean data showing the baseline discharge of group III and IV afferents in sham and CHF rats (A) and the responses of group III and IV afferents to static contraction induced by electrical stimulation of L5 ventral root in sham and CHF rats (B). Mean data showing the discharge of group III and IV afferents in response to two levels of passive stretch **(C)** and two doses of capsaicin **(D)** respectively in sham and CHF rats. Data are expressed as Mean ± SE. $^{*}P < 0.05$ vs. sham, $^{\dagger}P < 0.05$ vs. lower level of stretch or lower dose of capsaicin. [Reprinted from Wang et al. (2010a). Copyright @ 2010 The Physiological Society. Used with permission.]

EFFECT OF ExT ON THE EPR IN HEALTH AND CHF

Over the past decade numerous clinical trials and small randomized studies have demonstrated that long-term regular exercise is safe in stable CHF patients and increases the quality of life as well as survival (Belardinelli et al., 1999; Khan and Sinoway, 2000; Jankowska et al., 2007; Mueller, 2007a; Wisloff et al., 2007). Therefore, ExT has been recommended as a non-pharmacological treatment for CHF, ischemic heart disease and hypertension by the American Heart Association (Fletcher et al., 1996; Halbert et al., 1997; Fletcher et al., 2001; Pina et al., 2003). Furthermore, several clinical and experimental studies have also suggested that ExT effects EPR function in health and disease states. However, the mechanisms underlying the effect of ExT on EPR function in both health and disease remain largely unknown.

EFFECT OF ExT ON THE EPR IN HEALTH

An earlier study by Sinoway et al. (1996) reported that 4-week forearm training reduced sympathetic responses and mean AP rises during rhythmic voluntary handgrip exercise in normal subjects. Following study from this group (Mostoufi-Moab et al., 1998) demonstrated that forearm exercise conditioning paradigm also attenuated the pressor response to ischemic rhythmic exercise and decreased lactate accumulation and venous pH values, suggesting that muscle metaboreceptor afferent activity may be reduced because of a decrease in metabolite accumulation in the trained muscle. However, during such voluntary exercise, it has been difficult to distinguish between possible training-induced changes in central command, and the different muscle afferent inputs to the response. Therefore, Fisher and White (1999) used two exercise modes to re-evaluate the effects of ExT on central command and the EPR in healthy subjects. The first exercise mode was voluntary muscle contraction, which potentially involves central command as well as muscle mechanoreceptor and muscle metaboreceptor stimulation, and the second was electrically evoked contraction (involuntary) at the same force level. In this instance, central command was removed but muscle receptor activity should remain at the same level as in the voluntary exercise mode. Both forms of exercise were followed by PCCA where muscle metaboreceptor activity predominates. These data demonstrated that 6-week calf muscle training had no effect on the muscle afferent input to the pressor response to electrically evoked contraction in the untrained limb, since cardiovascular responses were unchanged both during exercise and PCCA. However, during voluntary contraction of the untrained limb, diastolic blood pressure and HR rises

were attenuated after training, but neither were altered from pre-training values during PCCA. Therefore the changes can only be explained by decreased central command during exercise. In animal experiments, we (Wang et al., 2010b) recently reported that although 8–10 week of treadmill ExT tended to reduce the blood pressure, HR and renal sympathetic activation responses to involuntary static contraction by electrical stimulation of ventral roots in a decerebrate rat model where central command was removed, this training effect did not reach statistical significance (**Figure 4**), indicating that ExT has less effect on the EPR in healthy animals. Direct evidence from muscle afferent recording experiments (Wang et al., 2012) also supports that training has less effect on the sensitivity of group III and IV afferents in healthy rats (**Figures 5–7**). Taken together the evidence suggests that it is very likely that ExT attenuates cardiovascular activity during exercise mainly via affecting central command rather than muscle afferent input in normal subjects or animals.

EFFECT OF ExT ON THE EPR IN CHF

The beneficial effect of ExT on the exaggerated EPR in CHF has also been previously demonstrated. Piepoli et al. (1996) reported that in patients with CHF (8–38 months, New York Heart Association (NYHA) class II–III), there is an exaggerated exercise-evoked sympatho-excitation, vasoconstrictor, and ventilatory drive characteristic of this population of patients, which is partially reversed by 6-week forearm training. These findings indicate a potential beneficial effect of ExT on the abnormal muscle reflex function in CHF patients. However, due to intrinsic limitations of human research, the study of Piepoli et al. could not distinguish between possible training-induced changes in central command, and the different muscle afferent inputs to the response. In animal experiments, using a decerebrate model to remove the cortical structures from which central command originates, we recently (Wang et al., 2010b) observed that 8–10 weeks of treadmill ExT initiated at an early stage of CHF (i.e., 2 weeks after coronary ligation) prevents the exaggerated HR, pressor and

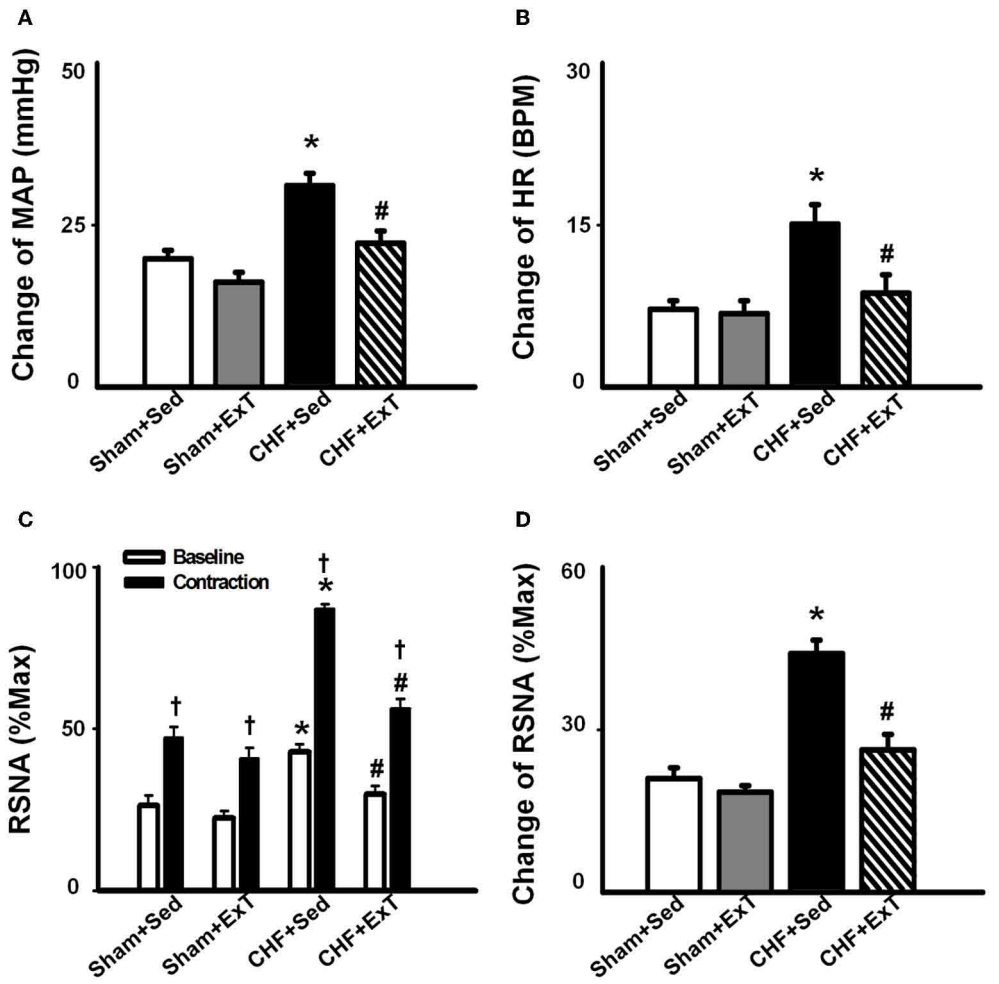

FIGURE 4 | Mean data showing the effects of ExT on pressor (A), heart rate (B) and sympatho-excitatory (D) responses to a 30-s static contraction in sham and CHF rats. Both baseline and contraction-induced RSNA were compared in sham+Sed, sham+ExT, CHF+Sed and CHF+ExT rats (C). Values are Mean ± SE, $^{*}P < 0.05$ vs. sham+Sed and sham+ExT, $^{\dagger}P < 0.05$ vs. baseline RSNA, $\#P < 0.05$ vs. CHF+Sed. [Reprinted from Wang et al. (2010b). Copyright @ 2010 American Heart Association. Used with permission.]

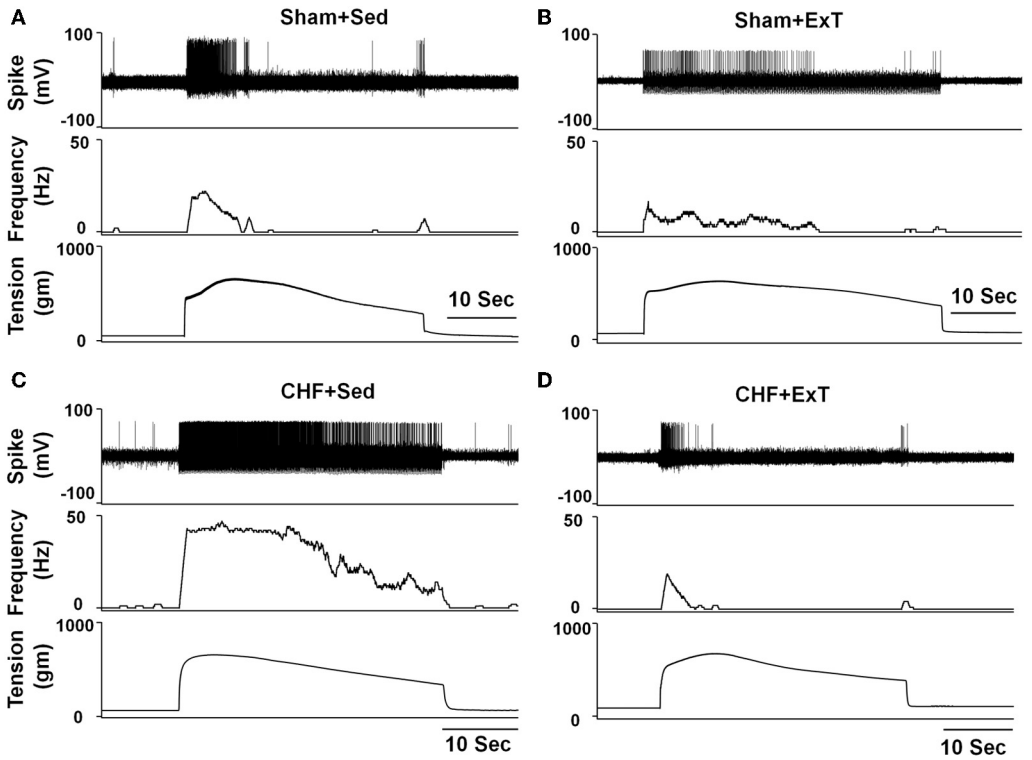

FIGURE 5 | Representative recordings showing the discharge of group III afferents in response to static contraction induced by electrical stimulation of L5 ventral root in sham+Sed (CV, 4.1 m/s, A), Sham+ExT(CV, 3.0 m/s, B), CHF+Sed (CV, 4.7 m/s, C) and CHF+ExT rats (CV, 5.3 m/s, D). [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

sympatho-excitatory responses to either static contraction or passive stretch (a purely mechanical stimulus) and partially prevents the blunted cardiovascular responses to injection of exogenous capsaicin (a chemical stimulus) in CHF rats (**Figure 4**). These findings indicate that ExT at an early stage of CHF has a beneficial effect on the exaggerated EPR. More recently, we (Wang et al., 2012) further demonstrated that this training protocol prevents the sensitization of group III afferents and partially prevents the blunted sensitivity of group IV afferents in CHF rats (**Figure 8**), suggesting that the beneficial effects of ExT on the exaggerated EPR is at least, in part, mediated by preventing the abnormal sensitization of muscle afferents in the CHF state.

MECHANISMS UNDERLYING THE BENEFICIAL EFFECT OF ExT ON THE EXAGGERATED EPR IN CHF

Although the beneficial effects of ExT on the exaggerated EPR has been demonstrated in CHF patients and animals (Piepoli et al., 1996; Piepoli, 2006; Wang et al., 2010b, 2012), the underlying mechanisms have not been completely identified. For example, in addition to muscle afferents, whether other components of the reflex arc are also affected by ExT in CHF remains unknown. We previously demonstrated that ExT also attenuated the exaggerated sympatho-excitation at rest in CHF animals (Liu et al., 2000; Gao et al., 2007). The effects of ExT on sympatho-excitation in CHF is, in part, mediated by effects on central neural structures such as the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitaries (NTS) (Mueller and Hasser, 2006; Gao et al., 2007). Therefore, it is possible that ExT affects the EPR in CHF via a central mechanism. In addition, in a previous study (Gao et al., 2007) we demonstrated that the effects of ExT on central neural structures such as the RVLM also contribute to an improvement of the blunted arterial baroreflex function in CHF. The latter and the EPR are well known to modify one another functionally during exercise. For example, it has been demonstrated that the cardiovascular response to activation of the EPR is enhanced in normotensive baro-denervated cats and rats (Waldrop and Mitchell, 1985; Smith et al., 2006b). Therefore, we speculate that a blunted baroreflex in CHF might contribute to the genesis of the exaggerated EPR whereas the ExT-mediated improvement of the blunted arterial baroreflex might ameliorate the exaggerated EPR. However, direct evidence for these hypotheses is absent.

Previous studies (Drexler et al., 1992; Mancini et al., 1992) have shown that peripheral skeletal myopathy develops in CHF (e.g., muscle atrophy, decreased peripheral blood flow, fiber-type transformation and reduced oxidative capacity). These abnormalities in the peripheral musculature in CHF may alter the environment around muscle afferent endings to sensitize muscle afferents. Because ExT has been reported to reverse skeletal myopathy in CHF (Howald et al., 1985; Hambrecht et al., 1997), this effect may play a critical role in the ExT-mediated improvement of the abnormal sensitization of muscle afferents in CHF.

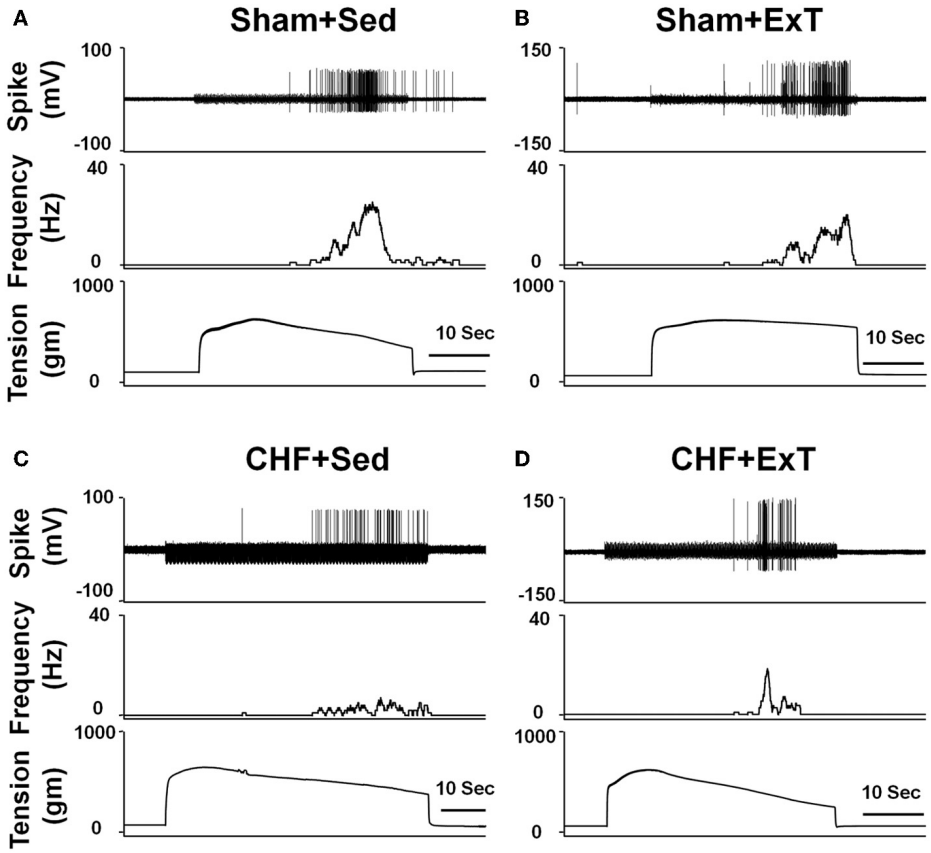

FIGURE 6 | Representative recordings showing the discharge of group IV afferents in response to static contraction induced by electrical stimulation of L5 ventral root in sham+Sed (CV, 1.2 m/s, A), Sham+ExT(CV, 0.82 m/s, B), CHF+Sed (CV, 0.73 m/s, C) and CHF+ExT rats (CV, 1.0 m/s, D). [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

In addition, a chronic reduction in skeletal muscle perfusion in CHF patients may alter muscle metabolism and cause excessive accumulation of metabolites during exercise. As such, the potential chronic exposure to excess metabolites could result in the sensitization of muscle afferents. It has been well documented that ExT leads to an increased perfusion of skeletal muscle in CHF patients (De Matos et al., 2004). This combined with an increased ability of the muscle to maintain aerobic metabolism leads to a decreased reliance on anaerobic metabolism. We speculate that this will lead to lower interstitial concentration of metabolites, evoking less muscle afferent stimulation.

ExT REVERSED MUSCLE TYPE SHIFT IN CHF

Iwamoto and Botterman (1985) reported that contraction of fast-twitch fibers (type II) evoked a larger pressor response to static contraction compared to slow-twitch fiber (type I) contraction. Furthermore, the study by Wilson et al. (1995) demonstrated that chronic low-frequency electrical stimulation of the tibial nerve of one hindlimb of adult rabbits, which converted the gastrocnemius (predominately type II) to a muscle that was primarily type I, decreased the pressor response to static contraction. These two studies suggested that type II fiber contraction may activate a larger number of muscle afferent receptors. In the CHF state, a muscle fiber-type shift from type I to type II could cause an exaggerated EPR. Since muscle fiber-type transformation in CHF can be reversed by ExT (Howald et al., 1985; Hambrecht et al., 1997), the improvement of abnormal fiber-type shift by ExT may subsequently affect muscle afferent function, and eventually ameliorate the exaggerated EPR function in the CHF state. Clearly, further research is needed in this area.

PURINERGIC RECEPTORS ARE INVOLVED IN THE MECHANISM BY WHICH ExT PREVENTS THE SENSITIZATION OF GROUP III AFFERENTS IN CHF

Purinergic (P) ligand-gated ion channels have been localized to both group III and IV muscle afferent neurons (Vulchanova et al., 1996, 1997, 1998). Skeletal muscle contraction triggers the release of purines such as adenosine and interstitial ATP, which act as ligands for P1 and P2X receptors, respectively (Hellsten et al., 1998; Li et al., 2003, 2005). Previous studies (Costa and Biaggioni, 1994; Middlekauff and Chiu, 2004) demonstrated that ATP is a potential metabolic stimulator of the EPR via the P2X receptor whereas adenosine and the P1 receptor is not involved in the modulation of the EPR. For example, an intra-arterial administration of α,β-methylene ATP (a P2X receptor agonist) into the hindlimb of decerebrated cats elevates BP and enhances

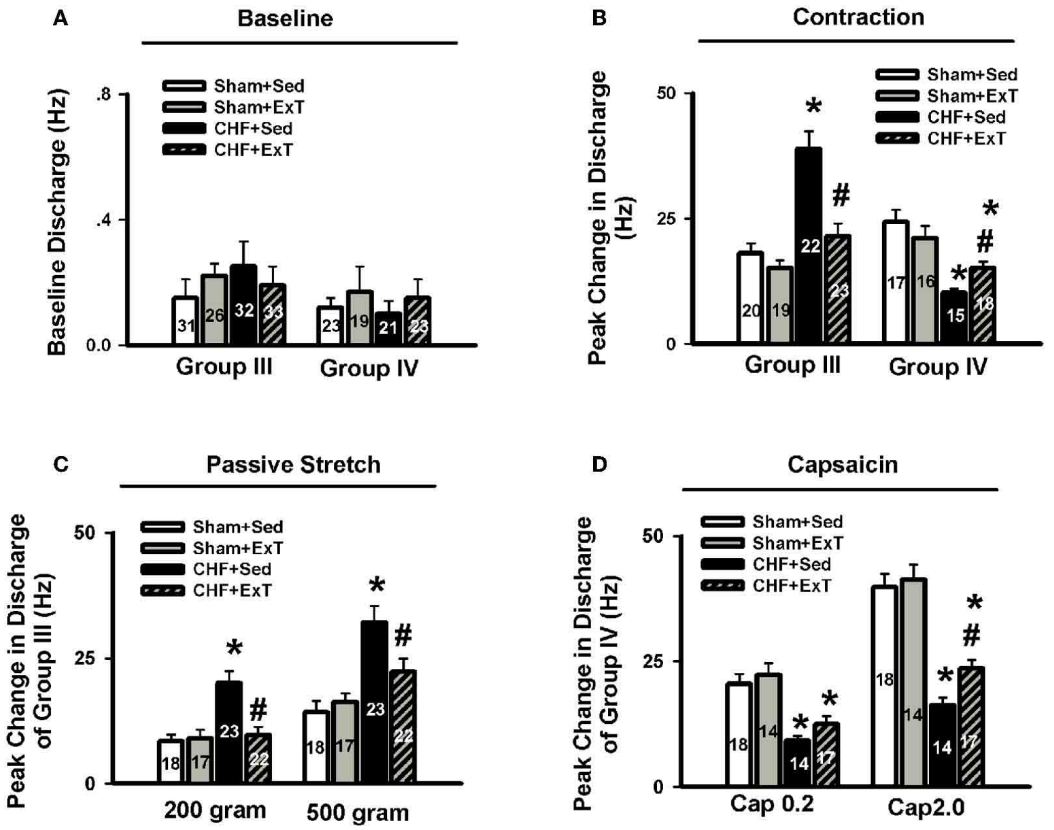

FIGURE 7 | Mean data showing the baseline discharge of group III and IV afferents (A) and the responses of group III and IV afferents to static contraction induced by electrical stimulation of L5 ventral root (B) in Sham+Sed, Sham+ExT, CHF+Sed and CHF+ExT rats. Mean data showing the discharge of group III and IV afferents in response to two levels of passive stretch **(C)** and two doses of capsaicin **(D)** respectively in Sham+Sed, Sham+ExT, CHF+Sed and CHF+ExT rats. The digit in the bar graph indicates the number of recording fibers. Data are expressed as Mean ± SE. $*P < 0.05$ vs. sham + Sed, $\#P < 0.05$ vs. CHF + Sed. [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

afferent impulses from group IV fibers by 67% (Hanna and Kaufman, 2004). Furthermore, the arterial administration of the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS) attenuates the pressor response to static muscle contraction by 38% in cats and reduces the pressor response to post-contraction circulatory occlusion (Hanna and Kaufman, 2003). Furthermore, ATP may function as a metabolite that sensitizes mechanoreceptors. Kindig et al. (2006) reported that PPADS attenuated the responses of group III muscle afferents to static contraction as well as to tendon stretch in decerebrate cats, suggesting that P2 activation sensitizes group III afferents in the normal state. Recently, we (Wang et al., 2010a) demonstrated that (1) PPADS attenuated the response of group III muscle afferents to either static contraction or passive stretch in CHF rats to a greater extent than in sham rats; (2) protein expression of P2X3 receptors in the DRG was significantly increased in CHF rats compared with sham rats; (3) increased protein expression of P2X3 receptors in DRG was located on both IB4-positive (C fiber marker) and NF200-positive (A fiber marker) neurons. These findings suggest that ATP and P2X receptors are involved in the mechanism underlying the sensitization of group III afferents in CHF state. Furthermore, we (Wang et al., 2012) found that (1) the increased antagonistic effect of PPADS on the sensitivity of group III afferents observed in CHF rats was prevented by ExT (**Figure 8**) and (2) ExT prevented the upregulation of P2X3 receptors in both A- and C-fiber DRG neurons in CHF rats (**Figures 9** and **10**), indicating that ExT prevented the sensitization of group III afferents, at least in part, by the normalization of the upregulated P2X receptors in the CHF state.

THE TRPV1 RECEPTOR IS INVOLVED IN THE MECHANISM BY WHICH ExT PREVENTS THE DESENSITIZATION OF GROUP IV AFFERENTS IN CHF

Transient receptor potential vanilloid 1 (TRPV1) receptors are predominantly localized to group IV fibers (Michael and Priestley, 1999; Wang et al., 2010a, 2012). Intra-arterial injection of capsaicin, a TRPV1 receptor agonist, markedly increases BP, HR, and SNA by stimulating group IV afferents (Crayton et al., 1981; Kaufman et al., 1982, 1983; Wang et al., 2010a,b). TRPV1 receptors are sensitive to changes in muscle temperature, increases in extracellular hydrogen ion concentration (pH <5.7), and inflammatory products such as bradykinin and prostaglandins (Tominaga et al., 1998; Guenther et al., 1999; Jordt et al., 2000; Welch et al., 2000). These potential activators of the TRPV1

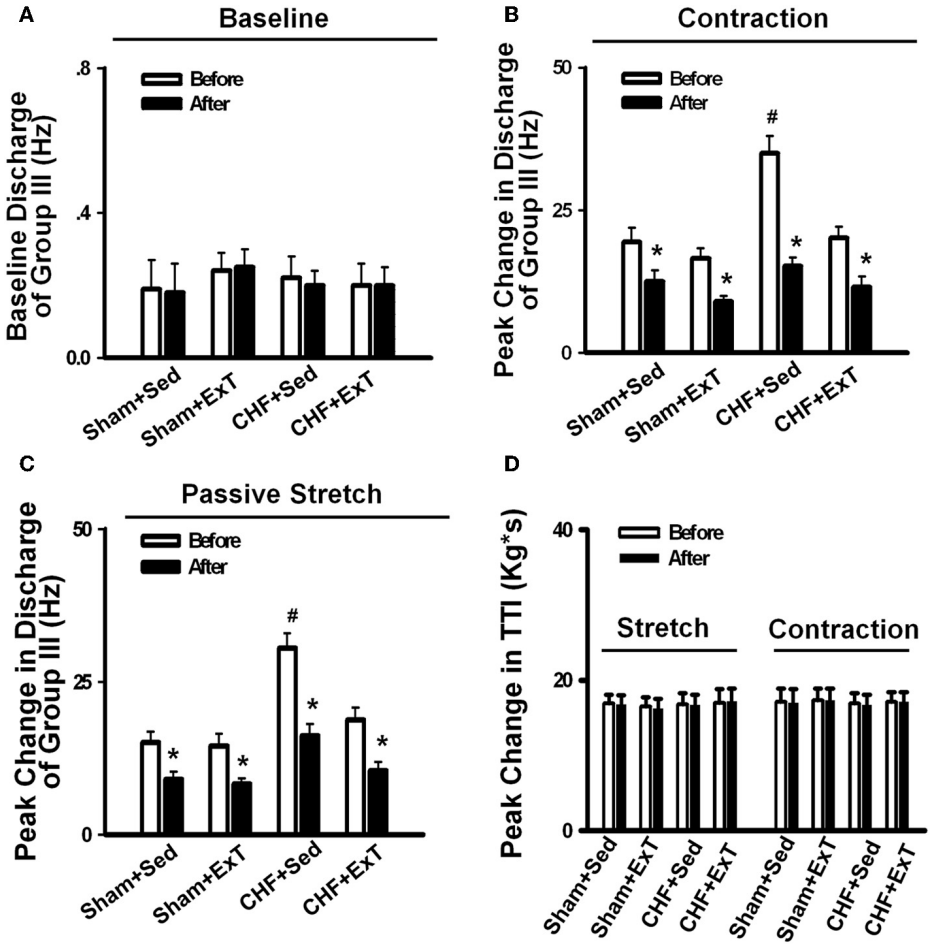

FIGURE 8 | Mean data showing the effect of PPADS, a P2X antagonist, on the baseline discharge (A) and the responses of group III afferents to either static contraction (B) or passive stretch (C) in Sham+Sed, Sham+ExT, CHF+Sed, and CHF+ExT rats. (D) The effect of PPADS on the TTI produced by passive stretch or static contraction in Sham+Sed, Sham+ExT, CHF+Sed and CHF+ExT rats. Data are expressed as Mean ± SE. $n = 8 - 10$/each group. $^*P < 0.05$ vs. before, $\#P < 0.05$ vs. Sham+Sed. [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

receptor are present during exercise. An earlier study by Kindig et al. (2005) demonstrated that TRPV1 blockade failed to prevent the pressor response to static contraction in decerebrated cats, indicating that TRPV1 plays little role in evoking the EPR. On the contrary, Smith et al. (2010) recently reported that TRPV1 blockade attenuated the pressor response to static contraction in decerebrate rats, indicating that TRPV1 plays an important role in evoking the EPR. The discrepancy among studies is not readily apparent. However, the majority of studies do raise the possibility that the activation of TRPV1 receptors by skeletal muscle metabolites (e.g., protons) may contribute to the excitation of the skeletal muscle metaboreflex during exercise. In CHF animals, several reports by Smith and colleagues (Smith et al., 2005b, 2006a, 2010) demonstrated that (1) TRPV1 activation by capsaicin caused a blunted cardiovascular response in CHF rats compared to sham rats, which was confirmed by our recent study (Wang et al., 2010b); (2) chronic deletion of TRPV1 receptors in normal rats recapitulates the exaggerated EPR observed in CHF rats, indicating that the loss of TPRV1 receptors may be an important contributor to the development of the exaggerated EPR in CHF; and (3) the mRNA level of TRPV1 in the DRG and in skeletal muscle was decreased in CHF rats compared to sham rats. Recently, we (Wang et al., 2010a) further demonstrated that (1) the response of group IV afferents to exogenous TRPV1 activation by capsaicin was blunted in CHF rats and (2) protein expression of TRPV1 receptors in the DRG was significantly decreased in C-fiber DRG neurons of CHF rats. These findings suggest that the TRPV1 receptor plays an important role in causing the blunted group IV sensitivity in the CHF state. More recently, we (Wang et al., 2012) demonstrated that ExT partially prevents the blunted sensitivity of group IV afferents in response to either static contraction or to administration of capsaicin in CHF rats (**Figure 7**). This was associated with an improvement in the decrease in protein expression of TRPV1 receptors in C-fiber DRG neurons of CHF+ExT rats (**Figures 10** and **11**). These findings indicate that ExT improves the blunted sensitivity of group IV afferents, in part, by preventing the downregulation of TRPV1 receptors in muscle afferent neurons in the CHF state. It

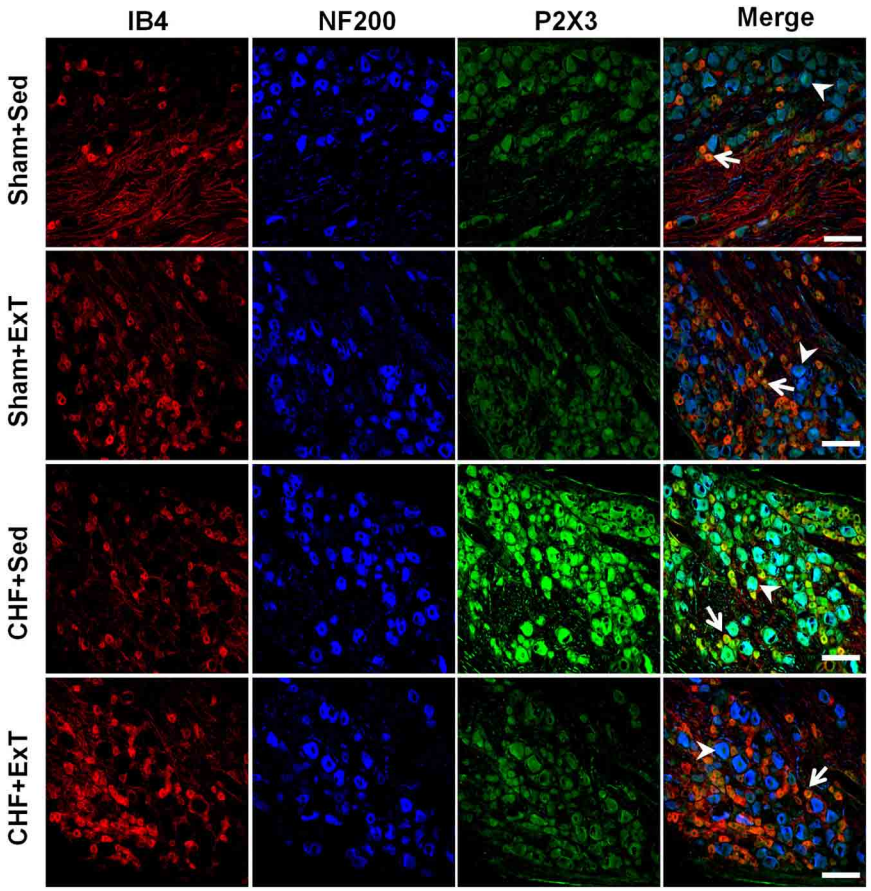

FIGURE 9 | Immunohistochemical data showing the protein expression of P2X3 receptors in L4/L5 dorsal root ganglion (DRG) in Sham+Sed, Sham+ExT, CHF+Sed and CHF+ExT rats. Isolectin B4 (IB4), a C-fiber neuron marker; NF200, an A-fiber neuron marker. White Bar = 100 μm. White arrow represents double staining of P2X3 with IB4, white arrowhead represents double staining of P2X3 with NF200. [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

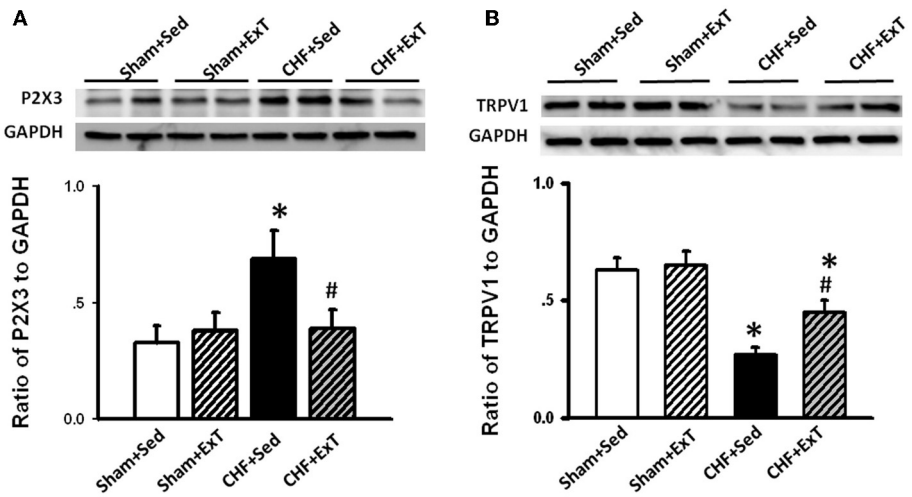

FIGURE 10 | Western blot data showing the protein expression of P2X3 (A) and TRPV1 (B) receptors in L4/L5 dorsal root ganglion (DRG) in Sham+Sed, Sham+ExT, CHF+Sed, and CHF+ExT rats. Data are expressed as Mean ± SE. $n = 6$/each group. $^*P < 0.05$ vs. sham+Sed, $\#P < 0.05$ vs. CHF+Sed. [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

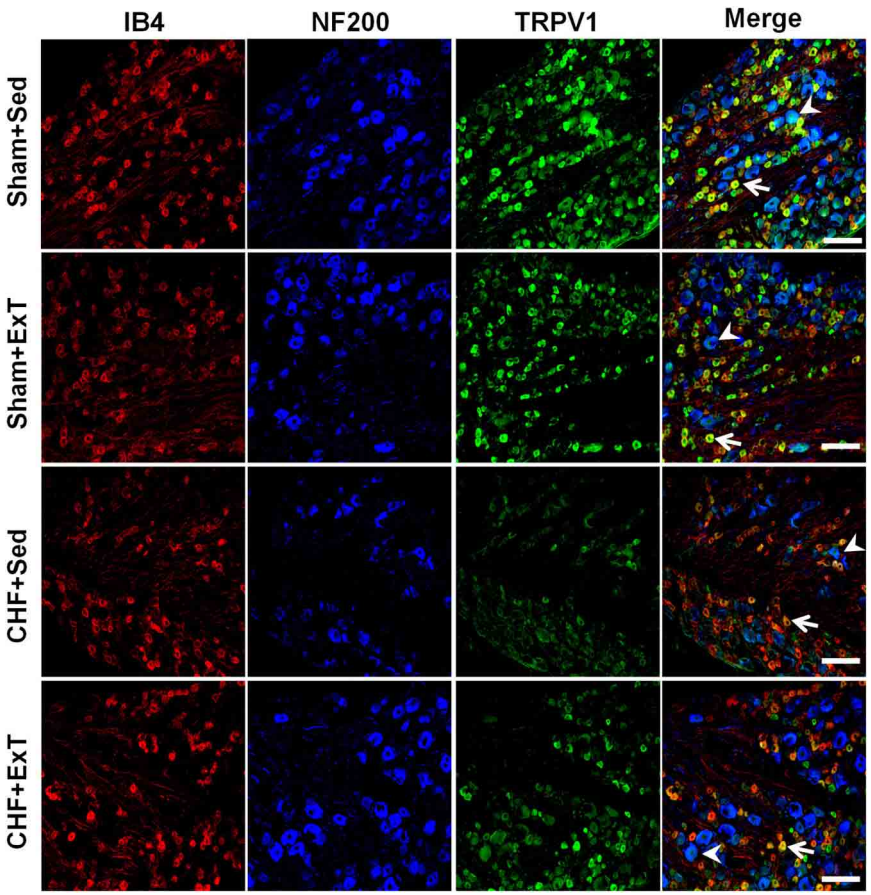

FIGURE 11 | Immunohistochemical data showing the protein expression of TRPV1 receptors in L4/L5 dorsal root ganglion (DRG) in sham and CHF rats. IB4, a C-fiber neuron marker; NF200, an A-fiber neuron marker. White Bar = 100 μm. White arrow represents double staining of TRPV1 with IB4, white arrowhead represents double staining of TRPV1 with NF200. [Reprinted from Wang et al. (2012). Copyright @ 2012 American Heart Association. Used with permission.]

should be noted that similar to TRPV1 receptors, acid-sensing ion channels (ASICs), which open when exposed to an extracellular pH of 7.0 or less, are also localized to group IV fibers and contribute to the metaboreceptor component of the EPR (Chen et al., 1998; Zhang and Canessa, 2001; McCord et al., 2009). However, the role of ASIC channels in mediating the blunted metaboreflex as well as the desensitization of group IV afferents in CHF is largely unknown. Whether ASIC channels are involved in the ExT-induced improvement of the blunted metaboreflex as well as the densitization of group IV afferents in CHF remains unclear.

OTHER POTENTIAL MECHANISMS

Other peripheral mechanisms may play a role in mediating the beneficial effects of ExT on reversing abnormal muscle afferent activity in CHF. For example, due to underperfusion of skeletal muscle in CHF, there is release of reactive oxygen species and inflammation. Augmented ROS production is strongly associated with endothelial dysfunction and may contribute to the exaggerated sympatho-excitatory response to exercise in CHF (Thomas et al., 2001; Thomas and Segal, 2004). In normal rats, we demonstrated that hindlimb infusion of a superoxide dismutase inhibitor increased ROS production within the skeletal muscle and augmented the pressor response to static muscle contraction (Wang et al., 2009). This sympatho-excitatory response was significantly attenuated by intra-arterial infusion of either a superoxide dismutase mimetic Tempol or an NADPH-oxidase inhibitor apocynin (Wang et al., 2009), indicating that ROS plays an excitatory role in modulation of the EPR. We recently demonstrated (Wang et al., 2011) that the both Tempol and a membrane permeable superoxide dismutase, polyethylene glycol-superoxide dismutase (PEG-SOD) attenuated sodium channel activity in muscle afferent neurons in rats. Because we provided evidence that sodium channels in muscle afferent neurons are critical for the genesis of the EPR (Wang et al., 2011), the inhibitory effect of ROS scavengers (Tempol and PEG-SOD) on sodium channel activity in muscle afferent neurons indicates that ROS modulates the EPR by affecting sodium channel activity in muscle afferents. In contrast, the studies from Koba et al. (2009) and McCord et al. (2011) found that injection of Tempol into hindlimb and trapping the circulation to maximize the local effects of the drug were unable to verify that Tempol attenuated the pressor response to static contraction in normal rats, indicating that local ROS

in skeletal muscle did not modulate the EPR in normal state. However, it should be noted that these investigators did not measure the EPR function immediately after stopping the trap protocol but rather during a 30-min of reperfusion after trapping. This may exhaust or minimize the pharmacologic effect of Tempol by either dynamic metabolism or ischemic-reperfusion. Therefore, whether local ROS in skeletal muscle is involved in the modulation of the EPR in the normal state is still controversial. Direct evidence from muscle afferent recording is needed to address this discrepancy. However, in rats with CHF induced by myocardial infarction, the entrapment of Tempol within the hindlimb circulation did produce a marked reduction in BP, HR, and renal SNA in response to the activation of the EPR (Koba et al., 2009). Collectively, these data suggest that increases in ROS generation (oxidative stress) in the hindlimb skeletal muscle contributes to the exaggerated cardiovascular response to stimulation of the EPR in CHF. With regard to the antioxidant and anti-inflammatory effects of ExT (Linke et al., 2005; Batista et al., 2010) in CHF, it is reasonable to speculate that the decreased muscle ROS level by ExT may bring the exaggerated EPR close to normal. Clearly, further research is needed in this area.

FUTURE DIRECTIONS

The exaggerated sympatho-excitation during exercise potentially increases cardiovascular risk and contributes to exercise intolerance during physical activity in CHF patients (Grassi and Mancia, 1999; Piepoli et al., 1999; Smith et al., 2006a). A therapeutic strategy for preventing or slowing the progression of the exaggerated EPR may significantly improve symptoms of exercise intolerance and reduce cardiovascular risk in CHF patients. This review has summarized the evidence from human and animal experiments suggesting that that long-term ExT has a beneficial effect on the exaggerated EPR in the CHF state. Evidence from our animal studies indicates that ExT in the early stage of CHF (2 weeks after coronary ligation) has a "protective" effect (rather than a "curative" effect) on the exaggerated EPR-evoked sympatho-excitation during exercise in CHF since the exaggerated EPR has not developed (Smith et al., 2003) at that time point. In addition, this training strategy also has a protective effect on the elevated resting sympathetic tone in CHF. An important clinical relevance of these findings is that patients recovering from myocardial infarction can take advantage of this early ExT strategy to slow or improve the symptoms associate with CHF. However, whether the benefits of this strategy can be applied for all degrees of CHF patients is unclear. Clearly, further studies are necessary for to determine which CHF patients will derive maximal benefit from ExT. It will also be important to determine what dose (i.e., duration and intensity) of ExT can be tolerated safely in CHF patients and still be effective. Furthermore, whether ExT can improve functional capacity of patients with more established CHF and an exaggerated EPR remains unclear. Piepoli et al. (1996) reported that 6-weeks of forearm training partially reversed the exaggerated exercise-evoked sympatho-excitation, vasoconstrictor response and ventilatory drive in patients with CHF. Whether central command is also involved in these ExT-mediated benefits remains largely unknown. Further animal studies are necessary to isolate the contribution of central command to the ExT-mediated benefits during exercise.

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Selective enhancement of glutamate-mediated pressor responses after $GABA_A$ receptor blockade in the RVLM of sedentary versus spontaneous wheel running rats

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Overactivity of the sympathetic nervous system (SNS) is a hallmark of many cardiovascular diseases. It is also well-known that physical inactivity independently contributes to cardiovascular diseases, likely in part via increased SNS activity. Recent work from our laboratory has demonstrated increased SNS responses in sedentary animals following either direct activation or disinhibition of the rostral ventrolateral medulla (RVLM), an integral cardiovascular brainstem region. These data led us to hypothesize that the interaction between excitation and inhibition of the RVLM is altered in sedentary versus physically active animals. To test this hypothesis, we recorded mean arterial pressure (MAP) and lumbar sympathetic nerve activity (LSNA) in Inactin anesthetized rats that were housed for 8–12 weeks with or without access to a running wheel. Pressor responses to direct activation of the RVLM with glutamate were similar between groups under intact conditions. However, blockade of γ-aminobutyric acid $(GABA)_A$ receptors with bicuculline selectively enhanced pressor responses to glutamate in sedentary animals. Interestingly, LSNA responses to glutamate were not enhanced in sedentary versus active animals in the presence or absence of tonic GABAergic tone. These results suggest that sedentary compared to active conditions enhance GABAergic inhibition of glutamate-sensitive neurons in the RVLM that are involved in blood pressure regulation, and by mechanisms that do not involve LSNA. We also speculate that regular physical activity has differential effects on SNS activity to specific vascular beds and may reduce the risk of developing cardiovascular diseases via changes occurring in the RVLM.

Keywords: blood pressure, exercise, brainstem, glutamate, GABA

INTRODUCTION

There is now convincing evidence that cardiovascular diseases including hypertension and heart failure are associated with overactivity of the sympathetic nervous system (SNS) (Zucker et al., 2001; Esler et al., 2003; Fisher et al., 2009; Malpas, 2010). Since one of the primary risk factors for cardiovascular disease is a lack of regular exercise (i.e., a sedentary lifestyle), it is not surprising that studies have also demonstrated evidence of sympathetic overactivity in sedentary versus physically active individuals (Meredith et al., 1991; Mueller, 2010). In particular, we and others have proposed that changes in sympathetic regulation in sedentary versus physically active animals are due to alterations in the rostral ventrolateral medulla (RVLM), an important brainstem region involved in sympathetic outflow (Becker et al., 2005; Martins-Pinge et al., 2005; Mueller, 2010). Indeed, RVLM neurons receive a variety of cardiovascular and exercise-related inputs (Dampney, 1994a,b; Guyenet and Stornetta, 2004). Ionotropic glutamate receptors in the RVLM appear to mediate pressor response to activation of skeletal muscle afferents by contraction or direct stimulation (Bauer et al., 1989; Kiely and Gordon, 1993; Ally, 1998). Despite the evidence in favor of a role for the RVLM in the acute response to exercise, the mechanisms by which chronic exercise (or lack thereof) alters control of SNS activity at the level of the RVLM remain to be fully elucidated.

The activity of RVLM neurons is regulated primarily by the excitatory neurotransmitter glutamate and by the inhibitory neurotransmitter γ-amino butyric acid (GABA), with contributions from other neurotransmitters as well (Dampney, 1994a; Guyenet and Stornetta, 2004; Pilowsky et al., 2008). Recent data from our laboratory and others have suggested that sedentary versus physically active conditions produce a relative enhancement in the responsiveness of RVLM neurons to direct excitation by glutamate but not all neurotransmitters (Becker et al., 2005; Martins-Pinge et al., 2005; Mueller, 2007). In addition, alterations in GABAergic neurotransmission at the level of the RVLM have been proposed to contribute to changes in sympathoexcitation in a variety of animal models (Adams et al., 2007; Huber and Schreihofer, 2011), including models of physical activity and inactivity (Moffitt et al., 2002; Mueller, 2007). Although under acute experimental conditions, glutamate and GABA can provide parallel excitation and inhibition, respectively in the RVLM (Miyawaki et al., 1996), there are no studies to our knowledge that have examined this interaction in a chronic model of physical (in)activity.

The primary purpose of this study was to examine the influence of sedentary versus physically active conditions on the interaction between glutamate-mediated excitation and GABAergic-mediated inhibition at the level of the RVLM. Similar to our previous studies (Mueller, 2007; Mischel and Mueller, 2011), we hypothesized that sedentary versus physically conditions would result in enhanced pressor and sympathoexcitatory responses to direct excitation of the RVLM and that these responses would be further augmented by blockade of tonic GABAergic transmission. To test these hypotheses, we recorded blood pressure, heart rate (HR), and lumbar sympathetic nerve activity (LSNA) responses to RVLM microinjections in sedentary and physically active rats.

MATERIALS AND METHODS

DRUGS

Inactin, L-glutamate, and bicuculline methiodide were obtained from Sigma Chemical (St. Louis, MO). Drugs used for microinjection were dissolved in artificial cerebrospinal fluid (aCSF) in which pH had been adjusted to 7.3–7.5 using sodium hydroxide or hydrochloric acid.

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of Wayne State University and conducted in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals." All animals received food (Purina Lab Diet #5001, Purina Mills, Richmond, IN) and tap water *ad libitum*.

DAILY SPONTANEOUS RUNNING

Forty-nine male Sprague–Dawley rats (initially 75–100 g, Harlan, Indianapolis, IN) were used for these studies. Animals were housed individually in cages with (physically active group, $n = 24$) or without running wheels (sedentary group, $n = 25$) for 8–12 weeks. Running wheels were purchased from a national vendor (Tecniplast, Eaton, PA; wheel diameter 34 cm diameter). Running distances were measured via bicycle computers (Sigma Sport, Olney, IL) calibrated to the diameter of the running wheel and recorded daily by laboratory personnel. Previous studies have demonstrated that rats allowed to run spontaneously on running wheels exhibit increases in maximal oxygen consumption, increases in heart weight-to-body weight ratio, and other exercise-related adaptations (Nelson et al., 2010).

SURGICAL PROCEDURES

Following 8–12 weeks of sedentary or physically active conditions, animals were instrumented acutely for brainstem microinjections while recording mean arterial pressure (MAP), HR, and LSNA (Mueller, 2007; Mischel and Mueller, 2011). Briefly, animals were anesthetized with isoflurane (2% in 100% O2) so that arterial and venous femoral catheters could be implanted to measure arterial pressure and for the administration of drugs, respectively. Following a midline abdominal incision, the abdominal aorta was gently retracted and a section of the lumbar chain was isolated caudal to the renal vein. The nerve was carefully placed on silver wire electrodes before encasing in polyvinylsiloxane gel (Darby Dental Supply Co., Inc., Westbury, NY). The abdominal incision was sutured around the electrode as it exited the peritoneal cavity. A ground wire was attached to the incision to reduce noise. A tracheostomy was performed and the animals were ventilated artificially with a mixture of isoflurane and oxygen (2% in 100% O2) via an endotracheal tube. Rats were placed in a prone position in a stereotaxic device (Kopf, Tujunga, CA) and following a midline incision at the level of the occipital bone, the brainstem was exposed by retracting the underlying muscles, performing a partial occipital craniotomy, and removing the atlanto-occipital membrane.

Once all surgical procedures were complete, an initial infusion of Inactin (thiobutabarbital sodium, 0.025 ml/min, 100 mg/kg, i.v.) was performed over 20–30 min during which the isoflurane was slowly reduced. Following the infusion, Inactin was given in supplemental doses (5 mg, i.v.) until an appropriate level of anesthesia was maintained in the absence of isoflurane. Anesthesia was deemed appropriate by lack of a withdrawal reflex to firm pinch. Animals continued to be ventilated (60–80 breaths/min) with a mixture of 100% O_2 and room air. Arterial blood gases were determined and were maintained in the physiological range ($P_{O_2} > 100$ mmHg, P_{CO_2} between 35 and 40 mmHg) by adjusting the rate or volume of the ventilator. Body temperature was maintained near 37°C via the use of a heating pad and measured throughout the experiment with a rectal thermometer. In order to diminish outside electrical noise, experiments were performed inside a Faraday cage.

MICROINJECTIONS

We performed microinjections based on previously published techniques in our laboratory (Mueller and Hasser, 2006; Mueller, 2007). Briefly, once the animal was in the stereotaxic frame, the head was positioned at a downward angle such that calamus scriptorius was 2.4 mm caudal to the interaural line and the brainstem oriented in the horizontal plane (Kiely and Gordon, 1994; Moffitt et al., 2002). Triple-barrel glass micropipettes were drawn by a pipette puller to an outside tip diameter of 30–60 μm, filled with appropriate drugs, and inserted into the dorsal surface of the brainstem with the use of a surgical dissecting microscope. The RVLM was located with the following coordinates using calamus scriptorius as a reference point: 0.9–1.1 mm rostral and 1.7–2.2 mm lateral to calamus scriptorius, and 3.5–3.7 mm ventral to the dorsal surface of the medulla. Micropipettes were connected via polyethylene tubing to a commercially available picoejection system (Toohey Company, Fairfield, NJ). The volume of microinjections was monitored directly by visualizing the meniscus in each barrel with the aid of a compound microscope (150×) that contained a calibrated reticule. The RVLM was functionally identified by observing pressor and sympathoexcitatory responses to a standard dose and volume of glutamate (10 mM, 30 nl). Microinjection sites were marked with 2% Chicago sky blue dye (30 nl). At the end of the experiments, animals were overdosed with Fatal-Plus euthanasia solution (Vortech, Dearborn, MI, 0.2 ml) and brains were removed and placed in 4% phosphate buffered formalin solution. Following post fixation, brains were transferred to 30% sucrose for a minimum 48-h infiltration. The hindbrain was frozen and cut into 30 μm sections on a cryostat. Coronal sections were mounted on gel-coated slides and a

bright-field microscope was used to determine the center of the dye spot and its location in the brainstem with the aid of a rat brain atlas (Paxinos and Watson, 2007). The dye spot location was represented graphically on a modified diagram from the rat atlas (Paxinos and Watson, 2007).

PROTOCOL #1—EFFECT OF INCREASING CONCENTRATIONS OF GLUTAMATE ON RESPONSES TO EXCITATION OF THE RVLM IN SEDENTARY AND PHYSICALLY ACTIVE RATS

We tested the hypothesis that responses to excitation of the RVLM were enhanced in sedentary animals compared to animals that ran spontaneously on running wheels. Unilateral microinjections of specific concentrations of glutamate (1, 10, and 100 mM) were performed at a constant volume of 30 nl (30, 300, and 3000 pmol total, respectively). Specific concentrations were ejected from individual barrels of the triple-barrel micropipette, performed in a random order, and a minimum of 5 min of recovery time was allowed between responses. Control injections of vehicle (i.e., aCSF) produced little or no response in MAP, HR, and LSNA (see Results).

PROTOCOL #2—EFFECT OF $GABA_A$ RECEPTOR BLOCKADE ON RESPONSES TO GLUTAMATE

We hypothesized that responses to glutamate would be enhanced in sedentary rats in the absence of tonic GABAergic tone compared to animals that ran spontaneously on running wheels. To test this hypothesis, GABA receptors were blocked unilaterally with the $GABA_A$ receptor antagonist, bicuculline (5 mM, 60 nl, or 300 pmol total) prior to subsequent injections of glutamate. Responses to glutamate were compared before and 5, 15, 30, and 45 min after bicuculline. The concentration of bicuculline used was based on previous studies in which bicuculline was shown to inhibit $GABA_A$ receptor activation in the RVLM (Miyawaki et al., 2002; Moffitt et al., 2002; Horiuchi et al., 2004) or block arterial baroreflex-mediated changes in sympathetic nerve activity (Sun and Guyenet, 1985; Blessing, 1988; Dampney et al., 1988; Guyenet and Stornetta, 2004; Mueller, 2007).

PROTOCOL #3—EFFECT OF CONTROL INJECTIONS ON RESPONSES TO GLUTAMATE

Microinjections of glutamate were tested in the presence and absence of aCSF (60 nl) to determine the influence of vehicle, volume, or time on responses to repeat microinjections of glutamate. The time course and size of injections were similar to those used for bicuculline in Protocol #2.

DATA COLLECTION AND ANALYSIS

A computer data acquisition system (Power Lab, ADInstruments, Colorado Springs, CO) was used to collect all experimental data. Raw LSNA was monitored on a Tektronix oscilloscope and a Grass preamplifier (P511) was used to amplify (20,000×) and filter LSNA (3 kHz low pass and 30 Hz high pass filter). LSNA was electronically rectified, integrated, and averaged using a time constant of 28 ms. Changes in LSNA were calculated as a percentage of control prior to each microinjection. The average amplified voltage (20,000×) was also compared between all animals as an estimate of baseline sympathetic nerve activity. Hexamethonium (30 mg/kg) and atropine methyl bromide (1 mg/kg, i.v.) were administered as ganglionic blocking agents to determine background noise. Background noise was subtracted from each animal and the remaining signal was defined as LSNA.

STATISTICAL ANALYSIS

Baseline hemodynamic variables, body weights, and organ weights were analyzed by Student's *t*-test. MAP, HR, or LSNA changes to specific concentrations of glutamate were analyzed by Two-Way analysis of variance (ANOVA) with repeated measures. When ANOVA indicated a significant interaction, differences between individual means were assessed by *post-hoc* Holm-Sidak test according to a commercially available software package (SigmaStat 3.0, SPSS Inc., Chicago, IL). In one instance square root transformations were performed on MAP/Glutamate dose response data to achieve normality before Two-Way ANOVA. The transformation did not change the statistical outcome of the test ($p > 0.05$).

For protocols 2 and 3 (bicuculline and aCSF, respectively), data were combined initially and subjected to multivariate and repeated measures analyses using the General Linear Model (GLM) program in IBM SPSS for Windows (Version 19.0 Armonk, NY: IBM Corp). Based on both theory and the literature regarding the time course of bicuculline effects, our expectation was that any significant effects over the time points measured in both protocols (control, 5′, 15′, 30′) would be represented by the quadratic trend component (i.e., the two middle values would be higher than the first and fourth). Although preliminary analyses using both the multivariate and the repeated measures options gave similar results, the quadratic effects accounted for almost all of the between time variance. In the repeated measures analysis for the quadratic scores, we found both a significant time by experiment interaction and a time by group interaction ($p < 0.05$ for each). The former interaction justified testing the individual protocols (Bicuculline and aCSF) separately. We then performed simple main effects *t*-tests on the quadratic scores. As expected and borne out by the analysis, all of the effects revealed by ANOVA were due to bicuculline. As follow up to the significant group by quadratic time interaction (for bicuculline only), we analyzed the simple main effects of group at each time point using Holm–Sidak *post-hoc* tests provided by the Two-Way RM ANOVA. The results of these analyses are provided in the graphs and justify separation of the two protocols graphically.

For all analyses, a probability of $p < 0.05$ was considered statistically significant and *p*-values less than or equal to 0.1 for main effects are reported for clarity (Curran-Everett and Benos, 2004). Data are expressed as mean ± SEM.

RESULTS

SEDENTARY VERSUS PHYSICALLY CONDITIONS

Table 1 contains baseline characteristics in sedentary versus physically active animals. Physically active animals ran for an average of 10.4 ± 0.3 weeks which resulted in total distances ran of over 200 km (**Table 1**). Sedentary animals remained in their cages for similar periods of time without access to running wheels. Body weight was higher in sedentary animals ($p < 0.05$); however, there were no significant differences in baseline MAP, HR, or the average amplified LSNA voltage between groups. Similarly,

Table 1 | Baseline characteristics of sedentary versus physically active animals.

	Body weight (g)	MAP (mmHg)	HR (bpm)	LSNA (mV.s)	Total running distance (km)
Sedentary ($n = 25$)	407 ± 7*	102 ± 2	279 ± 4	1.22 ± 0.11	–
Physically active ($n = 24$)	385 ± 9	105 ± 2	284 ± 4	1.18 ± 0.17	220 ± 24

*MAP, mean arterial pressure; HR, heart rate; LSNA, average amplified lumbar sympathetic nerve voltage; *$p < 0.05$ compared to physically active animals.*

Table 2 | Baseline values for individual protocols.

	MAP (mmHg)	HR (bpm)	LSNA (mV.s)
GLUTAMATE DOSE-RESPONSE			
Sedentary ($n = 17$)	103 ± 2	276 ± 5	1.08 ± 0.11
Physically active ($n = 16$)	104 ± 2	278 ± 5	1.30 ± 0.25
GLUTAMATE ± BICUCULLINE			
Sedentary ($n = 10$)	106 ± 2	294 ± 6	1.61 ± 0.17
Physically active ($n = 10$)	105 ± 2	289 ± 8	1.18 ± 0.21
GLUTAMATE ± ACSF			
Sedentary ($n = 8$)	107 ± 3	299 ± 7	1.73 ± 0.28
Physically active ($n = 7$)	97 ± 6	280 ± 6	1.65 ± 0.39

MAP, mean arterial pressure; HR, heart rate; LSNA, average amplified lumbar sympathetic nerve voltage; ACSF, artificial cerebrospinal fluid. There were no significant differences in any baseline variable between groups for any of the protocols.

baseline MAP, HR, and average amplified LSNA voltage was not significantly different between groups prior to each protocol (**Table 2**).

PROTOCOL #1—EFFECT OF INCREASING CONCENTRATIONS OF GLUTAMATE IN THE RVLM OF SEDENTARY VERSUS PHYSICALLY ACTIVE RATS

Representative MAP and LSNA responses in one sedentary and one physically active rat to RVLM microinjection of glutamate (30 nl, 10 mM) are shown in **Figures 2A,C**. Averaged peak changes in MAP, HR, and LSNA to microinjections of glutamate (30 nl, 1–100 mM) are shown in **Figure 1**. As in our previous studies (Mueller, 2007; Mischel and Mueller, 2011), glutamate produced concentration-dependent increases in MAP, HR, and LSNA ($p < 0.001$, main effect of dose for all three variables). The pressor responses to increasing concentrations of glutamate were not statistically different between sedentary and physically active animals ($p = 0.10$ for main effect) and the lack of a significant interaction did not allow for testing for differences at individual concentrations between groups ($p = 0.872$). HR responses to glutamate microinjections into the RVLM were small in general (<20 bpm) and there was neither a main effect of sedentary condition ($p = 0.215$) nor a significant interaction ($p = 0.489$) that would allow for testing differences at individual doses. Lastly, unlike our previous studies in which we demonstrated enhanced sympathoexcitation in splanchnic (Mischel and Mueller, 2011) or lumbar sympathetic nerves (Mueller, 2007) of sedentary animals, increases in LSNA were not significantly different between sedentary and physically active animals ($p = 0.576$ for main effect; $p = 0.646$ for interaction).

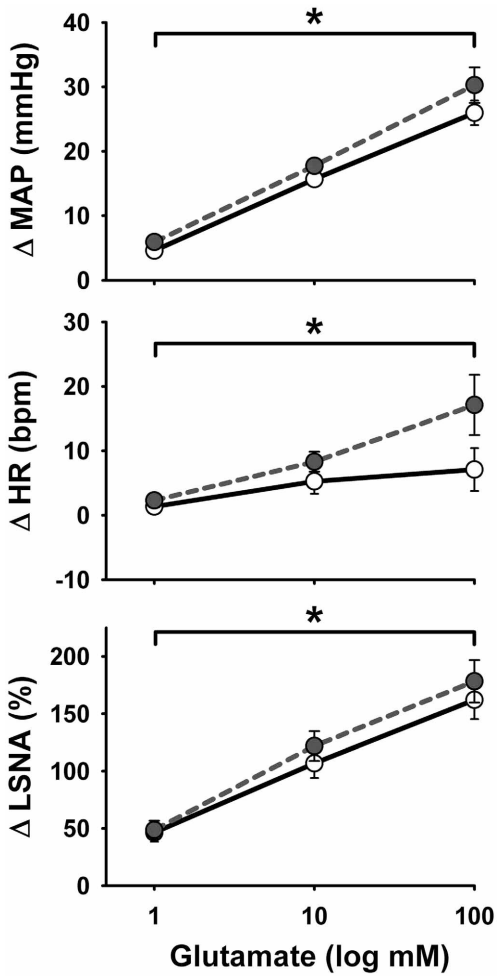

FIGURE 1 | Peak mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) responses to activation of the rostral ventrolateral medulla with increasing concentrations of glutamate (30 nl) in sedentary (filled circles, $n = 16$) and physically active rats (open circles, $n = 17$). There was a significant effect of glutamate concentration to increase MAP, HR, and LSNA (*$p < 0.05$) but responses did not differ between groups.

PROTOCOL #2—EFFECT OF $GABA_A$ RECEPTOR BLOCKADE ON RESPONSES TO GLUTAMATE

Representative MAP and LSNA responses in one sedentary and one physically active rat to RVLM microinjection of glutamate (30 nl, 10 mM) before and after bicuculline are shown in **Figure 2**. Averaged peak changes in MAP, HR,

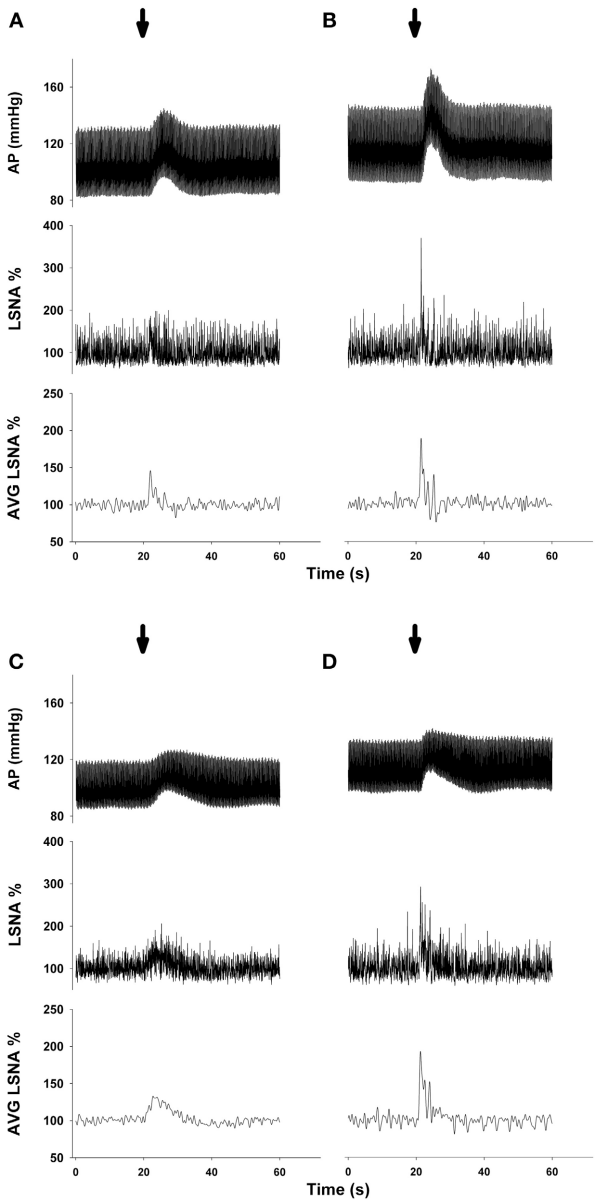

FIGURE 2 | Representative examples of arterial pressure (AP) and LSNA responses to unilateral microinjections of glutamate (30 nl, 10 mM) into the rostral ventrolateral medulla before and after microinjection of the $GABA_A$ antagonist bicuculline (60 nl, 5 mM). **(A)** Control response to glutamate in one sedentary rat. **(B)** Response to glutamate in the same sedentary rat 5 min after bicuculline. **(C)** Control response to glutamate in one physically active rat. **(D)** Response to glutamate in the same physically active rat 5 min after bicuculline. Note enhanced pressor response only in sedentary rat after bicuculline and enhanced LSNA response in both sedentary and physically active rats following bicuculline. Arrow represents timing of glutamate microinjection. Abbreviations are as defined in **Figure 1**.

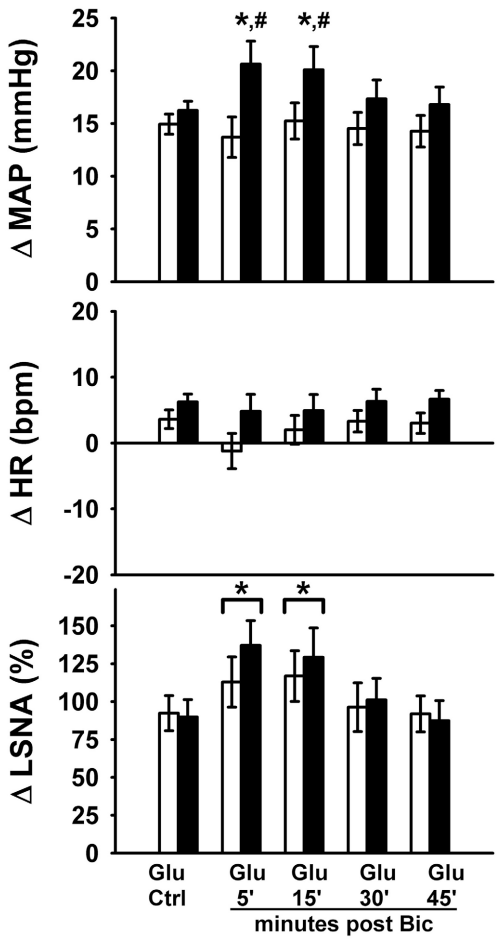

FIGURE 3 | Peak MAP, HR, and LSNA responses to activation of the rostral ventrolateral medulla with glutamate (10 mM, 30 nl) in the presence or absence of $GABA_A$ receptor blockade with bicuculline (5 mM, 60 nl). Sedentary (filled bars, $n = 10$) and physically active rats (open bars, $n = 10$) had similar pressor and sympathoexcitatory response to glutamate prior to bicuculline (Glu Ctrl). Following bicuculline, sedentary animals exhibited a selective enhancement in pressor responses to glutamate at 5 and 15 min ($^*p < 0.05$), resulting in significantly greater pressor responses compared to physically active animals ($^\#p < 0.05$ at 5 and 15 min). LSNA response were similarly enhanced in both groups at 5 and 15 min (*with bracket, $p < 0.05$). Responses in both groups returned to control within 30 min. The small HR responses were also significantly different between groups ($p < 0.05$). Abbreviations are as defined in **Figure 1**.

and LSNA to microinjections of glutamate (30 nl, 10 mM) with and without bicuculline are shown in **Figure 3**. As in Protocol #1, control responses to glutamate (30 nl, 10 mM) produced increases in MAP and LSNA and small changes in HR (<10 bpm) in both sedentary and physically active animals. Unilateral injections of bicuculline increased baseline MAP (Sedentary $\Delta 9 \pm 2$ mmHg; Physically Active $\Delta 13 \pm 2$ mmHg), HR (Sedentary $-\Delta 7 \pm 6$ bpm; Physically Active $\Delta 5 \pm 2$ bpm), and LSNA (Sedentary $\Delta 3 \pm 4\%$; Physically Active $\Delta 9 \pm 3\%$) prior to the subsequent injection of glutamate at 5 min. These changes were not significantly different between groups ($p > 0.05$). Similar to control injections of glutamate, microinjection of glutamate 5 min following bicuculline also produced increases in MAP, HR, and LSNA. However, increases in MAP produced by glutamate injections at both 5 and 15 min after

bicuculline were selectively enhanced in sedentary animals such that responses were significantly greater in sedentary versus physically active animals ($p < 0.05$ for main effects, interaction, and *post-hoc* tests). Glutamate-induced pressor responses at 30 and 45 min were similar to glutamate control responses suggesting recovery of responses and consistent with the time course of action of bicuculline in producing disinhibition of the RVLM (Miyawaki et al., 2002; Mueller, 2007). Interestingly, glutamate-induced increases in LSNA were also enhanced by bicuculline at 5 and 15 min in both groups ($p < 0.05$ for main effect and multiple comparisons within main effect) but were not significantly different between sedentary and physically active animals ($p > 0.05$ for main effect). At 30 and 45 min after bicuculline, increases in LSNA produced by glutamate were not significantly different than control glutamate responses ($p > 0.05$ for multiple comparisons within main effect) and were not different between groups, also suggesting full recovery of responses from bicuculline in a manner consistent with its time course of action. Lastly, bicuculline produced a slight but significant main effect on the small HR responses ($p < 0.05$ for main effect), but there was neither a main effect of sedentary condition nor interaction to allow testing for further differences ($p > 0.05$ for both).

PROTOCOL #3—EFFECT OF CONTROL INJECTIONS ON RESPONSES TO GLUTAMATE (FIGURE 4)

To control for potential volume-, vehicle-, or time-related effects of bicuculline injections on responses to glutamate microinjections, we performed a series of experiments utilizing microinjections of the bicuculline vehicle (i.e., aCSF) before and after repeat microinjections of glutamate into the RVLM. As in Protocols 1 and 2, control responses to glutamate (30 nl, 10 mM) produced increases in MAP and LSNA and small changes in HR (<10 bpm) which were not different between sedentary and physically active animals. Similar to previous studies (Mueller, 2007), microinjections of aCSF (60 nl) had little or no effect on baseline MAP (Sedentary $-\Delta 2 \pm 2$ mmHg; Physically Active $\Delta 0 \pm 1$ mmHg), HR (Sedentary $-\Delta 1 \pm 3$ bpm; Physically Active $-\Delta 1 \pm 1$ bpm), or LSNA (Sedentary $\Delta 1 \pm 3\%$; Physically Active $\Delta 0 \pm 1\%$). Responses to repetitive microinjections of glutamate at 5, 15, and 30 min were also not significantly different than control glutamate microinjections for increases in MAP and LSNA, and small changes in HR ($p > 0.05$ for main effect of aCSF). Lastly, responses to repetitive microinjections were not significantly different between groups ($p > 0.05$ for main effect of physically active condition).

HISTOLOGY (FIGURE 5)

Microinjection sites marked with 2% Chicago sky blue dye (30 nl) were recovered in 16 sedentary and 17 physically active animals used in these studies. As in our previous studies (Mischel and Mueller, 2011), all microinjections sites were near the caudal pole of the facial nucleus and located ventral to the nucleus ambiguus, lateral to the pyramidal tract, and medial to the spinal trigeminal tract, which characterize the boundaries of the RVLM established by previous studies (Guyenet, 2006; Schreihofer and Sved, 2011).

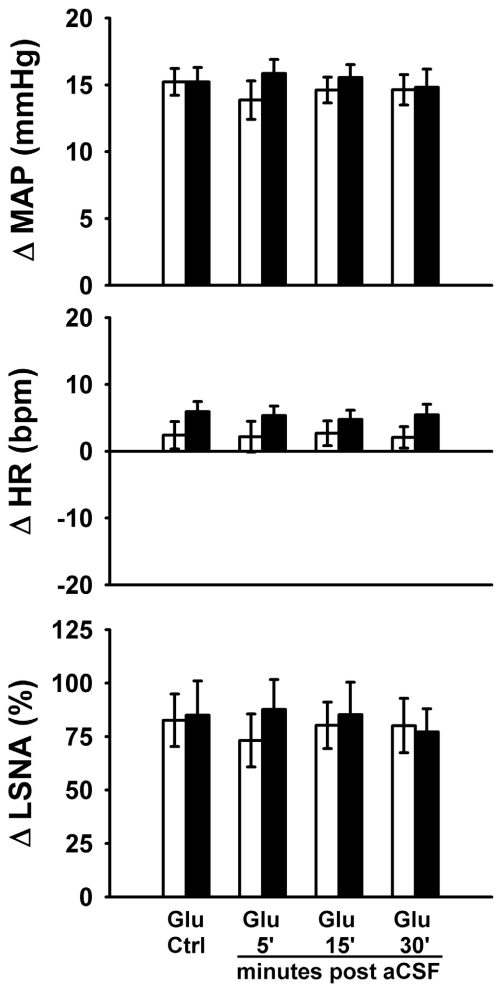

FIGURE 4 | Peak MAP, HR, and LSNA responses to activation of the rostral ventrolateral medulla with glutamate (10 mM, 30 nl) in the presence or absence of artificial cerebrospinal fluid injections (aCSF, 60 nl). aCSF had no significant effect on responses in either sedentary (filled bars, $n = 8$) or physically active rats (open bars, $n = 7$) over the entire time course of injections and there were no significant differences between groups. Abbreviations are as defined in **Figure 1**.

DISCUSSION

The primary purpose of this study was to examine the influence of sedentary versus physically active conditions on the interaction between glutamate-mediated excitation and GABAergic-mediated inhibition at the level of the RVLM. We hypothesized that sedentary versus physically conditions would result in enhanced pressor and sympathoexcitatory responses to direct excitation of the RVLM and that these responses would be further augmented by removal of tonic GABAergic transmission. Our hypothesis was confirmed in part by the selective enhancement in the pressor responses to glutamate in sedentary animals following GABA receptor blockade in the RVLM. In contrast, and contrary to our hypothesis, lumbar sympathetic nerve responses to direct excitation of the RVLM were similar in both groups under intact conditions or following

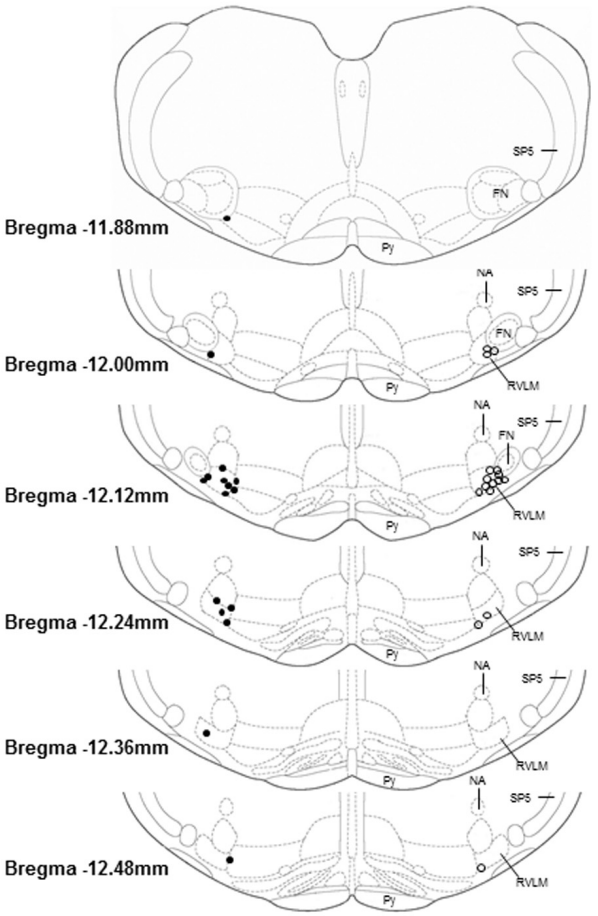

FIGURE 5 | Histological representation of microinjection sites plotted on brainstem sections that have been modified from a standard rat atlas (Paxinos and Watson, 2007). Unilateral injection sites from sedentary (filled circles, $n = 16$) and physically active animals (open circles, $n = 16$) are plotted on opposite sides for clarity. All microinjection sites were associated with pressor and sympathoexcitatory responses to glutamate; were confined within the general boundaries of the rostral ventrolateral medulla (RVLM); and were distributed similarly between groups. FN, facial nucleus; NA, nucleus ambiguus; Py, pyramidal tract; SP5, spinal trigeminal tract.

$GABA_A$ receptor blockade. Collectively, these data provide several important new findings that increase our understanding of alterations in neural control of the circulation between sedentary and physically active animals. (1) Sedentary versus physically active conditions result in enhanced GABAergic modulation of glutamate-sensitive neurons in the RVLM that are involved in blood pressure regulation. (2) Sedentary versus physically active conditions appear to enhance the excitability of glutamate-sensitive neurons in the RVLM in the absence of GABAergic modulation. (3) Enhanced pressor responses observed in sedentary animals following $GABA_A$ receptor blockade do not appear to be mediated by lumbar sympathetic nerves. We speculate that sedentary versus physically active conditions produce alterations in RVLM neurons which lead to differential changes in regulation of blood pressure and may contribute to the increased prevalence of cardiovascular disease in sedentary individuals.

One of the most important and novel observations in our study was the enhanced pressor response to activation of the RVLM following blockade of $GABA_A$ receptors in sedentary but not physically active animals. These data suggest two important concepts regarding the influence of sedentary versus physically active conditions on the regulation of blood pressure at the level of the RVLM. First, GABA appears to modulate glutamate-mediated increases in arterial pressure produced at the level of the RVLM differently in sedentary versus physically active animals. Second, in the absence of modulation by $GABA_A$ receptors, RVLM neurons involved in blood pressure regulation appear to be more sensitive to glutamatergic excitation in sedentary animals. Enhanced GABAergic modulation in sedentary animals appears to restrain excessive blood pressure responses from occurring under intact conditions since responses were similar in physically active animals. However, when GABAergic inhibition is removed (via receptor blockade in this study), the enhanced pressor response to glutamate is allowed to be fully expressed resulting in significantly larger pressor responses compared to physically active animals. Consistent with this finding are previous studies in which enhanced sympathoexcitation has been observed in sedentary animals under conditions in which GABAergic transmission would be expected to be reduced. For example, enhanced sympathoexcitation occurs in sedentary animals during baroreceptor unloading (DiCarlo and Bishop, 1988; Negrao et al., 1993) and inhibition of the nucleus tractus solitarius (Mueller and Hasser, 2006). By performing microinjections directly into the RVLM, our experiments provide evidence consistent with changes occurring at the level of the RVLM. Taken together, evidence from our study and others (Smith and Barron, 1990; Huber and Schreihofer, 2011) indicate that altered GABAergic modulation at the level of the RVLM plays an important role in changes in the regulation of blood pressure under physiological and pathophysiological states.

The observation of enhanced pressor responses to glutamate after blockade of $GABA_A$ receptors also supports others' and our previous contention that sedentary conditions alter excitatory as well as inhibitory mechanisms at the level of the RVLM (Becker et al., 2005; Martins-Pinge et al., 2005; Mueller, 2010). Indeed, greater blood pressure or sympathoexcitatory responses to microinjections of glutamate in the RVLM have been observed in sedentary compared to physically active animals (Martins-Pinge et al., 2005; Mueller, 2007; Mischel and Mueller, 2011). The fact that responses to other excitatory neurotransmitters, such as angiotensin II, are not enhanced in sedentary animals (Becker et al., 2005) suggests that alterations in excitatory neurotransmission may be more specific to glutamatergic mechanisms; however, this remains to be fully established. The functional consequence of greater glutamatergic excitation of the RVLM may be reflected in an increased activation of the RVLM under various sympathoexcitatory conditions. For example, previous studies have demonstrated a greater number of Fos activated RVLM neurons in sedentary versus physically active animals following acute exercise (Ichiyama et al., 2002) and acute stress (Greenwood et al., 2003). As mentioned above, by activating the RVLM directly with

microinjections of glutamate our studies corroborate enhanced activation at the level of the RVLM observed in previous studies. In combination, these results and ours suggest important alterations at the level of the RVLM that contribute to differences between sedentary and physically active animals.

Since lumbar sympathetic nerve responses to glutamate were equally enhanced in both groups by pretreatment with bicuculline, these data imply that lumbar sympathetic nerves are not responsible for the enhanced pressor responses observed in sedentary animals. One of the more straightforward explanations is that activation of other sympathetic nerves during glutamate microinjections in the RVLM could contribute to the enhanced pressor responses in sedentary animals. Indeed, several different sympathetic nerves can be activated by stimulation of the RVLM (Adams et al., 2007; Mueller et al., 2011 for example). Further studies involving a broader survey of sympathetic nerves would be required to confirm which sympathetic nerve(s) contribute to the enhanced pressor responses.

It is also possible that enhanced blood pressure responses in sedentary animals could be related to other pressor-related mechanisms produced by activation of the RVLM. For instance, we have previously shown that increases in blood pressure produced by intravenous administration of the α_1-receptor agonist phenylephrine are enhanced in sedentary animals, suggesting some level of enhanced peripheral vasoconstriction (Mischel and Mueller, 2011). Since we did not observe a concentration-dependent enhancement of the pressor response under intact conditions even at the highest concentration of glutamate used (100 mM) we speculate that the greater pressor response is more likely due to enhanced activation of neurons controlling non-lumbar-mediated sympathetic nerve activity. Our studies emphasize the need to consider the highly integrative nature of centrally mediated changes in blood pressure and the multiple factors that could contribute to differences observed in various models (Osborn and Fink, 2010; Stocker et al., 2010; Mischel and Mueller, 2011; Osborn et al., 2011; Silva and Schreihofer, 2011; Osborn and Kuroki, 2012).

The lack of enhanced lumbar sympathetic nerve responses to activation of the RVLM in sedentary animals is in contrast to our previous study in which lumbar sympathetic nerve responses were significantly augmented in sedentary rats when compared to animals that were endurance trained on a treadmill (Mueller, 2007). It has been reported that rats allowed to run spontaneously on running wheels exhibit increases in maximal oxygen consumption, increases in heart weight-to-body weight ratio, and other exercise-related adaptations (Nelson et al., 2010). However, direct comparisons between treadmill and spontaneous wheel running studies should be done carefully given the number of differences between the models (e.g., voluntary vs. forced running; intensity, duration, length, and number of exercise bouts). Although animals in the current study ran over two kilometers per day on average and had lower body weights, these data alone do not imply that that these animals performed an equivalent type of exercise or have the same exercise capacity compared to treadmill trained animals in previous studies. Ultimately, these data highlight the importance of examining sympathetic nerve activity in the context of the different models of physical activity and inactivity.

To our knowledge there are only two other animal studies that have specifically examined the effects of chronic exercise on regulation of LSNA. Chen and colleagues reported modest changes in baroreflex control of LSNA in sedentary versus spontaneous wheel running rats (Chen and DiCarlo, 1996). Burgi and coworkers recently reported that resting lumbar nerve activity is reduced in treadmill trained versus sedentary Wistar–Kyoto rats (Burgi et al., 2011). In humans, the effects of exercise and inactivity on muscle sympathetic nerve activity appear to be fairly equivocal in terms of both resting (Ray and Hume, 1998) and reflex-mediated changes (Shoemaker et al., 1999; Fadel et al., 2001; Alvarez et al., 2005). Again these studies demonstrate the need for additional studies on the mechanisms by which different types of physical activity and inactivity influence regulation of sympathetic nerve activity to skeletal muscle.

TECHNICAL CONSIDERATIONS

Although previous studies have reported significant differences in baseline sympathetic nerve activity in various models of cardiovascular disease (Huber and Schreihofer, 2011; Mischel and Mueller, 2011; Silva and Schreihofer, 2011), comparisons of absolute levels of sympathetic nerve activity across different groups of animals warrants careful consideration (Guild et al., 2010; Burke et al., 2011). Since there were no differences in LSNA voltages between groups, we chose to express our data in terms of percent change to allow for an easier interpretation of the relative changes in sympathetic nerve activity. Furthermore, with our repetitive microinjection protocols we were able to examine nerve activity responses within animals over a time course that also demonstrated recovery of responses back to control values. Understanding that several important factors contribute to the voltage signal in a given recording (Guild et al., 2010; Burke et al., 2011) and group differences in the present study weren't significant, we have presented our nerve activity data in the more traditional and more easily interpreted format of percent change.

In order to complete our concentration response and repetitive microinjection protocols in the RVLM of rats while recording MAP, HR, and LSNA, we felt the use of anesthesia was necessary in these studies. The use of anesthesia allowed us to localize our RVLM microinjections more readily and successfully in a given animal since we were able to adjust the location of injection when necessary to achieve responses typically elicited from the RVLM. Furthermore, the use of triple-barrel glass micropipettes allowed us to inject different concentrations of glutamate or inject bicuculline or aCSF before and after multiple injections of glutamate without having to remove the micropipette from its position in the RVLM. Notwithstanding these advantages, we are fully aware that our use of anesthesia may have altered responses compared to those observed in conscious animals. Since we have been able to identify significant differences in our other studies using nearly identical preparations (Mueller and Hasser, 2006; Mueller, 2007; Mischel and Mueller, 2011), we are confident in the relative similarities and differences observed in the present study.

Given the time-consuming nature of our microinjections studies and the practicalities of generating the number of animals required for this study, we grouped experiments from animals that ran between 8 and 12 weeks. This can be considered a

limitation of our study since it did not allow us to distinguish between potential differences between separate groups of animals that ran for 8 vs. 12 weeks. The time course by which functional alterations occur in brain regions important in cardiovascular regulation in response to physical (in)activity is worthy of consideration for future studies.

One caveat to our studies is that we used a volume and concentration of bicuculline based on previously published studies (Miyawaki et al., 2002; Moffitt et al., 2002; Horiuchi et al., 2004), including a study from our own laboratory in which we rigorously tested the effectiveness of $GABA_A$ receptor blockade in the RVLM of physically active and sedentary rats (Mueller, 2007). Our expectation was that a similar volume (60 nl) and identical concentration (5 mM) of bicuculline used unilaterally would produce a similar level of blockade as to that used bilaterally in these previous studies. This line of thinking was reinforced by our experimental design in which we tested a higher volume of bicuculline (60 nl) against a smaller volume of glutamate (30 nl), both in the same micropipette, in order to maximize the chances that glutamate was only activating neurons in a region that had been affected by the antagonist. Nonetheless, the fact is that we did not perform experiments in the current study to confirm whether we achieved similar levels of blockade in both groups of animals. Consequently we cannot eliminate the possibility that $GABA_A$ receptors were blocked to a lesser extent in the wheel running group and provide an explanation for the reduced effects of bicuculline on the blood pressure response to glutamate. We contend, however, that it is hard to reconcile the collective results of the current study with this possibility. For example, bicuculline produced similar effects on baseline MAP and SNA in both groups while producing differential effects on the glutamate-induced pressor response. Bicuculline also similarly enhanced the LSNA responses in both groups in a time course consistent with the known actions of bicuculline observed in previous studies (Miyawaki et al., 2002; Moffitt et al., 2002; Horiuchi et al., 2004; Mueller, 2007). Thus, it seems hard to conclude that these different effects could all occur because bicuculline produced less effective blockade in the wheel running group.

SUMMARY

The results of this study are reflective of others which have implicated important neural mechanisms in cardiovascular disease states (Esler et al., 2001; Schlaich et al., 2004; Guyenet, 2006; Fisher et al., 2009). Indeed, altered regulation of SNS activity from brainstem and hypothalamic cardiovascular nuclei have been demonstrated in several animal models of cardiovascular disease that are sensitive to physical activity or inactivity (Moffitt et al., 2002; Mueller, 2010; Patel and Zheng, 2012). Interestingly, altered glutamatergic or GABAergic signaling in the RVLM appears to be common to many of these disease states (Moffitt et al., 2002; Sved et al., 2003; Wang et al., 2009; Mueller, 2010; Huber and Schreihofer, 2011). To our knowledge, this is the first study to demonstrate selectively enhanced blood pressure responses to activation of the RVLM following blockade of tonic GABAergic inhibition in sedentary versus physically active animals. These data suggest that both glutamatergic and GABAergic regulation of RVLM neurons involved in blood pressure regulation are altered under different physical activity conditions and we speculate that these may play important roles in the development of cardiovascular diseases that are more prevalent in sedentary individuals (Blair, 2009; Danaei et al., 2009). In addition, since elevated sympathetic activity has detrimental effects on the cardiovascular system via direct and indirect mechanisms (Fisher et al., 2009; Grassi et al., 2011), this study and others highlight the need for more effective therapies which can lower sympathetic output specifically from the CNS (Mueller, 2010; Grassi et al., 2011; Osborn et al., 2011; Osborn and Kuroki, 2012).

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Exercise pressor reflex function following acute hemi-section of the spinal cord in cats

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Cardiovascular disease is a leading cause of morbidity and mortality in patients post spinal cord injury (SCI). The prescription of exercise as a therapeutic modality for disease prevention in this population is promising. It is logical to suggest that the sooner an exercise program can begin the more benefit the patient will receive from the therapy. However, the time point after injury at which the requisite circulatory responses needed to support exercise are viable remains largely unknown. The skeletal muscle exercise pressor reflex (EPR) significantly contributes to cardiovascular control during exercise in healthy individuals. Experiments in patients with a chronic lateral hemi-section of the spinal cord (Brown-Séquard syndrome) suggest that the EPR, although blunted, is operational when examined months to years post injury. However, whether this critically important reflex remains functional immediately after lateral SCI or, in contrast, experiences a period of reduced capacity due to spinal shock has not been established. This study was designed to assess EPR function after acute lateral transection of the spinal cord. The EPR was selectively activated in seven decerebrate cats via electrically stimulated static contraction of the triceps surae muscles of each hindlimb before and after lateral hemi-section of the T_{13}–L_2 region of the spinal cord. Compared to responses prior to injury, increases in mean arterial pressure (MAP) were significantly decreased when contracting the hindlimb either ipsilateral to the lesion (MAP $= 17 \pm 3$ mmHg before and 9 ± 2 mmHg after) or contralateral to the lesion (MAP $= 22 \pm 5$ mmHg before and 12 ± 4 mmHg after). The heart rate (HR) response to stimulation of the EPR was largely unaffected by induction of acute SCI. The findings suggest that the EPR maintains the ability to importantly contribute to cardiovascular regulation during exercise immediately following a Brown-Séquard-like injury.

Keywords: blood pressure, heart rate, muscle afferents, spinal cord injury, cardiovascular disease

INTRODUCTION

Spinal cord injury (SCI) often leads to a reduction in physical activity and a loss of muscle mass as well as the generation of significant autonomic cardiovascular dysfunction (Hagen et al., 2012). In part as a result of these maladaptive responses to injury, the development of cardiovascular disease post SCI is a major concern and is now recognized as a leading cause of morbidity and mortality in the SCI patient (Myers et al., 2007). It has long been known that various forms of exercise can improve physical and cardiovascular fitness in individuals with SCI (Hicks et al., 2011; Phillips et al., 2011). Thus, the prescription of exercise as a therapeutic modality to prevent the development of cardiovascular disease after SCI is promising. It stands to reason that the earlier an exercise program can be undertaken in this patient group, the greater the impact the physical training will have as a preventative and health promoting measure. This being stated, the time point after injury at which the requisite cardiovascular adjustments needed to support exercise are operative remains relatively unknown. Therefore, understanding both the timing at which cardiovascular regulation is viable and the mechanisms underlying this control after SCI is critically important to the design and prescription of exercise in this patient population.

During exercise, two primary neural inputs are engaged that regulate the cardiovascular response to physical activity. The first, central command, is a feed-forward mechanism originating within the cerebral cortex that activates both descending motor neurons and cardiovascular control circuits in a parallel fashion (Goodwin et al., 1972). The second, the exercise pressor reflex (EPR), is a feed-back mechanism that likewise activates cardiovascular control centers when mechanically and chemically sensitive Group III and IV afferent fibers originating in skeletal muscle are stimulated during contraction (Kaufman et al., 1983; Mitchell et al., 1983; Hayes et al., 2005). Combined input from these two neural sources mediates the requisite circulatory

adjustments needed to meet the metabolic demands of working skeletal muscle; primarily, sympathetically mediated increases in blood pressure and cardiac function (Smith et al., 2006; Murphy et al., 2011). Given the significance of both central command and the EPR to neural cardiovascular control, it is important to understand the contribution of each during exercise after SCI.

To this end, a previous study was performed in patients designed to determine the contribution of central command and the EPR to the cardiovascular response to static exercise post SCI (Winchester et al., 2000). The investigation utilized individuals with Brown-Séquard syndrome; a condition characterized by a unique lateral hemi-section of the spinal cord which leaves the ipsilateral side of the body below the lesion with reduced motor function and normal sensation while the contralateral side typically has normal motor function and decreased sensation to stimuli transduced by small diameter afferent fibers. In the study, the cardiovascular responses elicited by both voluntary and electrically stimulated contractions of the limbs led the investigators to conclude that central command and the EPR remained operative maintaining their ability, at least in part, to induce the circulatory adjustments required to perform physical activity. While the investigation provided significant insight into the mechanisms of neural cardiovascular control in Brown-Séquard patients, it did so in a patient population that was months to years post injury. Whether this control is adequate to support exercise immediately following this type of SCI remains unknown. The EPR could be particularly susceptible to dysfunction directly after lateral spinal hemi-section due to the development of spinal shock; a condition in which reflexes originating caudal to the site of the spinal cord lesion are transiently and sometimes permanently depressed (Ditunno et al., 2004).

Given this latter concern, the current study was designed to assess the contribution of the EPR to the cardiovascular response to exercise immediately after induction of an acute Brown-Séquard-like lesion. The study utilized an animal model (the cat) in which a lateral spinal hemi-section could be readily induced and easily verified. In addition, the model allowed for the activation of the EPR-independent of central command. It was hypothesized that the cardiovascular response to activation of the EPR would be attenuated, to some extent, post induction of SCI. That being stated, it was further postulated that despite reductions in reflex function, the EPR would maintain the ability to significantly contribute to cardiovascular regulation during exercise.

MATERIALS AND METHODS

Experiments were performed on seven mongrel cats of either sex weighing between 3 and 5 kg. The procedures outlined were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas. All studies were conducted in accordance with the United States Department of Health and Human Services National Institutes of Health Guide for the Care and Use of Laboratory Animals.

GENERAL SURGICAL PROCEDURES

Animals were initially anesthetized with isoflurane gas (2–5% in 100% oxygen). Cats were subsequently intubated and artificially ventilated with a mechanical respirator (model 661, Harvard Apparatus). Levels of inhalant gas were increased as indicated by a withdrawal reflex to pinching of the hind paw, presence of a corneal reflex, and/or spontaneous increases in heart rate (HR). Fluid-filled catheters were placed within an external jugular vein for the administration of fluids and a common carotid artery for the measurement of arterial blood pressure (ABP). Arterial blood gas levels were periodically monitored using an automated blood gas analyzer (model ABL5, Radiometer) and were maintained within the following: arterial PO_2, >80 mmHg; arterial PCO_2, 35–40 mmHg; pH, 7.3–7.4. Body temperature, as assessed by rectal thermometer, was kept within 37–38°C by a water-perfused heating pad and an external heat lamp. A urinary catheter was inserted to monitor urine output throughout the experiment. Following cannulation, a laminectomy was performed in order to isolate the ventral roots from L_7–S_1. Briefly, the dorsal aspect of the L_7–S_1 vertebrae were removed and the dorsal and ventral roots were carefully separated. The ventral roots were transected along the peripheral ends and positioned on a bipolar stimulating electrode. A small pouch was formed from the skin of the exposed area and the neural tissue submersed in mineral oil. The triceps surae muscles were isolated in both legs. The calcaneal bone of each leg was sectioned and the Achilles tendons connected to force transducers (Grass FT10). Cats were then placed in a stereotaxic frame (Kopf Instruments) with hip spikes positioned to secure the animal within the frame. A mid-collicular decerebration was performed and anesthesia was withdrawn. At the conclusion of each experiment, animals were humanely euthanized by administration of sodium pentobarbital (120 mg/kg iv).

EPR TESTING

The triceps surae muscles of either the right or left hindlimb were contracted for 30 s with a 20 min recovery period between each contraction. Each hindlimb was contracted a minimum of two times. Contractions were produced via electrical stimulation of the L_7 and S_1 ventral roots at a frequency of 30–40 Hz at 3 × motor threshold with a pulse duration of 0.1 ms. This procedure has been shown to selectively stimulate both the mechanically and chemically sensitive components of the EPR-independent of central command activation (Mitchell et al., 1983). Following, a lateral hemi-section of the spinal cord was made in the T_{13} to L_2 region with fine forceps. After a stabilization period of 1 h, the contraction protocol was repeated in each limb. Finally, the cardiovascular responses observed post SCI were confirmed as neural in nature by repeating the contraction protocol in both limbs after severing the dorsal and remaining ventral roots caudal to L_4.

HISTOLOGY

At the conclusion of each experiment, sections of the injured spinal cord were harvested and fixed for histological examination. The spinal cord was sectioned transversely. Each section was 50 μm in thickness. Sections were mounted on slides and stained using Luxol blue to evaluate white matter. These procedures were performed to verify the extent of the experimentally-induced lesion in each animal.

DATA ACQUISITION AND STATISTICAL ANALYSES

ABP was continuously monitored via a pressure transducer (model P23 ID, Statham). Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was derived from the ABP pulse using a biotachometer (Gould Instruments). A force transducer (model FT10, Grass Instruments) was attached to the Achilles tendon, and used to quantify contraction-induced tension development. All data were subject to analog-to-digital conversion (micro 1401, Cambridge Electronic Design) using commercially available software (Spike 2 version 3, CED) and recorded on a personal computer (550-MHz Pentium III, Dell Computer). All statistical tests were performed using SigmaStat 2.03 for Windows (SPSS). Comparisons between experimental procedures were analyzed using repeated measures analysis of variance (ANOVA) with a Student-Newman-Keuls *post hoc* test employed when the ANOVA was found to be significant. Results are presented as means ± SE. The level of significance was set at $P < 0.05$.

RESULTS

Representative tracings from one cat of the MAP responses to electrically-induced static muscle contraction before and after spinal cord hemi-section are presented in **Figure 1**. Histological confirmation of the extent of the SCI for each animal studied is presented in **Figure 2**. Baseline blood pressures prior to muscle contraction were not different before or after hemi-section of the spinal cord whereas the induction of SCI significantly raised baseline HR (**Table 1**). In all conditions, muscle contraction-induced significant increases in MAP, HR, and muscle tension from baseline values (**Table 1**). The pressor response (12 ± 4 mmHg) to activation of the EPR during contraction of the limb contralateral to the lesion (ipsilateral sensory projection intact) was significantly attenuated compared to the change in pressure evoked by contraction prior to the induction of SCI (23 ± 5 mmHg). Similarly, the HR response to contraction after lateral hemi-section tended to be reduced although statistical significance was not obtained (**Figure 3**). When contracting the limb ipsilateral to the lesion (contralateral sensory projection intact), the MAP response prior to spinal cord hemi-section (16 ± 3 mmHg) was significantly greater than the change in pressure observed after surgical lesioning (9 ± 2 mmHg). Again, there was a tendency for the HR response to activation of the EPR to be less after induction of injury although a statistical difference was not observed (**Figure 4**). Interestingly, the magnitude of the reduction in the pressor response to activation of the EPR post-spinal cord hemi-section was similar regardless of the limb contracted in relation to the location of the spinal cord lesion. For example, the MAP response to static contraction was diminished by a little over 40% when contracting the limb either ipsilateral or contralateral to the lesion. In other words, over 50% of the pressor response evoked by muscle contraction prior to injury remained after the induction of injury. Regardless of the limb contracted in relation to the spinal cord lesion, bi-lateral dorsal root transection almost completely abolished the pressor and cardioaccelerator responses to static contraction (**Figures 3** and **4**).

DISCUSSION

This study demonstrates, in cats, that the EPR significantly contributes to the pressor and cardioaccelerator responses to muscle contraction immediately following lateral spinal cord hemi-section independent of central command. The MAP responses to either electrically-induced static contraction of the limb contralateral to the lesion (ipsilateral sensory projection intact) or ipsilateral to the lesion (contralateral sensory projection intact) were reduced after SCI. That being stated, a significant proportion of the pressor response (over 50%) remained post hemi-section regardless of the limb contracted in relation to the site of the lesion. Interestingly, with regard to HR, the induction of acute SCI had no significant effect on the tachycardic response to activation of the EPR. These findings suggest that the EPR

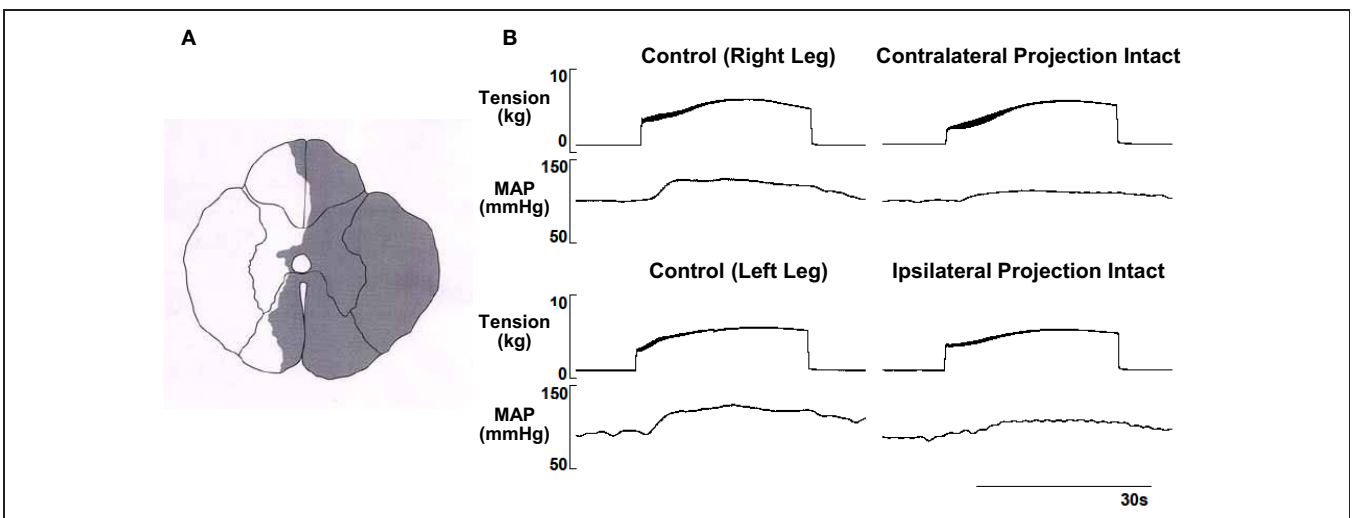

FIGURE 1 | (A) Histological reconstruction of the Brown-Séquard like lesion in the lower spinal cord of one animal. **(B)** Electrically-induced static contraction increased MAP before (left tracing) and after (right tracing) induction of the Brown-Séquard like lesion in both the leg ipsilateral to the injury site (contralateral sensory projection intact) and contralateral to the injury site (ipsilateral sensory projection intact).

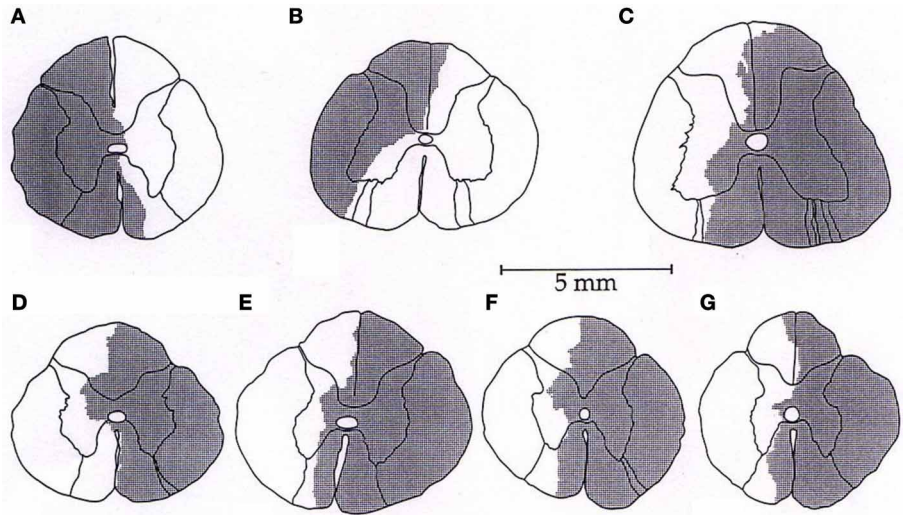

FIGURE 2 | (A–G) Histological reconstructions of the Brown-Séquard like lesion in the lower spinal cord of each animal used in the investigation.

Table 1 | Cardiovascular responses to contraction before and after hemi-section of the spinal cord.

Variable	Condition	Contralateral projection intact		Ipsilateral projection intact	
		Control	Post hemi-section	Control	Post hemi-section
MAP	Baseline	140 ± 8	136 ± 9	137 ± 10	133 ± 8
(mmHg)	Peak	156 ± 11*	144 ± 9*†	160 ± 14*	145 ± 11*†
HR	Baseline	198 ± 22	228 ± 16†	196 ± 23	234 ± 15†
(beats·min^{-1})	Peak	217 ± 22*	245 ± 17*†	217 ± 22*	250 ± 14*†
Tension	Baseline	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
(kg)	Peak	5.1 ± 1.0*	5.0 ± 1.0*	4.5 ± 0.7*	4.3 ± 0.7*

Data are means ± S.E.M. (n = 7). MAP, mean arterial pressure; HR, heart rate.

**Significantly different from baseline.*

†Significantly different from control. P < 0.05.

largely maintains the ability to elicit the pressor and tachycardic responses required to support exercise directly after a lateral injury to the spinal cord. The results further suggest that depression of the EPR by acute spinal shock is minimal if present at all.

Previously, Winchester et al. (2000) recruited four patients with Brown-Séquard syndrome (i.e., lateral spinal cord hemisection) to assess the contributions of central command and the EPR to the cardiovascular response to static exercise. Due to the fact that these patients presented with a loss of sensation and intact motor control on one side of the body and intact sensation and a loss of motor control on the other side of the body, these investigators were able to perform a series of contractions under four unique conditions: (1) voluntary contraction of the sensory loss/motor intact leg (central command activated, EPR input minimal); (2) electrical stimulation of the sensory loss/motor intact leg (no central command, EPR input minimal); (3) attempted voluntary contraction of the sensory intact/motor loss leg (central command and EPR activated); and (4) electrical stimulation of sensory intact/motor loss leg (no central command, EPR activated). Isometric knee extension exercise-induced increases in ABP and HR, albeit reduced in magnitude compared to that reported in the literature for normal healthy individuals, under each of these conditions with the exception of electrical stimulation of the sensory loss/motor intact leg. In their interpretation of these findings, the investigators suggested that both central command and the EPR maintained, to some extent, their ability to regulate the cardiovascular system during exercise in a chronic SCI patient population. With regard to the EPR, the results of the current investigation suggest that the findings of Winchester and colleagues may be extended to the acute phase of lateral SCI.

In agreement with a previous report based on a small number of animals (Iwamoto et al., 1984), the findings of the current investigation suggest that, in cats, the spinal pathway mediating the EPR control of MAP is bilateral in nature implying a level of redundancy within this species. The extent of this pathway redundancy appears to be incomplete with regard to blood pressure as the absence of either of its components (i.e., contralateral or ipsilateral sensory projection) attenuates the pressor response to static muscle contraction. That being stated, the data also suggest that neither component of the bilateral pathway is dominant as interrupting either the ipsilateral or contralateral sensory

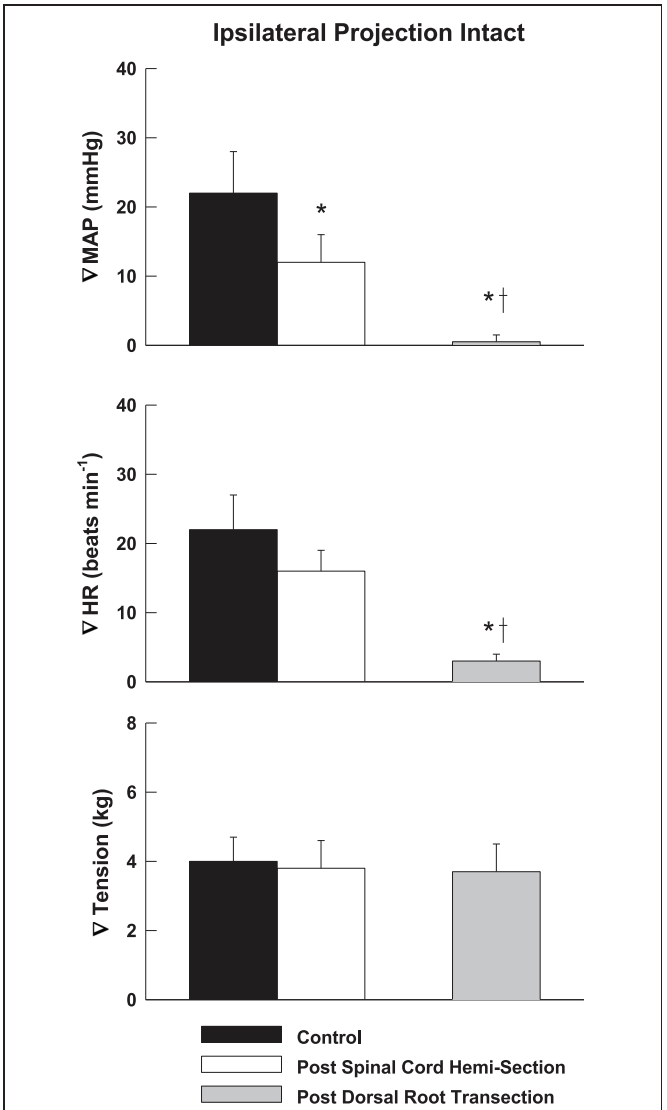

FIGURE 3 | After lateral hemi-section of the spinal cord, the exercise-induced increases in MAP were attenuated compared to control when contracting the leg contralateral to the lesion (ipsilateral sensory projection intact). HR responses were largely unaffected. Sectioning the dorsal roots innervating the activated hindlimb skeletal muscle almost completely abolished the cardiovascular response to exercise. Asterisk indicates significantly different from control condition. Cross indicates significantly different from spinal cord hemi-section condition ($P < 0.05$).

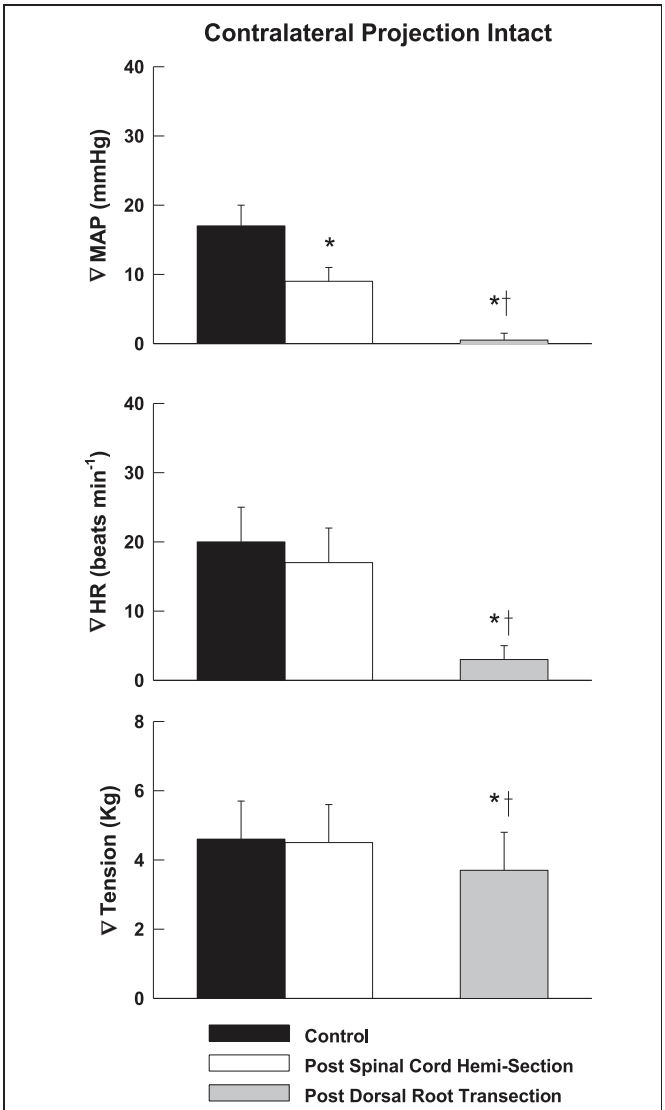

FIGURE 4 | After lateral hemi-section of the spinal cord, the exercise-induced increases in MAP were attenuated compared to control when contracting the leg ipsilateral to the lesion (contralateral sensory projection intact). HR responses were largely unaffected. Sectioning the dorsal roots innervating the activated hindlimb skeletal muscle almost completely abolished the cardiovascular response to exercise. Asterisk indicates significantly different from control condition. Cross indicates significantly different from spinal cord hemi-section condition ($P < 0.05$).

projection in relation to the exercising limb produces the same decrement in the MAP response to contraction. These conclusions do not appear to be applicable to the tachycardic response to EPR activation as it was predominately unaffected by spinal cord lesioning.

Unlike the cat, the EPR pathway in humans appears to be predominately unilateral in nature (Winchester et al., 2000). This must be considered when interpreting and translating the findings of the current investigation to humans suffering an acute lateral hemi-section of the spinal cord. That being stated, the known characteristics of spinal cord pathways may aid in relating the functional, physiological findings in cats to humans with a Brown-Séquard lesion. Three pathways are of principal interest: the spinothalamic, spinoreticular, and spinomesencephalic (including that to the periaqueductal gray) tracts. Each of these pathways is generally characterized (with some variation depending on target nucleus) by a strong component which ascends contralaterally from spinal levels in the primate and cat with a lesser ipsilateral projection (Willis and Coggeshall, 2004). At least in the case of the spinothalamic tract, it is clear that the ipsilateral projection in the primate (a close relative of humans) is proportionally smaller than in the cat (Willis and Coggeshall,

2004). This may account, in part, for the lack of a cardiovascular response evoked during electrically-induced exercise in the limb contralateral to the lesion in the Brown-Séquard patient study previously discussed (Winchester et al., 2000). It is somewhat more difficult to make this association with the other tracts, but the differences between the cat and the primate pathways (and hence possibly humans) appear to be similar to that noted in the spinothalamic tract (Willis and Coggeshall, 2004). However, it is evident from the current study that ipsilateral projections in the cat are capable of mediating a strong EPR-mediated cardiovascular response. As mentioned, in the human Brown-Séquard investigation, patients were studied months to years after their initial lesion in direct contrast to the acute injuries-induced in the present experiments (Winchester et al., 2000). Although contralateral projections predominate in the spinal tracts of primates, lesser ipsilateral projections exist that may also be present in humans (Willis and Coggeshall, 2004). It is unknown whether activation of such ipsilateral pathways by the EPR could elicit cardiovascular responses after acute injury in patients as has been demonstrated in the cats of the current study. Likewise, it is unknown what effect beginning exercise training shortly after the occurrence of SCI might have on surviving ipsilaterally projecting cells in humans. Although highly speculative, it is possible that enhancing the activity of ipsilateral projections in the EPR pathway shortly after injury in humans could induce a functional plasticity that effectively compensates for the loss of the more dominant contralateral projections. Similar plasticity has been demonstrated in efferent projections to the phrenic motor nucleus in which respiratory challenge facilitates activity in contralateral neurons that restores function to the phrenic motor neurons on the side ipsilateral to the lesion (Fuller et al., 2006). As a result, diaphragmatic activity is recovered. Increasing our understanding of how the cat utilizes these ipsilateral EPR pathways after acute spinal cord hemi-section may facilitate our ability to induce similar plasticity in humans shortly after injury.

It should likewise be noted that the findings of the current investigation in cats and those previously reported in Brown-Séquard patients (Winchester et al., 2000) are primarily confined in application to individuals experiencing a complete lateral hemi-section of the spinal cord. Circumstances in which the spinal cord is only partially lesioned in any portion or completely transected would most likely produce different results. On the other hand, it is possible that EPR-mediated cardiovascular responses might not be different under these specific SCI conditions if extraspinal pathways (perhaps carried in the sympathetic trunk) are utilized. Studies in which EPR function is assessed after complete transection of the spinal cord could address this possibility. In addition, the level of the spinal cord in which the injury is sustained may also affect the function of the EPR. That being stated, the Brown-Séquard patients studied previously displayed similar cardiovascular responses to exercise under various conditions with hemi-sections ranging from the C5 to T8 neurological levels.

Limitations that could affect the interpretation of results in this study are acknowledged. To begin, the contribution of central command to the cardiovascular response to exercise after acute spinal cord hemi-section was not examined in this investigation. The importance of this input to circulatory regulation during physical activity immediately following SCI warrants further investigation. Secondly, due to technical reasons, arterial PCO_2 was maintained within the range of 35–40 mmHg. This is slightly elevated from the PCO_2 values reported in normal conscious cats which tend to be in the lower 30 mmHg range (Herbert and Mitchell, 1971; Lovering et al., 2003). As a result, sensory input from central and peripheral chemoreceptors that effect both respiratory and cardiovascular function could have influenced the hemodynamic responses to muscle contraction reported. That being stated, as arterial PCO_2 was elevated only minimally, this concern is relatively minor. Thirdly, both baseline HR and MAP tended to be on the high end of the range reported commonly for decerebrated cats (Iwamoto et al., 1985; Hayes and Kaufman, 2001; Hanna and Kaufman, 2003). It is possible that such elevated basal hemodynamics may have limited the magnitude of the EPR-induced pressor and tachycardic responses masking the full impact of the spinal cord hemi-section. For example, changes in MAP are commonly reported to be in the range of 30–40 mmHg in response to muscle contraction in decerebrated cats (Iwamoto et al., 1985; Hayes and Kaufman, 2001; Hanna and Kaufman, 2003). The group means for the MAP responses to EPR activation prior to hemi-section in the current study were approximately 15–25 mmHg. In contrast, the HR responses elicited were similar to those previously reported (Iwamoto et al., 1985; Hayes and Kaufman, 2001). Finally, this study was conducted without the inclusion of a time control (i.e., conduction of the protocol over the full time period without the induction of spinal hemi-section). That being stated, previous studies in decerebrate cats have reported no decrement in the pressor or tachycardic response to EPR activation for several hours after initial stimulation (Hayes and Kaufman, 2001). Similar results have been reported in anesthetized cats (Smith et al., 2005).

CLINICAL IMPLICATIONS

As stated previously, injuries to the spinal cord, whether complete or incomplete, often disrupt cardiovascular control and induce a host of complications. The autonomic dysfunction that develops can be associated with a number of conditions that increase the risk for cardiovascular disease including abnormalities in blood pressure related to central and peripheral vascular dysfunction, irregularities in HR variability, generation of arrhythmias and abnormal temperature control (Gao et al., 2002; Jacobs and Nash, 2004; Myers et al., 2007; Brown and Macefield, 2008; Alexander et al., 2009). In addition, the cardiovascular response to exercise is often blunted resulting in decreased exercise capacity which can subsequently increase inactivity (Myers et al., 2007). Physical inactivity in itself can lead to an elevated risk for thromboembolism, due to the development of coagulation disorders and venous stasis, as well as obesity and metabolic syndromes (Waring and Karunas, 1991; Groah et al., 2001; Myers et al., 2007; Ploumis et al., 2009). Prevention and/or successful treatment of these cardiovascular complications following SCI could clearly reduce the incidence of cardiovascular disease in this patient population. To this end, evidence supports

the use of exercise as an effective therapy for enhancing physical capacity, muscular strength, and vascular function in the SCI patient (Gerrits et al., 2001; Hopman et al., 2002; DeGroot et al., 2005; Thijssen et al., 2005, 2006; Zbogar et al., 2008; Hicks et al., 2011; Phillips et al., 2011). It is logical to propose that the earlier the exercise therapy begins, the more effective the treatment. The results of the current investigation suggest that regulation of the cardiovascular system by the EPR is relatively maintained immediately after acute lateral hemi-section of the spinal cord and can likely support the metabolic demands of physical activity. The findings increase our understanding of both the mechanisms of neural cardiovascular control during exercise post SCI as well as the time at which this control is operative. The results may prove beneficial in the development of training interventions specifically designed to optimize the efficacy of physical activity as a therapeutic modality in these patients.

SUMMARY

In cats with an acute lateral hemi-section of the spinal cord, activation of the EPR by electrically-induced static exercise increases MAP and HR. The responses can be evoked by contracting skeletal muscle in either the limb contralateral to the lesion (ipsilateral sensory projection intact) or ipsilateral to the lesion (contralateral sensory projection intact). These findings suggest that the EPR maintains the potential to contribute significantly and importantly to the cardiovascular response to exercise immediately following spinal cord hemi-section.

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Carotid baroreflex responsiveness in normotensive African Americans is attenuated at rest and during dynamic leg exercise

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Evidence suggests differences between African Americans (AAs) and Caucasian Americans (CAs) in cardiovascular responsiveness to physiological stressors. This study tested the hypothesis that carotid baroreflex (CBR) control of heart rate (HR) and blood pressure is reduced in AAs compared to CAs during exercise. Mean arterial pressure (MAP) and HR were continuously recorded at rest and during leg cycling in 23 non-hypertensive male subjects (12 AA; 11 CA; age 19–26 years). CBR control of HR and MAP was assessed with 5-s pulses of neck pressure (NP, simulated hypotension) and neck suction (NS, simulated hypertension) ranging from +45 to −80 Torr. Across all NS stimuli (−20, −40, −60, −80 Torr) at rest, the AA group demonstrated attenuated CBR-mediated reductions in HR (AA, -8.9 ± 1.9 vs. CA, -14.1 ± 2.3 bpm; $P < 0.001$) and MAP (AA, -6.4 ± 1 vs. CA, -7.8 ± 0.8 mmHg; $P < 0.05$). Despite similar gain and magnitude of resetting observed in the modeled stimulus response curves, an attenuation among AAs persisted in HR (AA, -8.2 ± 1.6 vs. CA, -11.8 ± 3 bpm; $P < 0.05$) and MAP (AA, -6.8 ± 0.9 vs. CA, -8.2 ± 1.1 mmHg; $P < 0.05$) responses to NS during exercise. No differences in CBR-mediated HR and MAP responses to NP were detected between groups at rest or during exercise. These data suggest impairment in the ability to defend against a hypertensive challenge among AAs during steady-state exercise compared to their CA counterparts.

Keywords: blood pressure, heart rate, racial differences, hypertension, exercise

INTRODUCTION

Hypertension affects African Americans (AAs) at a rate that is among the highest in the world (Lloyd-Jones et al., 2010). AAs are at 1.8X greater risk of fatal stroke related to hypertension and are over 4X more likely to develop end-stage renal disease associated with hypertension (NHLBI, 2004). Accumulating evidence indicates augmented blood pressure responses to emotional and physical stimuli in AAs compared to Caucasian American (CAs), (Light et al., 1987; Anderson et al., 1988; Treiber et al., 1990; Calhoun et al., 1993; Terrell and Manuck, 1996; Calhoun and Mutinga, 1997; Barnes et al., 2000; Kelsey et al., 2000; Bond et al., 2001; Arthur et al., 2004). AAs also exhibit exaggerated blood pressure responses to exercise relative to CAs (Alpert et al., 1981; Thomas et al., 1987; Ekelund et al., 1990; McAdoo et al., 1990; Walker et al., 1992; Duey et al., 1997). While many studies support racial differences for blood pressure responses to the aforementioned stimuli, mechanisms associated with dynamic reflex control of blood pressure (e.g., arterial baroreflex function) have not been thoroughly investigated.

Regulation of beat-to-beat changes in arterial blood pressure is largely determined by arterial baroreflex-mediated changes in autonomic neural activity to the heart and vasculature. Some differences between AAs and CAs in neural control of arterial blood pressure during rest have been reported (Ray and Monahan, 2002; Franke et al., 2004; Hinds and Stachenfeld, 2010; Holwerda et al., 2011). In addition to blunted declines in cardiac output and total peripheral conductance in AA compared to CA in response to baroreceptor unloading (i.e., lower-body negative pressure) at rest (Franke et al., 2004), augmented transduction of sympathetic nerve activity to vascular resistance in AAs compared to CAs has also been reported under similar conditions (Ray and Monahan, 2002). We recently reported impaired carotid baroreflex (CBR) responsiveness among AAs compared to CAs (Holwerda et al., 2011). Our primary observation was a distinct impairment in CBR-mediated reductions in heart rate (HR) in response to simulated hypertension among AAs at rest. However, it is not clear how the potential racial differences in arterial baroreflex function may manifest during exercise, a condition in which the arterial baroreflex plays an important role in the regulation of cardiovascular adjustments.

An intact arterial baroreflex is essential for appropriate blood pressure responses to exercise (Melcher and Donald, 1981; Walgenbach and Donald, 1983; Walgenbach and Shepherd, 1984; Scherrer et al., 1990). Resetting of the reflex during exercise allows for continued control of arterial blood pressure across a wide spectrum of exercise intensity (Potts et al., 1993; Papelier et al., 1994; Fadel and Raven, 2012). While greater blood pressure responses have been reported in AA compared to CA during

dynamic (Alpert et al., 1981; Thomas et al., 1987; Ekelund et al., 1990; Walker et al., 1992) and static exercise (McAdoo et al., 1990; Duey et al., 1997), inadequate baroreflex buffering of exercise-induced increases in blood pressure, such as those mediated by the exercise pressor reflex, would theoretically lead to inappropriate cardiovascular responses [e.g., inadequate blood flow distribution due to inadequate baroreflex buffering of the sympathetic neural outflow (Joyner, 2006)].

This study tested the hypothesis that CBR control of HR and blood pressure is reduced in AAs compared to CAs during exercise. To test this hypothesis, we examined CBR-mediated changes in HR and Mean arterial pressure (MAP) to wide range of simulated hypertensive and hypotensive stimuli at rest and during dynamic leg cycle exercise in normotensive AAs, and age, BMI and fitness-matched normotensive AAs.

METHODS

SUBJECTS

Twenty three adult, non-hypertensive male subjects (12 AAs; 11 CAs; age 19–26 years) were recruited from the University of Texas at Arlington. All subjects were free of known cardiovascular and respiratory diseases, non-smokers, and recreationally active (low to moderate intensity activity). Following the recruitment of AA subjects, CA subjects [i.e., similar fitness (VO_{2MAX} within 7 ml/kg/min), age (within 3 years) and body mass index (within 15%)] were recruited. 8 of the 12 AAs and 4 of the 11 CAs have previously volunteered as research subjects in our laboratory (Holwerda et al., 2011). Completely new experiments were performed on the previously studied volunteers. Each subject signed an informed consent that was approved by the Institutional Review Boards at the University of Texas at Arlington. Prior to participation, all subjects were familiarized with the testing protocols. Subjects were advised to not consume alcohol within the 24 h prior and not to consume caffeine within 12 h prior to the scheduled experiment. Subjects were also advised not to vigorously exercise for 48 h prior to the scheduled experiment. Subjects were not using prescription or over-the-counter medications, and known family history of hypertension was recorded for all subjects.

PRIOR TO EXPERIMENTATION

Each subject completed a medical health history questionnaire and a resting blood pressure screening. Subjects underwent a maximal exercise test on a recumbent leg cycle ergometer while obtaining continuous measurements of respiratory gases (TrueOne 2400, Parvo Medics) to establish maximal oxygen uptake for the determination of cardiorespiratory fitness and steady-state exercise workloads. Due to variability in the anatomical location of the carotid sinus, each subject's carotid sinuses were confirmed as appropriate for the neck chamber by Doppler ultrasound. Resting trials of NP and NS were performed for familiarization with the experimental measurements and protocol.

EXPERIMENTAL MEASUREMENTS

Subjects were instrumented with a 3-lead electrocardiogram (ECG) and an automated sphygmomanometer (Tango$^+$, Suntech) for continuous HR and steady-state arterial blood pressure measurements, respectively. Beat-to-beat arterial blood pressure was measured using a servo-controlled finger-cuff photoplethysmograph (Finometer® Pro, Finapres Medical Systems, Amsterdam, Netherlands) from a finger on the right hand while it rested at the level of the right atrium. Beat-to-beat arterial blood pressure recordings were adjusted to absolute steady-state arterial blood pressure measurements as determined by an automated sphygmomanometer, which has been validated for use during dynamic exercise (Cameron et al., 2004). Continuous measurements of respiratory gases (TrueOne 2400, Parvo Medics) were taken during the exercise phase of the experiment. CBR control of HR and MAP was assessed through the use of 5-s periods of neck pressure (NP, simulated hypotension) and neck suction (NS, simulated hypertension) delivered to the region of the carotid sinuses encased by a properly sized malleable neck chamber. Pressure and suction pulses were generated by a variable pressure source and delivered to the neck chamber through two-way solenoid valves and controlled using custom software (NS3). Data were sampled at 200 Hz and stored for off-line analysis (AcqKnowledge, BioPac Systems Inc., Goleta, CA).

EXPERIMENTAL PROCEDURES

Maximal exercise test

Subjects were seated in a semirecumbent position on an examination table equipped with an electrically braked cycle ergometer (Corival Supine, Lode, Groningen, The Netherlands), and instrumented with a 3-lead ECG and an automated arterial blood pressure cuff. After a 3-min warm-up at 50 Watts and preferred pedal frequency between 50 and 70 rpm, exercise workload increased 25 Watts each minute until the subject could no longer maintain pedal frequency. All subjects received verbal encouragement, and reported a rating of perceived exertion at test termination.

Experimental day

Subjects returned for experimental measures after at least 48 h following the maximal exercise test. Subjects were instrumented with a 3-lead ECG, automated arterial blood pressure cuff, and a finger-cuff photoplethysmograph. After 20 min of supine rest, subjects were seated in a semi-recumbent position (∼60°upright) and were fitted with a properly sized malleable neck chamber.

CBR responsiveness was assessed by applying multiple trials of random-ordered single 5-s pulses of NP and NS ranging from +45 to −80 Torr (i.e., +45, +30, +15, −20, −40, −60, −80). Each pressure stimulus was delivered to the carotid sinus during a 15 s breath hold at normal end-expiration to minimize respiratory-related modulation of HR and MAP. The generated pressures within the neck collar were manually controlled and a pressure transducer (model DP45, Validyne Engineering, Northridge, CA, USA) was connected to a port on the collar to accurately quantify the stimulus applied. At least four trials of each magnitude of NS and NP were administered with a minimum of 45 s of recovery between trials to allow variables to return to pre-stimulus values (Ogoh et al., 2003b).

The electrically braked cycle ergometer was positioned for the exercise bout following the resting NP and NS trials. After a 5-min warm-up at 50 Watts and a preferred pedal frequency between 50 and 70 rpm, workload was adjusted to elicit steady-state oxygen

consumption levels of ∼50% of maximal oxygen uptake. To minimize the potential of cardiovascular drift, only 3–4 trials at each chamber pressure were performed during exercise (Norton et al., 1999). The end-expiratory breath hold was not performed during exercise to diminish the potential for chemoreflex activation.

DATA ANALYSIS

Absolute steady-state blood pressure measures

In order to account for the change in the systolic/diastolic period ratio as HR increases, absolute steady-state MAP values at rest and during exercise were calculated as a function of HR in place of the standard MAP equation [MAP = DBP + 1/3(SBP − DBP)] (Moran et al., 1995). First, the fraction of systole (St) of the cardiac cycle was related to HR using the following equation: St = 0.01exp (4.14–40.74/HR). Diastolic blood pressure (DBP) and pulse pressure (PP) were then adjusted for St in the following equation: MAP = DBP + St (PP).

Maximum MAP and HR responses

The maximum MAP response was determined by assessing the three cardiac cycle interval with the largest change in MAP relative to pre-stimulus (three cardiac cycle average) for each trial of NP and NS. This analysis has been described previously (Keller et al., 2006). For HR, the single maximum cardiac cycle response was compared to pre-stimulus (three cardiac cycle average) for each trial of NP and NS.

Carotid baroreflex function curves

Carotid-cardiac and carotid-vasomotor stimulus-response curves were determined by plotting the maximal changes in HR and MAP, respectively, elicited by NP and NS against the estimated carotid sinus pressure (ECSP), which was calculated as mean blood pressure minus neck chamber pressure. CBR stimulus-response data were fit for each subject to the logistic function model described by Kent et al. (1972): Dependent variable = $A_1\{1 + \exp[A_2(\text{ECSP-}A_3)]\}^{-1} + A_4$ where the dependent variable is HR or mean blood pressure, A_1 is the range of response of the dependent variable (maximum–minimum), A_2 is the gain coefficient, A_3 is the centering point or carotid sinus pressure required to elicit equal pressor and depressor responses, and A_4 is the minimum response.

The CBR operating point gain and maximal gain were calculated using the equations: $G_{op} = A_1A_2\exp[A_2(\text{ECSP}_{op} - A_3)]/\{1 + \exp[A_2(\text{ECSP}_{op} - A_3)]\}^2$ and $G_{max} = -A_1A_2/4$ where G_{op} is the gain of the CBR function curve at the operating point, G_{max} is the maximal gain of the CBR function curve, and ECSP_{op} is the ECSP at the operating point (i.e., prestimulus MAP). The G_{op} was calculated as the gain at the operating point and used to provide a measure of responsiveness at the operating point of the CBR function curve, whereas the G_{max} was calculated as the gain at the centering point and used as an index of overall CBR responsiveness. The threshold (THR) and saturation (SAT), described as the minimum and maximum ECSP, respectively, that elicits a reflex change in HR or MAP, were calculated using the following equation: $\text{THR} = -2.944/A_2 + A_3$ and $\text{SAT} = 2.944/A_2 + A_3$. The parameters for all subjects within an experimental condition were averaged to provide group mean responses.

The movement of the operating point away from the centering point was described by the following equation: OP-CP. The magnitude of baroreflex resetting during exercise (i.e., the upward and rightward movement of the carotid-cardiac and carotid-vasomotor curves) was determined by the sum of the changes in A_3, A_4, THR, and SAT from rest to exercise for each subject.

STATISTICAL ANALYSIS

Comparisons for changes in HR and BP from rest to exercise and descriptive characteristics (e.g., height, weight, BMI, VO_{2MAX}), were made between racial groups using unpaired *t*-tests. The statistical comparison of the baroreflex and cardiovascular response variables between racial populations (factor 1) and the various magnitudes of NP and NS (factor 2) at rest and exercise were made using a Two-Way ANOVA. For comparison of carotid-cardiac and carotid-vasomotor response curve parameters between racial groups and conditions (i.e., rest vs. exercise), Two-Way ANOVA was used. Two-Way analysis of covariance (ANCOVA) was also used to determine if differences existed between racial groups after controlling for known family history of hypertension. Each subject's corresponding covariate data were determined as either negative (no parental hypertension) or positive (one or more cases of parental hypertension). Family history of hypertension was determined for all subjects. When required, multiple comparison procedures were performed using the Holm–Sidak method. Statistical significance was set at $P < 0.05$.

RESULTS

Subject characteristics are described in **Table 1**. There were no significant group differences in age, height, weight, VO_2max, and BMI between racial groups. There were also no group differences in changes in HR, SBP, DBP, and MAP from rest to exercise.

Table 1 | Subject characteristics.

	AA ($n = 12$)	CA ($n = 11$)	*P*-value
Age (yr)	22 ± 2.3	22 ± 1.1	0.587
Height (m)	1.77 ± 0.08	1.81 ± 0.07	0.360
Weight (kg)	78.5 ± 13.4	78 ± 7.7	0.581
VO_2max (ml/kg/min)	40.1 ± 7	42.9 ± 7.7	0.350
BMI (kg/m^2)	25 ± 4	23.9 ± 1.8	0.428
ΔHR (bpm)	62 ± 3.9	64 ± 3.2	0.618
ΔSBP (mmHg)	49 ± 5.6	44 ± 3.8	0.404
ΔDBP (mmHg)	2.1 ± 3.4	−4.6 ± 3.6	0.144
ΔMAP[1] (mmHg)	18 ± 2.9	12 ± 2.8	0.100
ΔMAP[2] (mmHg)	29 ± 3.3	24 ± 3.3	0.232
Family history HTN	6(+), 6(−)	4(+), 7(−)	–

Values expressed as mean ± SD.

AA, African American; CA, Caucasian American; VO_2MAX, maximum oxygen consumption; BMI, body mass index; ΔHR, change in heart rate from rest to exercise, ΔSBP, change in systolic blood pressure from rest to exercise; ΔDBP, change in diastolic blood pressure from rest to exercise; ΔMAP[1], change in mean arterial pressure from rest to exercise {DBP + [(SBP − DBP)/3]}; ΔMAP[2], change in mean arterial pressure from rest to exercise corrected for systolic/diastolic period ratio (see "Methods"); HTN, hypertension.

CAROTID BAROREFLEX RESPONSIVENESS AT REST

Across all NP stimuli (15, 30, and 45 Torr), no significant differences ($P > 0.05$) were found in the magnitude of the HR responses (average value for all HR responses to NP stimuli at all pressures, AA, 6.5 ± 1.2 bpm; CA, 5.9 ± 1.1 bpm; **Figure 1A**) and MAP responses (AA, 7.2 ± 1.4 mmHg; CA, 7.5 ± 1.4 mmHg; **Figure 1B**). Similar findings were observed for HR and MAP responses to NP when controlling for family history of hypertension (Two-Way ANCOVA, both $P > 0.05$).

The magnitude of the HR response across all NS stimuli was attenuated in the AA group (−8.9 ± 1.9 bpm) compared to the CA group (−14.1 ± 2.3 bpm; $P < 0.001$; **Figure 1C**). The magnitude of the MAP response across all NS stimuli was also attenuated in the AA group (−6.4 ± 1 mmHg) compared to the CA group (−7.8 ± 0.8 mmHg; $P < 0.05$; **Figure 1D**). Similar findings were observed for HR and MAP responses to NS when controlling for family history of hypertension (Two-Way ANCOVA, both $P < 0.05$), despite a main effect of family history.

CAROTID BAROREFLEX RESPONSIVENESS DURING EXERCISE

Across all NP stimuli, no significant differences ($P > 0.05$) were found in the magnitude of the HR responses (AA, 3.3 ± 0.6 bpm; CA, 3.6 ± 0.6 bpm; **Figure 2A**) and MAP responses (AA, 8.1 ± 1.6 mmHg; CA, 6.5 ± 1 mmHg; **Figure 2B**). Similar findings were observed for HR and MAP responses to NP when controlling for family history of hypertension (Two-Way ANCOVA, both $P > 0.05$).

The magnitude of the HR response across all NS stimuli during exercise was attenuated in the AA group (−8.2 ± 1.6 bpm) compared to the CA group (−11.8 ± 3 bpm; $P < 0.05$; **Figure 2C**). The magnitude of the MAP response across all NS stimuli during exercise was also attenuated in the AA group (−6.8 ± 0.9 mmHg) compared to the CA group (−8.2 ± 1.1 mmHg; $P < 0.05$; **Figure 2D**). Similar findings were observed for HR and MAP responses to NS when controlling for family history of hypertension (Two-Way ANCOVA, both $P < 0.05$), despite a main effect for family history for only MAP responses.

CAROTID-CARDIAC AND CAROTID-VASOMOTOR STIMULUS-RESPONSE CURVES

Table 2 describes the logistic model parameters and derived variables that describe CBR control of HR (carotid-cardiac) and MAP (carotid-vasomotor) at rest and during exercise in both groups. The carotid-cardiac A_3, SAT, and magnitude of OP-CP

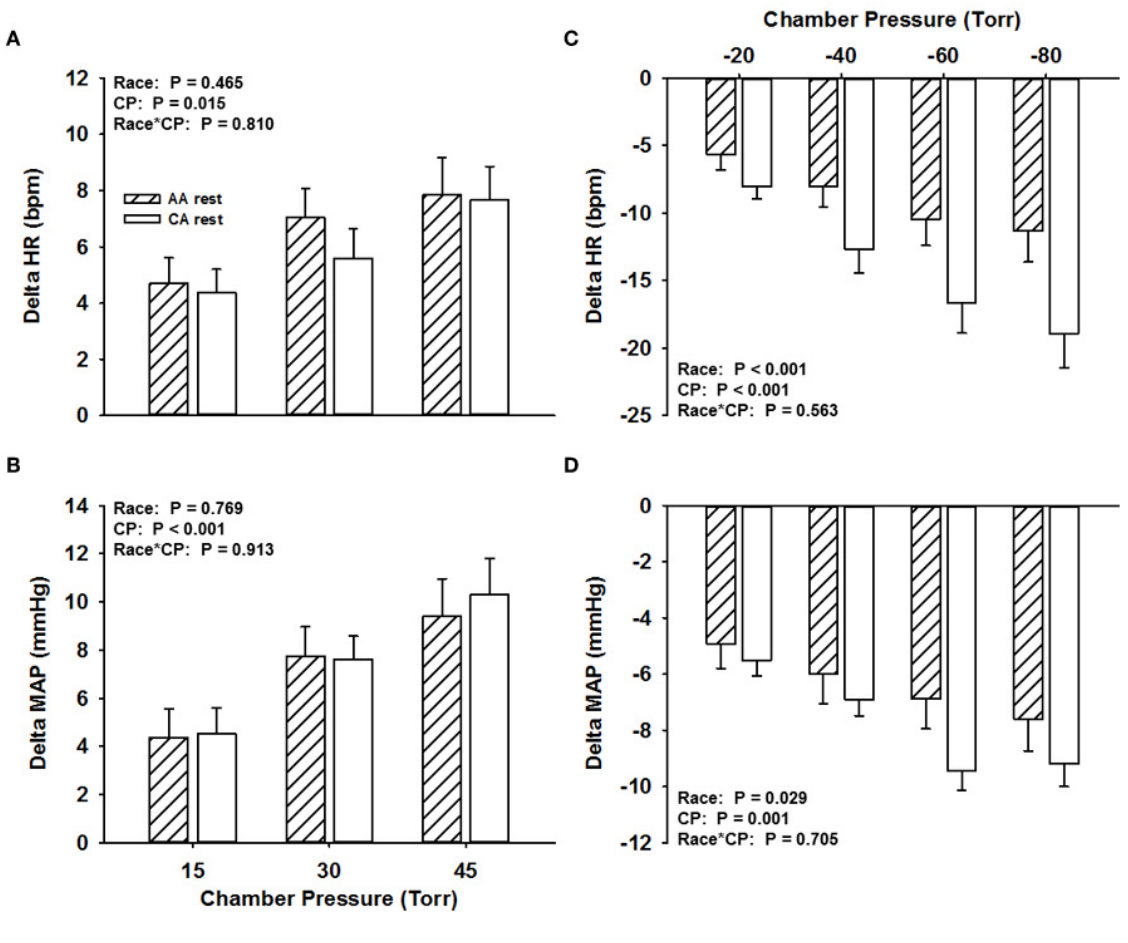

FIGURE 1 | Change in HR (A) and MAP (B) in response to NP and change in HR (C) and MAP (D) in response to NS in African Americans (AA, hatched bars) and Caucasian Americans (CA, open bars) at rest. CP, chamber pressure.

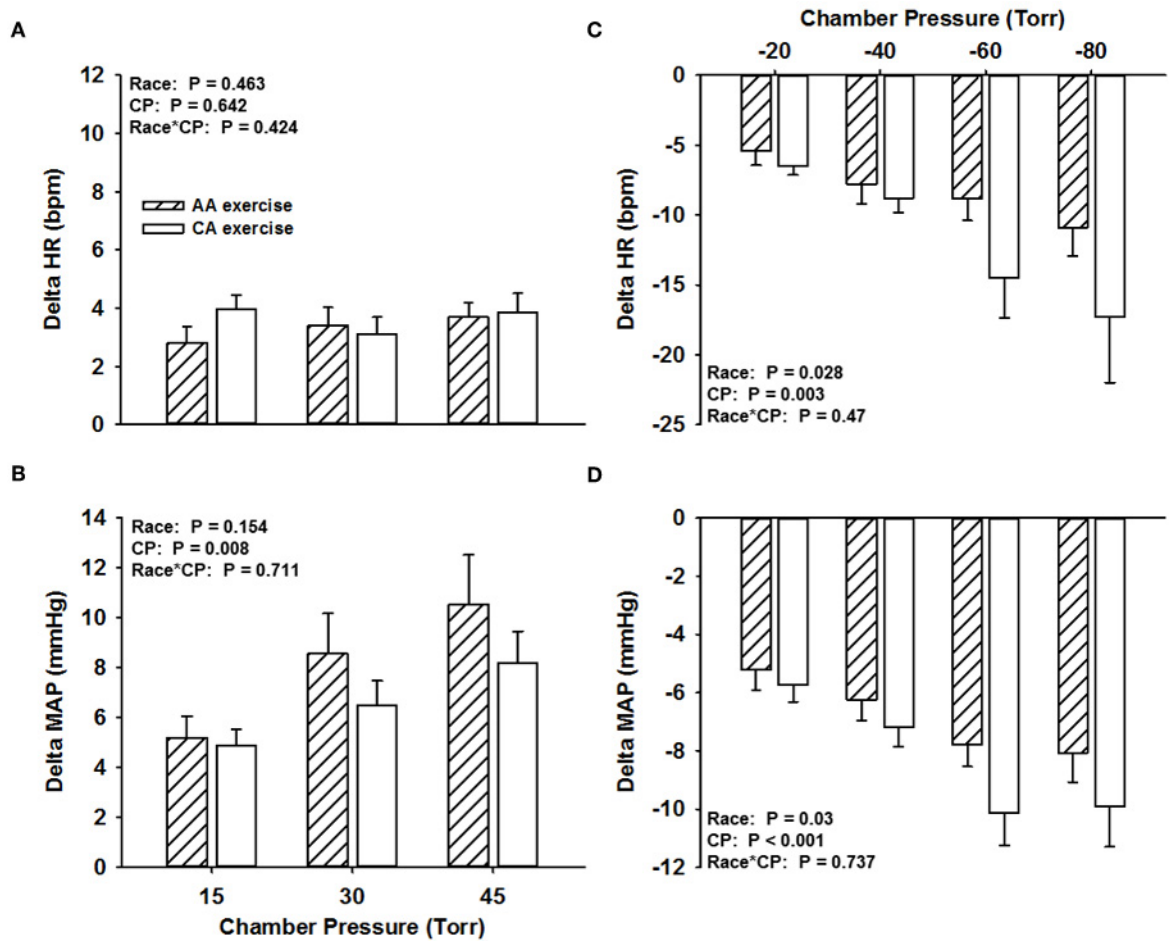

FIGURE 2 | Change in HR (A) and MAP (B) in response to NP and change in HR (C) and MAP (D) in response to NS in African Americans (AA, hatched bars) and Caucasian Americans (CA, open bars) during exercise. CP, chamber pressure.

was significantly less ($P < 0.05$) in the AA group compared to the CA group. Carotid-cardiac A_1 (responding range) in the AA group tended to be diminished (Two-Way ANOVA, $P = 0.059$), reaching a statistically significant difference when controlling for family history of hypertension (Two-Way ANCOVA, $P = 0.028$). No differences ($P > 0.05$) were detected between groups in maximal or operating point gain for both carotid-cardiac and carotid-vasomotor. The significant increase in A_3, A_4, THR, and SAT from rest to exercise in both groups (all $P < 0.05$) describes the upward and rightward resetting of the carotid-cardiac (**Figure 3A**) and carotid-vasomotor (**Figure 3B**) stimulus response curves. The magnitude of resetting (i.e., sum of the changes in A_3, A_4, THR, and SAT from rest to exercise) was not different between groups ($P > 0.05$). Findings for all stimulus response curve variables other than carotid-cardiac A_1 were similar when controlling for family history of hypertension (Two-Way ANCOVA).

DISCUSSION

In the present study, we have characterized for the first time CBR control of HR and blood pressure in AA subjects during exercise. The primary findings are that the CBR-mediated reductions in HR and MAP in response to NS (simulated carotid hypertension) were smaller in AAs compared to age, BMI, and fitness-matched CA subjects during rest and dynamic leg exercise. These findings indicate impairment in the ability of AAs to buffer acute hypertension at rest, and during steady-state exercise.

We previously demonstrated reduced HR responsiveness to separate 5-s trials of NS among AAs at rest compared to CA subjects (Holwerda et al., 2011). Consistent with these findings, we demonstrate in the present study attenuated maximal HR responses to NS in AAs at rest (**Figure 1**), and extend these findings with an observed attenuation of maximal HR responses to NS during exercise (**Figure 2**). An attenuated minimum response (A_4), saturation point, and magnitude of OP-CP detected with the carotid-cardiac function curves coupled with a trending reduction in the responding range (A_1) also support the limited carotid-cardiac response to hypertensive stimuli among AA subjects compared to CA subjects (**Table 2**). That is, CA subjects operated further from saturation, providing a greater ability to reduce HR, despite similar maximal and operating gain. While a reduction in CBR gain can

Table 2 | Logistic model parameters and derived parameters.

	Rest		Exercise	
	AA	**CA**	**AA**	**CA**
CAROTID-CARDIAC CURVE				
A_1, bpm	20.5 ± 3.4	27.7 ± 3.6	14.8 ± 2.6	20.7 ± 4
A_2, au	0.11 ± 0.04	0.07 ± 0.01	0.12 ± 0.02	0.08 ± 0.02
A_3, mmHg*†	86.8 ± 3	106.2 ± 4	130.8 ± 4.6	134.3 ± 6.9
A_4, bpm†	50.5 ± 2.3	40.2 ± 3.1	112.3 ± 4.7	107.8 ± 7.1
Threshold, mmHg†	59.7 ± 2.9	72.9 ± 6.5	109.2 ± 5.7	101.2 ± 3.6
Saturation, mmHg*†	113.8 ± 4	139.4 ± 4.1	152.5 ± 5.4	167.3 ± 12.6
G_{max}, bpm/mmHg	−0.39 ± 0.05	−0.47 ± 0.07	−0.38 ± 0.06	−0.34 ± 0.05
G_{op}, bpm/mmHg	−0.35 ± 0.05	−0.3 ± 0.04	−0.26 ± 0.05	−0.24 ± 0.04
OP-CP, mmHg*†	−0.49 ± 3.4	−15.9 ± 3.8	−13.8 ± 2.5	−21.8 ± 5.8
CAROTID-VASOMOTOR CURVE				
A_1, mmHg	17.7 ± 2	20.8 ± 2	22.4 ± 3	21.7 ± 1.8
A_2, au	0.12 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
A_3, mmHg†	80.4 ± 3.3	89.7 ± 3.9	115.3 ± 7.8	116.1 ± 6
A_4, mmHg†	79 ± 2.4	79.3 ± 2.5	109.9 ± 3.5	101.3 ± 3.8
Threshold, mmHg†	58.9 ± 5.1	62.4 ± 5.3	82.6 ± 10	78 ± 9.3
Saturation, mmHg†	102 ± 4.3	116.9 ± 5	148 ± 8.8	154.1 ± 8.8
G_{max}, mmHg/mmHg	−0.49 ± 0.07	−0.41 ± 0.04	−0.42 ± 0.09	−0.36 ± 0.08
G_{op}, mmHg/mmHg	−0.34 ± 0.06	−0.37 ± 0.04	−0.35 ± 0.07	−0.33 ± 0.08
OP-CP, mmHg	5.5 ± 2.1	−0.48 ± 2.7	3.2 ± 5.9	−3.2 ± 4.9

Values expressed as mean ± SE.

AA, African American; CA, Caucasian American; A_1, response range; A_2, gain coefficient; A_3, centering point; A_4, minimum response; G_{max}, maximal gain; G_{op}, operating point gain; OP-CP, operating point-to-centering point; au, arbitrary units.

**Main effect for race ($P < 0.05$); †Main effect of condition (rest vs. exercise, $P < 0.05$).*

lead to inappropriate neural cardiovascular responses to exercise, racial differences in CBR-mediated reductions in HR do not appear to be due to an impaired CBR gain. Impaired CBR-mediated reductions in HR among AA subjects is more likely attributed to other neural mechanisms that influence vagal and/or sympathetic activity, as altered control of vagal activity and impaired CBR-mediated reductions in sympathetic activity are both potential contributors to the blunted HR response to NS observed in AAs.

In addition to the impaired HR responses to NS, we observed attenuated CBR-mediated maximal MAP responses to NS in AAs at rest and during exercise when compared to CAs (**Figures 1**, **2**). Despite seemingly small differences, the attenuated MAP responses to NS among AAs may extend to a physiologically meaningful distinction. The arterial baroreflex relies significantly on changes in vasomotor activity to regulate blood pressure (Collins et al., 2001; Ogoh et al., 2003b), thus the findings in the present study suggest that AAs potentially have a diminished ability to withdraw sympathetic outflow to the vasculature during hypertensive stimuli. While BP is a function of cardiac output, the reduced bradycardic response may contribute to the attenuated MAP responses to NS seen in this group. However, although not reported, the latency for the maximum HR response to NS rarely, if at all, coincided with the latency for the maximal MAP response to NS, consistent with previous observations (Fisher et al., 2009). Thus, altered arterial baroreflex control of the vasculature in AAs likely contributes to the observed group differences in the maximal MAP response to NS.

On the other hand, no racial differences in maximal MAP responses to NP were detected at rest or during exercise. Although not measured in the current study, Ray and Monahan (2002) reported smaller changes in MSNA in response to lower-body negative pressure were associated with similar peripheral vascular responses in AA compared to CA, indicating potentially greater transduction from neural to end-organ responses. As well, other investigations have reported exaggerated pharmacologically-induced alpha-adrenergic responses in AA (Kelsey et al., 2010, 2012). A divergence between AAs and CAs in sympathetic vascular transduction is indeed an important consideration when comparing baroreflex control of blood pressure between groups. Future studies that include simultaneous assessment of vascular conductance and MSNA during CBR activation at rest and exercise are warranted.

The importance of examining the ability of the arterial baroreflex to respond to rises and falls in blood pressure separately has previously been discussed (Studinger et al., 2009; Fisher et al., 2010; Holwerda et al., 2011). In addition to CBR function curves, we provide an analysis of maximal HR and MAP responses to the separate 5-s trials of NP and NS. No racial differences in carotid-cardiac or carotid-vasomotor function curve parameters during exercise were detected. However, despite similar CBR gain, AAs demonstrated significantly smaller maximal HR and MAP

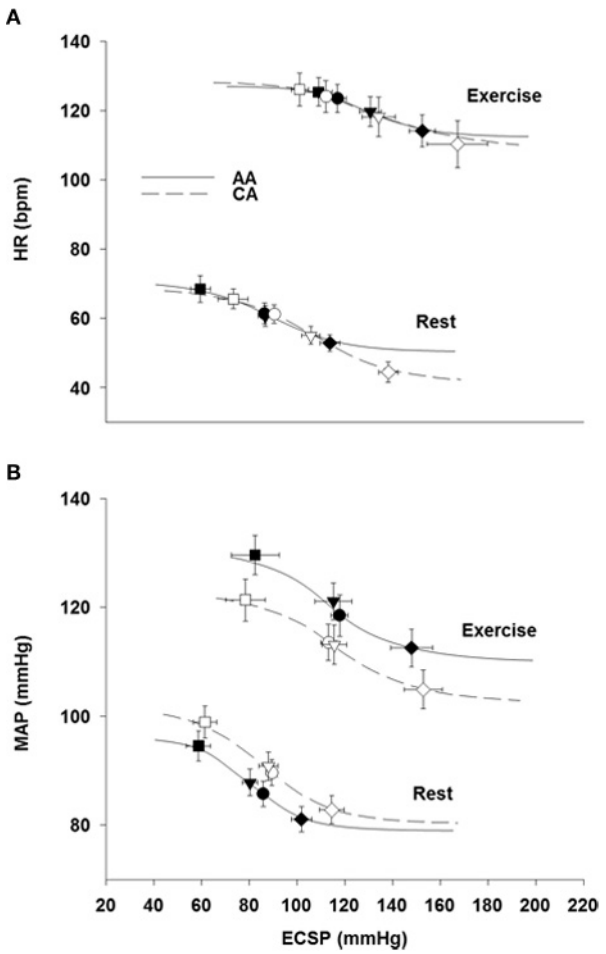

FIGURE 3 | Modeled carotid-cardiac (A) and carotid-vasomotor (B) baroreflex function curves at rest and during exercise in African Americans (AA, solid lines) and Caucasian Americans (CA, dashed lines). ECSP, estimated carotid sinus pressure. Filled symbols represent AA and open symbols represent CA. Circles represent operating points, inverted triangles represent centering points, squares represent carotid sinus pressure threshold, rotated squares represent carotid sinus pressure saturation.

responses to NS at rest and exercise, and no differences were observed in the maximal responses to NP. An examination of maximal responses to NP and NS unveiled important racial differences in acute buffering of hypertensive vs. hypotensive stimuli that may have otherwise been concealed by analysis of CBR function curves alone.

Previous studies have reported racial differences in BP responses to dynamic exercise (Alpert et al., 1981; Thomas et al., 1987; Ekelund et al., 1990; Walker et al., 1992). Statistical differences between groups in blood pressure changes from rest to exercise in the present study were not observed. However, upon removing one CA subject with an outlying BP response to exercise (MAP, $\Delta 43$ mmHg) compared to the rest of the CA group (MAP, $\Delta 22 \pm 2.7$ mmHg), a greater change in MAP was observed among AAs compared to the CAs ($P = 0.039$). The tendency for an exaggerated exercise-induced change in MAP among AAs was observed despite the relatively small total subject number compared to the vast total subject numbers seen in previous reports of racial differences in BP responses to dynamic exercise (Alpert et al., 1981; Thomas et al., 1987; Ekelund et al., 1990).

The use of 5-s trials of NP and NS used in the present study potentially elicits only an abbreviated range of HR and MAP responses. Although carotid baroreceptor stimulation with 5-s trials of NP and NS in the present study was sufficient to unveil group differences, perturbation of ∼20 s trials has previously been demonstrated to be required to develop a full response for MAP (Ogoh et al., 2003a). However, administration of 5-s periods of NP and NS eliminates pressure changes that would otherwise be sensed by aortic and cardiopulmonary baroreceptors, thus allowing for only carotid and, very likely, arterial baroreflex-mediated changes in HR and blood pressure to be observed.

PERSPECTIVES

A convincing body of evidence indicates augmented blood pressure responses to emotional and physical stressors in AAs compared to CAs (Light et al., 1987; Anderson et al., 1988; Treiber et al., 1990; Calhoun et al., 1993; Terrell and Manuck, 1996; Calhoun and Mutinga, 1997; Barnes et al., 2000; Kelsey et al., 2000; Bond et al., 2001; Arthur et al., 2004), and genetic studies have attempted to described the association between such sympathetic reactivity and the development of hypertension among this population. Alpha-adrenergic receptor gene polymorphisms (Kelsey et al., 2012) and genetic variations in beta-adrenergic receptors (Kelsey et al., 2010) have recently been associated with cardiovascular reactivity among AAs. Additional mechanisms linked to risk for hypertension among AAs include elevated salt sensitivity (Sowers et al., 1988) via altered amiloride-sensitive epithelial sodium channel (ENaC) function (Ambrosius et al., 1999; Pratt et al., 2002), and small nuclear polymorphisms associated with blood pressure (Adeyemo et al., 2009; Fox et al., 2011). While the relationship between risk factors such as adrenergic receptor variations or altered ENaC function and the development of hypertension among AAs has been considered, additional studies are warranted to determine whether impaired CBR responsiveness is indeed a neural cardiovascular risk factor in this population.

In summary, our findings suggest impairment in CBR ability to defend against a hypertensive challenge among AAs during exercise compared to their CA counterparts. Despite similar gain and magnitude of resetting of the carotid-cardiac and carotid-vasomotor logistic function curves, maximal HR and MAP responses to NS (simulated hypertension) were reduced in AA subjects. The reduction in maximal HR responses to NS observed in AA subjects are likely attributed to altered control of vagal activity, while the mechanisms associated with the reductions in the peak MAP responses to NS are less discernable. Given that an intact arterial baroreflex is important for appropriate cardiovascular responses to exercise, these findings lend insight to the potential mechanism(s) responsible for previously observed racial differences in cardiovascular responses to physical and mental stimuli observed among AAs.

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The arterial baroreflex resets with orthostasis

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The arterial baroreflexes, located in the carotid sinus and along the arch of the aorta, are essential for the rapid short term autonomic regulation of blood pressure. In the past, they were believed to be inactivated during exercise because blood pressure, heart rate, and sympathetic activity were radically changed from their resting functional relationships with blood pressure. However, it was discovered that all relationships between carotid sinus pressure and either HR or sympathetic vasoconstriction maintained their curvilinear sigmoidal shape but were reset or shifted so as to best defend BP during exercise. To determine whether resetting also occurs during orthostasis, we examined the arterial baroreflexes measured supine and upright tilt. We studied the relationships between systolic BP and HR (the cardiovagal baroreflex), mean BP, and ventilation (the ventilatory baroreflex) and diastolic BP and sympathetic nerve activity (the sympathetic baroreflex). We accomplished these measurements by using the modified Oxford method in which BP was rapidly varied with bolus injections of sodium nitroprusside followed 1 min later by bolus injections of phenylephrine. Both the cardiovagal and ventilatory baroreflexes were "reset" with no change in gain or response range. In contrast, the sympathetic baroreflex was augmented as well as shifted causing an increase in peripheral resistance that improved the subjects' defense against hypotension. This contrasts with findings during exercise in which peripheral resistance in active skeletal muscle is not increased. This difference is likely selective for exercising muscle and may represent the actions of functional sympatholysis by which exercise metabolites interfere with adrenergic vasoconstriction.

Keywords: cardiovascular regulation, standing, sympathetic nerve activity, heart rate, ventilation

INTRODUCTION

Standing up reduces venous return by translocating a large fraction of central blood volume, in excess of 500 ml in the adult human, to the dependent body parts. After mechanical equilibrium is re-established during continued standing, microvascular filtration from plasma to interstitium continues to reduce blood volume (Levick and Michel, 2010). Partial restitution of blood volume depends on lymphatic activity and reabsorption of interstitial fluid into the blood volume (Huxley and Scallan, 2011). Nevertheless, there is a net reduction in blood volume and venous return, and thus a net reduction in cardiac output (CO), cerebral blood flow, central blood volume, and stroke volume during quiet standing (Rowell, 1993). Total peripheral resistance (TPR), sympathetic nervous activity, and blood pressure are increased. Diastolic BP increases more than systolic blood pressure and the resultant decrease in pulse pressure coincides with the reduction in stroke volume when upright.

Orthostatic tolerance depends on intact intrinsic vascular structure and function, intact control of vasomotor function, adequate central blood volume and oxygen carrying capacity, and intact physical compensatory mechanisms including intact skeletal and respiratory muscle pumps (Wang et al., 1960; Miller et al., 2005). Rapid changes in blood pressure during orthostasis are primarily buffered by the arterial baroreflexes (Aviado and Schmidt, 1955; Mancia, 1975; Sanders et al., 1988) which comprise a negative feedback inhibitory stretch reflex (Sagawa, 1983). Therefore, a decrease in blood pressure reduces stretch of unmyelinated receptors in the carotid bifurcation and along the aortic arch causing a reflex increase in heart rate of primarily parasympathetic origin (Katona et al., 1970), and an increase in vasoconstriction of primarily sympathetic origin along with beta adrenergic mediated adrenal medullary secretion.

Increased arterial pressure during orthostasis should inhibit orthostatic sympathoexcitation which is instead increased (Burke et al., 1977). Prior investigations attribute this to a change in baroreflex sensitivity (Cooper and Hainsworth, 2001; Fu et al., 2006). Increased heart rate and adrenergic vasoconstriction are critical to maintaining blood pressure when upright and their absence is characteristic of autonomic failure causing neurogenic orthostatic hypotension (Freeman et al., 2011).

Changes in arterial baroreflexes have been found during exercise where it has been determined that the arterial blood pressure and heart rate stimulus-response derived from isolated carotid sinuses in dogs were displaced upward proportionate to exercise work load, with unchanged curve characteristics (Melcher and Donald, 1981); the same relationship was later seen in humans (Potts et al., 1993) where it was represents a resetting of the sigmoidal (Kent et al., 1972) cardiovagal and sympathetic baroreflex relations such that an upward and rightward shift of the stimulus-response (carotid sinus baroreflex pressure vs. heart rate,

sympathetic nerve activity, arterial pressure) occurs (Raven et al., 1997; Fadel et al., 2001; Fadel and Raven, 2012). Subsequent investigations indicate that baroreflex resetting during exercise is a consequence of interactions of the arterial baroreflex with central command, cardiopulmonary pressure reflexes, and the exercise pressor reflex (Fadel and Raven, 2012).

The decrease in cardiopulmonary pressure that occurs with upright posture (Victor and Mark, 1985) led us to hypothesize that similar arterial baroreflex resetting occurred during orthostasis. We tested this hypothesis by examining the cardiovagal baroreflex (HR/RR-interval vs. systolic blood pressure), and the sympathetic baroreflex [MSNA vs. diastolic blood pressure (DBP)] during upright tilt. Our recent work also indicated the importance of a ventilatory efferent arm of the arterial baroreflex [ventilation vs. mean arterial pressure (MAP)] which we also examined. This was included because of the demonstrated relation between MAP and ventilation (Stewart et al., 2011), the known relation between orthostasis and increased ventilation (Matalon and Farhi, 1979), and the importance of increased ventilation and hypocapnia to diverse forms of orthostatic intolerance (Novak et al., 1998; Lagi et al., 2001; Stewart et al., 2006; Thijs and van Dijk, 2007).

MATERIALS AND METHODS

SUBJECTS

Ten healthy volunteer subjects aged 18–25 years (median = 21 years, five female). Average weight (± SD) was 74 ± 18 kg, average height was 173 ± 13 cm, average BMI was 22 ± 3 kg/m^2. Subjects were free of all cardiorespiratory, autonomic, and systemic illnesses, took no medications and were non-smokers. There were no trained athletes. Informed consent was obtained. All protocols were approved by the Committee for the Protection of Human Subjects of New York Medical College.

INSTRUMENTATION

Subjects refrained from eating for at least 8 h and had no caffeinated beverages for at least 24 h prior to testing.

Testing began at 9:30 a.m. Subjects were familiarized with the procedures used in the study prior to any instrumentation. An intravenous catheter was placed in the left antecubital vein. Peroneal microneurography was performed to measure muscle sympathetic nerve activity (MSNA). In brief, multiunit recordings of efferent postganglionic MSNA were obtained using the technique established by Vallbo et al. (1979). A tungsten microelectrode was inserted into a muscle fascicle of the peroneal nerve, posterior to the fibular head (Wallin et al., 1996) using a unipolar tungsten electrode (uninsulated tip diameter 1–5 μm, shaft diameter 200 μm; Frederick Haer & Co.). Nerve activity was amplified with a gain of 100,000, then band pass filtered (0.7–2 kHz), and integrated using a 0.1 s lag (University of Iowa, Iowa City). A low impedance reference electrode was inserted a 1–2-cms away. Stable MSNA recordings were pulse synchronous, had increased burst counts during stage II of the Valsalva maneuver and were insensitive to sudden clapping or stroking of the skin. Integrated MSNA appear as upright "bursts." Identified bursts were expressed as burst count (bursts/min), and total MSNA (area under the curve for all bursts). Total MSNA was obtained after first normalizing each burst for any given subject by dividing all burst, supine, and upright by the largest burst amplitude during the baseline supine period and multiplying by 1,000. Total MSNA results were expressed as arbitrary units for each subject.

Subjects were instrumented for electrocardiogram (ECG), heart rate, and rhythm. Baseline beat to beat AP was collected while supine using finger photoplethysmography (Finometer, FMS, Amsterdam) calibrated against an oscillometric BP cuff. CO was estimated using the Finometer which models the circulation as an adaptive Windkessel and which was calibrated against an inert gas rebreathing apparatus (Innocor, Innovision, Denmark) using 0.1% sulfur hexafluoride (SF_6). Respirations were monitored by pneumotachography (Hans Rudolph Inc., MO, USA) using a tight fitting face mask. The pneumotachograph was calibrated using a 3-L syringe. End tidal carbon dioxide (ETCO2) was measured with a capnograph using nasal prongs connected to a side stream capnograph while oxygen saturation was measured using standard pulse oximetry (Capnocheck®Sleep Capnograph/Oximeter, Tri-Anim Health, and Sylmar, CA, USA). A chest impedance band (Respitrace, NIMS Inc., Miami, FL, USA) was also used to monitor relative respiratory excursions.

PROTOCOL

Following a 30 min acclimatization period, data were recorded throughout a 10 min supine baseline period. Baroreflex data were assessed using the modified Oxford method. An intravenous bolus injection of 100 μg sodium nitroprusside (SNP) was followed 1 min later by an intravenous injection of 150 μg phenylephrine (**Figure 1**). The method decreases AP by approximately 15–20 mmHg and subsequently increases AP by approximately 15–20 mmHg above baseline (Halliwill et al., 2003; Lipman et al., 2003). This results in an increase in heart rate (equivalently decrease in RR-interval), an increase in ventilation (Stewart et al., 2011), and an increase in sympathetic activity as shown in **Figure 1**.

Subjects were then allowed to recover and return to baseline over a 30 min period.

Following supine measurements subjects were tilted to 60° upright for 10 min. Heart rate, RR-interval, MSNA, respiratory, and AP data during minutes 1–5 were used for establishing a new upright baseline. The Oxford method was repeated at 5 min following the start of tilt tilt using the methods described above. After upright heart rate and AP returned to near pre-Oxford levels, subjects returned to the supine position. All subjects were able to tolerate the 10 min tilt and none reported symptoms of pre-syncope.

DATA ANALYSIS

Approximately 5 min of baseline "pre-Oxford" data were analyzed preceding performance of the Oxford method in the supine position. Also, upright baseline data were obtained during minutes 1–5 of HUT to avoid the fluid equilibration phase that occurs during the first minute of tilt (Wieling et al., 2007). Data were collected continuously during the hypotensive and hypertensive phases of the Oxford method in both supine and upright positions and included ECG, CO, systolic (SBP), and diastolic (DBP) blood pressure, and MAP calculated using the Finometer. TPR was computed using the formula TPR = MAP/CO (mmHg/L/min). RR-interval and heart rate were calculated from the EKG. MSNA

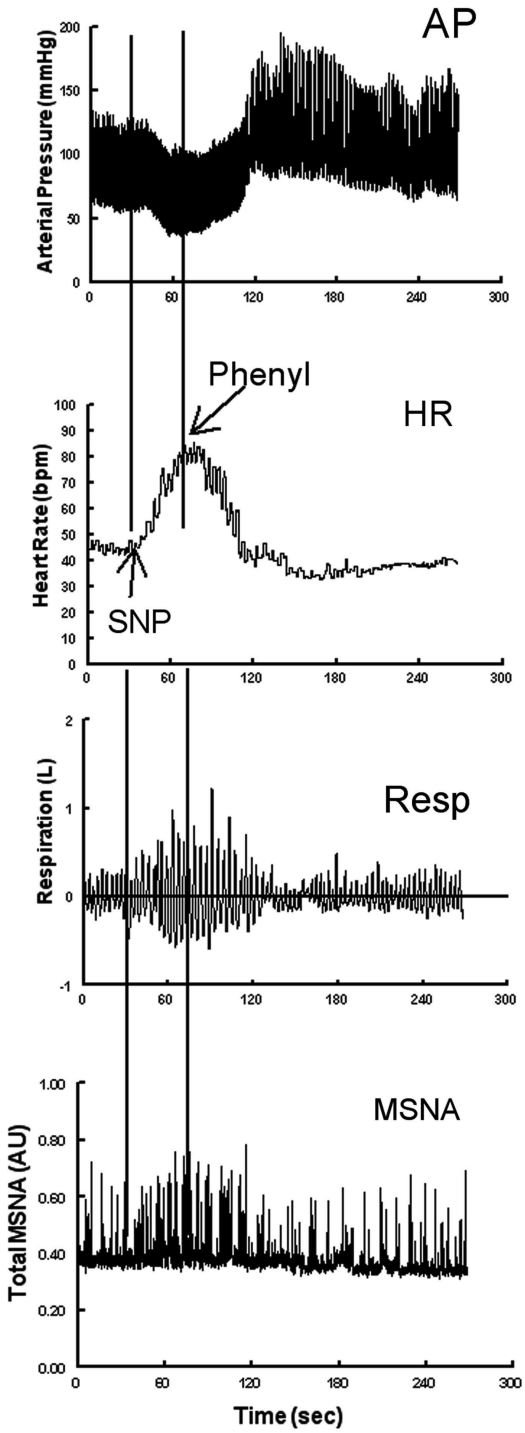

FIGURE 1 | Representative neurovascular and respiratory responses to the modified Oxford maneuver in which a sodium nitroprusside (SNP) bolus is followed 1 min later by phenylephrine. From top to bottom the figure shows arterial pressure, heart rate, respirations, and MSNA bursts.

bursts were detected by custom software that automated collection of bursts and burst counts with human operator oversight. All MSNA analysis was done by a single analyst. Data were sampled at 200 Hz using custom signal processing software and analyzed off-line.

CARDIOVAGAL BAROREFLEX DETERMINED BY THE MODIFIED OXFORD METHOD

Heart rate was plotted as a function of SBP to generate the cardiovagal baroreflex response during the modified Oxford method. The heart rate during each heart beat was calculated as 60,000/RR-interval (ms). Using all RR-intervals and SBP values from the onset of decrease in SBP until maximum SBP introduces "hysteresis" (Studinger et al., 2007); the same value of SBP during the decrease and increase of blood pressure can be associated with different RR-intervals. To avoid this complication we adopted the strategy of (Hunt and Farquhar, 2005) who restricted data acquisition to the rising arm of SBP (i.e., from the minimum until the maximum SBP was achieved).

BAROREFLEX MEDIATED SYMPATHETIC ACTIVATION DETERMINED BY THE MODIFIED OXFORD METHOD

Efferent baroreflex regulation of sympathetic activity was provided by the relationship between MSNA and DBP during the drug boluses. DBP correlates best with MSNA in humans (Sundlof and Wallin, 1978). MSNA bursts were time lagged by approximately 1.3 s from their respective triggering *R*-wave. Each burst was therefore shifted by the actual lag computed by our software. The shift needed to account for time lags varying from subject to subject.

A shifted and denoised file of bursts was created using normalized burst areas. Inter-burst activity was set to zero. Similarly, a file of burst counts was created such that each count comprised a discrete "delta" function at the peak of the burst with unit area corresponding to unit count at a given time. Thus, burst area per minute (total MSNA) or burst count per unit time could be simply obtained by taking the integral of burst area or burst count over the time in question.

VENTILATORY BAROREFLEX DETERMINED BY THE MODIFIED OXFORD METHOD

Efferent baroreflex regulation of ventilation was provided by the relationship between MSNA and MAP during the drug boluses. Respiratory volumetric changes were converted to expiratory minute volume (V_E) using a differencing procedure by which the volume of air exhaled was calculated for each breath and divided by the total time in seconds of that breath, then multiplied by 60 (s/min). As calculated from raw pneumotachometer data, this equaled the integrated flow during expiration divided by the breath duration from onset of inspiration to offset of expiration (=onset of the next breath) multiplied by 60. MAPs corresponded best to V_E. A time delay between MAP and ventilation would be expected based on neuromechanical issues. This was observed. Usually this varied between 0 and 3 s. Delays were related to the time to onset of the first breath following the start of decreasing blood pressure due to SNP used in the Oxford method. Delays were compensated by shifting respirations such that onset of change in respiration corresponded to onset of change in pressure. This is similar to practices used to align muscle sympathetic nerve recordings to corresponding RR-intervals (Delius et al., 1972).

Our data comprised pairs of time series indexed to the cardiac cycle: SBP and RR-interval, MAP and V_E, DBP, and MSNA bursts or total MSNA as delta functions. In each, the pressure (SBP, MAP, or DBP) in mmHg corresponding to each cardiac cycle was assigned to the entire cycle in the time series. Similarly we assigned the magnitude of the heart rate in beats per min, V_E in L/min, and MSNA bursts per min or total MSNA to their corresponding cardiac cycle, thus creating HR, V_E, MSNA, and pressure tachograms. A tachograms replaces a continuous set of values with its average for a given cardiac cycle. For a fiducial value such as diastole or systole, the tachygram assigns that value to the entire cardiac cycle. Thus, when the time series for a tachograms is closely inspected a series of steps of duration equal to the cardiac cycle and of amplitude equal to the measurement is obtained. For ventilation each step had only a portion of a respiratory cycle corresponding to it (within the particular cardiac cycle) or there could be overlap overlap with adjacent V_E steps. In either case a time average of the V_E falling within a particular cardiac cycle was performed. Thereafter, each cardiac cycle, pressure, and corresponding paired variable (HR, V_E, MSNA) were collapsed to a discrete (point) value corresponding to a single discrete cardiac cycle. These aligned digital sequences of pressure and paired variables were then sorted on pressure using the Quicksort algorithm (Hoare, 1962) from smallest to largest. We next binned pressures into pressure bins of 1–2 mmHg depending on data. Several values of any given pressure sequence typically fell within a given bin and were averaged to obtain an averaged bin pressure. Similarly, corresponding points of the paired variable (RR-interval, V_E, MSNA) point values were effectively binned to obtain an average of the paired over the specific bin. There were typically no empty bins because of the averaging procedure. We performed a weighted least squares fit of RR-interval against SBP where the weights were the number of points that fell into a specific bin divided by the total number of points in all bins.

Cardiovagal baroreflex curves (HR vs. SBP), ventilatory baroreflex curves (V_E vs. MAP), and sympathetic activity baroreflex curves (MSNA burst or total MSNA vs. DBP) were constructed for each subject while supine and during HUT by plotting pressure on the abscissa and the paired variable on the ordinate during the Oxford maneuver. A logistic (sigmoid) curve was fitted for each subject and each pressure pair during both positions using the Levenberg–Marquardt non-linear least squares algorithm (Marquardt, 1963) and gave a much better fit in the least squares sense compared to a straight line. An r^2 of 0.85 or greater was obtained with each fit. The logistic curve was determined by four parameters a_0, a_1, a_2, a_3, and calculated using the following equation:

$$\text{Paired variable} = a_0 + \frac{a_1}{1 + \exp\left[\frac{-(\text{AP} - a_2)}{a_3}\right]}$$

where:

a_0 is the lower asymptote or "threshold" of the fit,

$a_0 + a_1$ is the maximum asymptote or "saturation" of the fit,

a_2 is the value of AP at maximum slope (centering point),

$a_1/(4*a_3)$ determines the maximum slope or "sensitivity."

Baroreflex sensitivity corresponds to the maximum slope (or maximum gain) of the generated sigmoid curve, while the operating point (OP) was determined by the measured pressure and its paired variable when supine or upright. A slope was also calculated at the centering point from the logistic fit equation. The baroreflex threshold was identified as the minimum asymptote of the sigmoid curve and the saturation was identified as the maximum asymptote of the curve. The response range was calculated as the difference between the threshold and saturation. An idealized curve is shown in **Figure 2**. Note that a least squares algorithm was used to fit individual curves over a range of Oxford-generated pressures and its paired variables. Therefore the OP for each curve did not in general fall on the fit curve as shown.

STATISTICS

Because there were no discernible differences between the data from men and women, data from both groups were combined for analysis. Measurements made in the supine and HUT positions computed for each individual were compared using paired t-tests with a Bonferroni correction for multiple comparisons. Two-way analysis of variance for repeated measures was used for Oxford measurements comparing changes in response to SNP and phenylephrine in the supine and HUT positions. When appropriate, *post hoc* comparisons were performed using Tukey's test. Differences were considered significant when $P < 0.05$. All values are reported as means ± SD. Results were calculated using SPSS (Statistical Package for the Social Sciences) software version 11.0.

RESULTS

EFFECT OF HUT

The HR increased with HUT ($P < 0.001$) while systolic, diastolic, and MAP did not change significantly. CO, and TPR also did not change significantly but there were significant increases in total MSNA and in MSNA counts ($P < 0.01$). Expiratory minute volume increased ($P < 0.01$) while respiratory rate failed

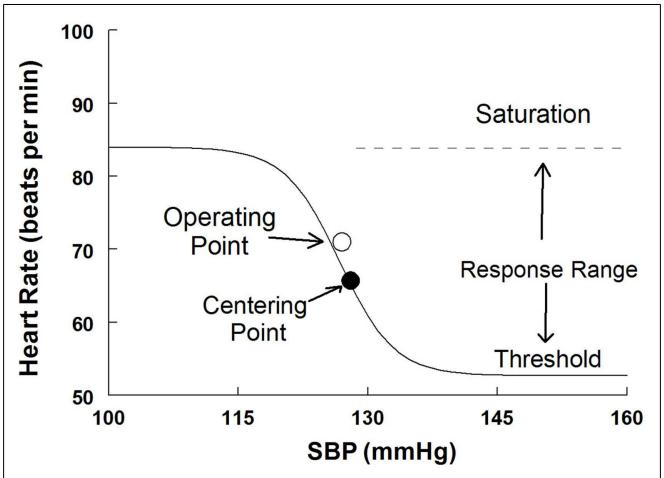

FIGURE 2 | A sigmoid fitted curve using the modified Oxford procedure to generate the HR vs. systolic blood pressure relation. The operating point, centering point, threshold, saturation, and response range are illustrated.

Table 1 | Supine and upright hemodynamics and MSNA pre-Oxford, after SNP bolus, and after phenylephrine.

Measurement	Supine	HUT
HR (bpm)		
Pre-Oxford	62 ± 4	86 ± 5†
SNP	94 ± 4*	121 ± 8*†
Phenylephrine	50 ± 3*	68 ± 4*†
SBP (mmHg)		
Pre-Oxford	122 ± 5	125 ± 5
SNP	98 ± 7*	96 ± 5*
Phenylephrine	137 ± 5*	138 ± 7*
DBP (mmHg)		
Pre-Oxford	67 ± 3	72 ± 2
SNP	50 ± 3*	46 ± 3*
Phenylephrine	78 ± 6*	83 ± 5*
MAP (mmHg)		
Pre-Oxford	82 ± 2	87 ± 3
SNP	68 ± 4*	64 ± 5*
Phenylephrine	92 ± 2*	91 ± 4*
CO (l/min)		
Pre-Oxford	4.8 ± 0.3	4.5 ± 0.4
SNP	6.8 ± 0.5*	6.4 ± 0.5*
Phenylephrine	3.6 ± 0.3*	3.7 ± 0.3*
TPR (mmHg/l/min)		
Pre-Oxford	16 ± 2	17 ± 2
SNP	11 ± 1*	12 ± 1*
Phenylephrine	26 ± 3*	23 ± 3*
Total MSNA (AU/min)		
Pre-Oxford	2112 ± 367	3153 ± 433†
SNP	7319 ± 656*	14677 ± 3018*†
Phenylephrine	1224 ± 311*	2023 ± 889*
MSNA counts (Bursts/min)		
Pre-Oxford	17 ± 2	33 ± 6†
SNP	40 ± 4*	52 ± 6*†
Phenylephrine	11 ± 2*	29 ± 4*†

*Values are mean ± SD. MSNA, muscle sympathetic nerve activity; HUT, head up tilt; SNP, sodium nitroprusside; SBP, systolic blood pressure; DBP, diastolic blood pressure; CO, cardiac output; TPR, total peripheral resistance; *P < 0.05 compared to pre-Oxford; †P < 0.05 compared with supine.*

to increase indicating an increase in tidal volume with HUT (**Tables 1** and **2**).

EFFECT OF THE MODIFIED OXFORD MANEUVER ON HEMODYNAMICS AND MSNA, SUPINE AND UPRIGHT

HR decreased with SNP ($P < 0.001$) and increased with phenylephrine ($P < 0.001$). Systolic, diastolic, and mean pressures decreased significantly ($P < 0.001$) with SNP and increased with phenylephrine. There were no differences when upright. In contrast CO increased with SNP and decreased with phenylephrine ($P < 0.01$) while TPR decreased with SNP and then increased with phenylephrine ($P < 0.01$). There were also no differences when upright. MSNA bursts and total MSNA increased with SNP and decreased to below baseline with phenylephrine. MSNA was significantly ($P < 0.01$) larger upright compared to supine (**Table 1**).

Table 2 | Supine and upright respiratory data pre-Oxford, after SNP bolus, and after phenylephrine.

Measurement	Supine	HUT
V_E (l/min)		
Pre-Oxford	7.8 ± 0.9	10.2 ± 1.0†
SNP	22.2 ± 7.1*	25.9 ± 7.8*
Phenylephrine	4.8 ± 0.6*	6.9 ± 0.8*†
Respiratory rate (breath/min)		
Pre-Oxford	13.6 ± 1.1	13.3 ± 1.6
SNP	12.3 ± 1.2	13.2 ± 1.6
Phenylephrine	13.1 ± 1.5	12.7 ± 1.6
$ETCO_2$ (Torr)		
Pre-Oxford	43 ± 1	42 ± 1
SNP	38 ± 1*	37 ± 1*
Phenylephrine	44 ± 1	44 ± 1

*Values are mean ± SEM. HUT, head up tilt; SNP, sodium nitroprusside; V_E, expiratory minute volume; $ETCO_2$, end tidal CO_2; *P < 0.05 compared to Pre-Oxford; †P < 0.05 compared with supine.*

EFFECT OF THE MODIFIED OXFORD MANEUVER ON RESPIRATORY DATA

The respiratory rate was unaffected by SNP and phenylephrine either supine or upright. However, expiratory minute volume V_E increased with HUT ($P < 0.05$). The expiratory minute volume V_E also increased markedly with SNP and decreased with phenylephrine ($P < 0.001$) implying a large increase in tidal volume. $ETCO_2$ was less affected with a smaller but significant ($P < 0.01$) fall in $ETCO_2$ as the result of short lived hyperpnea during SNP; this failed to empty carbon dioxide reserves (**Table 2**).

Baroreflex curves were fit for each cardiopulmonary parameter for each subject, while supine and upright using sigmoidal curvilinear fits as stated above. Goodness of fit using a squared correlation measure (r^2) varied from 0.80 to 0.98 with an average for each individual parameter of approximately of 0.90. For comparison linear fits resulted in squared correlation ranging from 0.4 to 0.8.

Cardiovagal baroreflex results supine and upright

Maximum cardiovagal baroreflex slope (sensitivity) is shown in **Table 3**. The slope is not statistically different supine or upright. The HR at maximum slope is significantly increased by HUT as are the threshold and saturation of the sigmoid curve. The response range is not different. Systolic BP at maximum gain is significantly increased by HUT ($P < 0.01$). **Figure 3** shows supine and upright curves created from the averaged values of **Table 3**. OPs averaged over all subjects are shown. Since response range and slope are unchanged, the curves can be created by translating the centering point to an increased heart rate thereby resetting the relationship without changing its sensitivity as shown in **Figure 3**.

Ventilatory baroreflex results supine and upright

Maximum ventilatory baroreflex slope (sensitivity) is shown in **Table 4**. The supine and upright maximum slopes are not significantly different. The expiratory minute volume, V_E, at maximum slope is not significantly increased during HUT nor are the threshold and saturation of the sigmoid curve. Thus, the response range

Table 3 | Calculated cardiovagal baroreflex sensitivity (slopes), threshold, saturation, and response range using the modified Oxford method.

Calculated logistic parameters	Supine	HUT
Slope (sensitivity, max gain, bpm/mmHg)	−2.7 ± 0.7	−3.4 ± 1.0
SBP at maximum gain (mmHg)	119 ± 5	127 ± 3†
HR at maximum gain (bpm)	73 ± 6	87 ± 5†
Threshold (bpm)	55 ± 3	66 ± 7†
Saturation (bpm)	91 ± 5	106 ± 6†
Response range (bpm)	36 ± 5	44 ± 7

Values are mean ± SD. bpm, Beats per minute; HUT, head up tilt; SBP, systolic blood pressure; †P < 0.05 compared to the supine.

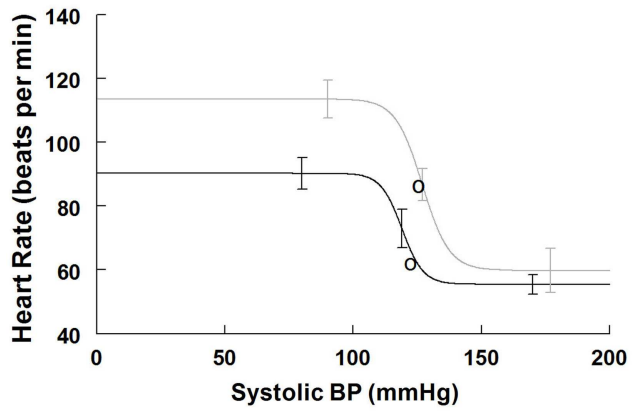

FIGURE 3 | Supine and upright cardiovagal baroreflex relationships were obtained by averaging data-fits over all subjects during the Oxford maneuver. The upright curve is shifted upwards and to the right from supine but the maximum slope (sensitivity) is unaffected by posture.

Table 4 | Calculated ventilatory baroreflex (slopes), threshold, saturation, and response range using the modified Oxford method.

Calculated logistic parameters	Supine	HUT
Slope (sensitivity, max gain, ms/mmHg)	−3.7 ± 0.7	−3.3 ± 0.8
MAP at maximum gain (mmHg)	68 ± 3	79 ± 3†
V_E at maximum gain (l/min)	31 ± 5	35 ± 4
Threshold (l/min)	8 ± 2	13 ± 4
Saturation (l/min)	51 ± 7	56 ± 6
Response range (l/min)	41 ± 8	41 ± 6

Values are mean ± SD. HUT, head up tilt; MAP, mean arterial pressure; V_E, expiratory, minute ventilation; †P < 0.05 compared to the supine.

is not different. **Figure 4** depicts supine and upright curves created from the averaged values of **Table 4**. OPs averaged over all subjects are shown. Since response range and slope are unchanged, the curves can be created by translating the centering point to an increased V_E, thereby resetting the relationship without changing its sensitivity as shown in **Figure 4**.

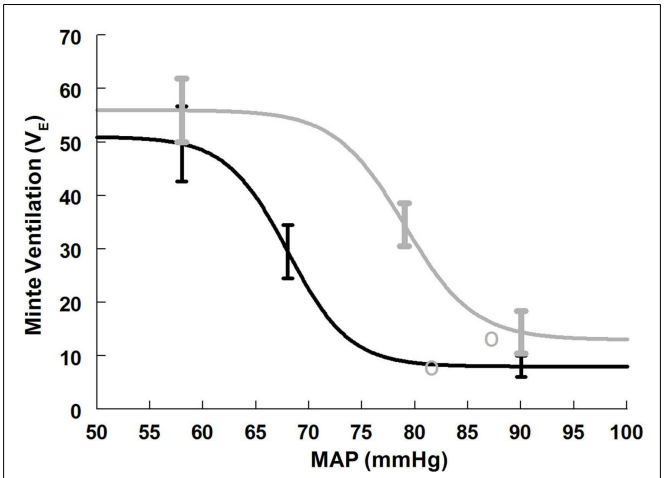

FIGURE 4 | Supine and upright ventilatory baroreflex relationships were obtained by averaging data-fits over all subjects during the Oxford maneuver. The upright curve is shifted to the right from supine but the maximum slope (sensitivity) is unaffected by posture.

Table 5 | Calculated total MSNA baroreflex sensitivity (slopes), threshold, saturation, and response range using the modified Oxford method.

Calculated logistic parameters	Supine	HUT
Slope (sensitivity, max gain, AU/mmHg)	−871 ± 167	−1084 ± 220†
DBP at maximum gain (mmHg)	60 ± 3	64 ± 3†
MSNA at maximum gain (AU/min)	5503 ± 582	11722 ± 2466†
Threshold (AU/min)	917 ± 282	698 ± 223
Saturation (AU/min)	10,080 ± 1871	22877 ± 3806†
Response range (AU/min)	9173 ± 1329	21457 ± 4306†

Values are mean ± SD. HUT, head up tilt; SBP, systolic blood pressure; †P < 0.05 compared to the supine.

Total MSNA baroreflex response supine and upright

Maximum total MSNA baroreflex slope (sensitivity) is shown in **Table 5**. The slope magnitude is significantly greater upright compared to supine ($P < 0.05$). There is no difference in threshold but the centering point is shifted to the right and upwards ($P < 0.01$), and both upright saturation and response range are twice as large as corresponding supine quantities ($P < 0.01$). **Figure 5** depicts supine and upright curves created from the averaged values of **Table 5**. Since both response range and slope are increased during HUT, the curves are not related by a simple translation of the centering point. Resetting in the sense of an unchanged shape of the sigmoid curve does not occur in the context of sympathetic activation during HUT as shown in **Figure 5**.

MSNA Count baroreflex response supine and upright

Maximum MSNA burst count slope (sensitivity) is shown in **Table 6**. The slope magnitude is significantly greater upright compared to supine ($P < 0.05$). There is no difference in threshold but the centering point is shifted to the right ($P < 0.01$).

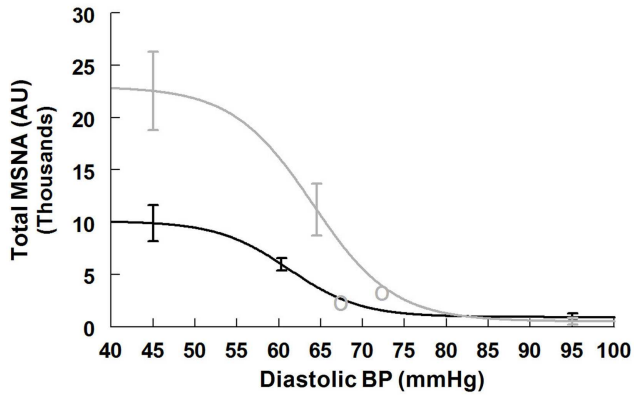

FIGURE 5 | Supine and upright total MSNA response relationships obtained by averaging data-fits over all subjects during the Oxford maneuver. The upright curve is shifted from supine but the maximum slope (sensitivity), response range, and saturation are also increased when upright.

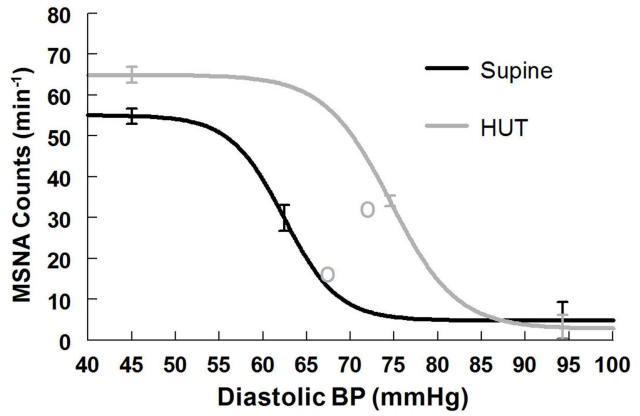

FIGURE 6 | Supine and upright MSNA burst count response relationships obtained by averaging data-fits over all subjects during the Oxford maneuver. The upright curve is shifted from supine but the maximum slope (sensitivity), response range, and saturation are also increased when upright.

Table 6 | Calculated MSNA counts.

Calculated logistic parameters	Supine	HUT
Slope (sensitivity, max gain, counts/mmHg)	-4.1 ± 0.9	$-5.3 \pm 1.0^{\dagger}$
DBP at maximum gain (mmHg)	62 ± 3	$74 \pm 3^{\dagger}$
MSNA at maximum gain (counts/min)	30 ± 3	$34 \pm 1^{\dagger}$
Threshold (counts/min)	5 ± 2	3 ± 2
Saturation (counts/min)	55 ± 4	$65 \pm 3^{\dagger}$
Response range (counts/min)	50 ± 5	$62 \pm 3^{\dagger}$

Values are mean ± SD. HUT, head up tilt; DBP, diastolic blood pressure; $^{\dagger}P < 0.05$ compared to the supine.

Upright saturation and response range are larger than corresponding supine quantities ($P < 0.025$) but difference in MSNA counts are far less dramatic compared to differences in total MSNA. **Figure 6** depicts supine and upright curves created from the averaged values of **Table 5**. Although response range and slope are still increased during HUT compared to supine this difference is relatively reduced compared to differences in total MSNA. The curves cannot be created by simply translating the centering point. Resetting in the context of sympathetic activity augments the response to changing diastolic pressure as shown in **Figure 6**.

DISCUSSION

On the one hand our results show cardiovagal and ventilatory baroreflex resetting when upright compared to supine. HR and expiratory minute volume increase when upright and respond similarly to a change of blood pressure whether supine or upright. On the other hand, the sympathetic baroreflex response is increased overall when upright compared to supine. The baroreflex function curves that are generated by the modified Oxford maneuver are displaced with respect to their centering points but are also changed in shape and thus in sensitivity and response range.

RESETTING OF THE CARDIOVAGAL ARTERIAL BAROREFLEX DURING ORTHOSTASIS

The resetting of the cardiovagal baroreflex can be understood in terms of the vagal withdrawal that occurs with upright posture. This resetting is similar to findings during exercise because similar vagal withdrawal occurs in both. Moreover, the increase of heart rate with upright posture does not normally exceed the capabilities of cardiac parasympathetic withdrawal to increase heart rate (Brouha et al., 1939). The OP of the upright cardiovagal baroreflex is at a higher heart rate and has shifted into the more linear portion of the sigmoid HR-baroreflex relation. A similar shifting occurs during exercise. The change in cardiovagal baroreflex centering or set point results from the reduction of central venous pressure on standing which unloads the cardiopulmonary baroreflexes (Pawelczyk and Raven, 1989). Stroke volume is markedly reduced during standing or HUT (Wang et al., 1960; Harms et al., 1999). Since $CO = SV \times HR$, the CO is less affected than stroke volume because heart rate increases when upright. Therefore the shift in the OP of the cardiovagal reflex and the resetting of the baroreflex curve favors and protects compensatory tachycardia without which chronotropic incompetence and orthostatic intolerance would supervene (Kawasaki et al., 2010).

RESETTING OF THE VENTILATORY ARTERIAL BAROREFLEX DURING ORTHOSTASIS

The resetting of ventilatory baroreflex is more difficult to explain since mechanisms remain hypothetical. Among potential mechanisms is the sympathoexcitation that occurs with orthostatic stress that can directly result in hyperventilation (Folgering, 1999). In this regard, large animal research indicates that projections from the arterial baroreceptors directly modulate respiratory activity (Gabriel and Seller, 1969; Richter and Seller, 1975), while sympathetic activation and vagal withdrawal stimulate peripheral and central chemoreflex activity (Heistad et al., 1975; Mancia, 1975).

Along with cardiovagal baroreflex resetting, ventilatory baroreflex resetting would protect CO by shifting the OP to a more linear portion of the V_E vs. MAP baroreflex curve when upright so that ventilation is enhanced. Hypotension results in increasing hyperpnea. The respiratory pump is engaged and is particularly effective in enhancing venous return to the heart during conditions of increased tidal volume (i.e., hyperpnea) by its effects on abdominal pressure, splanchnic emptying and femoral venous return (Takata et al., 1990).

RESETTING OF THE SYMPATHETIC ARTERIAL BAROREFLEX DURING ORTHOSTASIS

Our data indicate that the total MSNA – DBP relation is not similarly reset as are heart rate and ventilation. The upright curve is not simply a translated version of the supine curve. Rather it is potentiated by upright posture so that gain, saturation and response range are increased. Increased MSNA counts during standing has been previously reported (Burke et al., 1977) although linear data fits were used; it is unclear whether there could be enhanced unloading of the central compartment without any change in the functional relationship between blood pressure and MSNA (movement along a baroreflex functional curve) or a change in the functional relationship as we now report. Significantly increased total MSNA and burst count have been reported (Fu et al., 2004). During exercise, MSNA and leg vascular resistance appear to be dissociated (Shoemaker et al., 2000) even though similar increases in MSNA occur (Norton et al., 1999; Fadel et al., 2001; Ogoh et al., 2009). On the other hand increased MSNA also occurs with orthostasis but results in increased local peripheral resistance (Cooper and Hainsworth, 2001) which is sustained and correlates well with MSNA (Fu et al., 2006).

Thus, during orthostasis there is a functional relationship of MSNA with peripheral resistance; during exercise there is no well-defined relationship. This observation indicates certain fundamental differences in vascular regulation during exercise and orthostasis. Both stressors require adaptation of blood pressure control that includes resetting of the arterial baroreflexes; both maintain blood pressure stable or increased and defend against hypotension; and both produce generalized sympathetic activation comprising different strategies for dealing with these different vascular challenges. In dynamic exercise, vasodilation of actively exercising muscle occurs in response to metabolic and flow mediated factors promoting a large increase in active muscle blood flow. Increased blood flow depends on the decrease in peripheral resistance, on the actions of the skeletal and respiratory muscle pumps to enhance venous return to the heart, and on increased CO which is ensured by sympathetically mediated increase in cardiac contractility, by the Starling mechanism, and by cardiac afterload reduction (Kimball et al., 1993). Sympathetic vasoconstriction, in part mediated by the arterial baroreflex regulation, in part by central command, and in part by somatic reflexes such as the exercise pressor reflex (Donald et al., 1970), still occurs and would tend to counteract excessive skeletal muscle metabolic vasodilation. Local vasodilation and flow augmentation therefore reach a balance with sympathetic vasoconstriction. In addition, evidence suggests that sympathetic vasoconstrictive capability in actively exercising muscle is reduced in potency. Thus, for example, sympathetically denervated limbs may have similar vasodilation to exercise as sympathetically intact limbs (Beaconsfield, 1954). Also, functional sympatholysis – the blunting of vascular smooth muscle responsiveness to sympathetic vasoconstriction by metabolic factors in exercising muscle – can reduce MSNA-vascular smooth muscle transduction (Tschakovsky et al., 2002).

In orthostasis there is no sympatholysis, there is no pressor reflex, but there is central command evidenced by an increase in HR and BP in the seconds prior to standing or upright tilt. Standing up profoundly decreases venous return by almost instantly translocating a large fraction of central blood volume, in excess of 500 ml in the adult human, to the dependent body parts (Blomqvist and Stone, 1983). Because of this, until reflex vasoconstriction is established, there is a substantial transient decrease in blood pressure referred to as "initial orthostatic hypotension" (Wieling et al., 2007). Even with normal autonomic activation there is a decrease of CO by approximately 20%. Because there is no sympatholysis, transduction of MSNA to peripheral vasoconstriction occurs in monotonic fashion to provide a steady increase of sympathetic activity and adrenergic vasoconstriction with orthostatic stress. That is precisely what happens during upright stance. Sympathetic baroreflex curves show that when supine, both total MSNA and burst counts operate near the centering point where sensitivity is at maximum. With orthostasis, sympathetic sensitivity (gain) increases, and the OP for total MSNA shifts so as to remain near the centering point of the new upright shifted and augmented sigmoid curve. Burst counts appear to be close to their maximum which may best afford the overall increase in total MSNA. Thus, any decrease in blood pressure is prevented by a compensatory increase in MSNA operating over the optimum linear range while upright. This fits well with teleology: sympathetic activity is diminished when it is an impediment and enhanced when it is an advantage toward blood pressure maintenance. In the absence of effective sympathetic vasoconstriction, neurogenic orthostatic hypotension supervenes within seconds to a few minutes and syncope follows (Freeman et al., 2011).

LIMITATIONS

A modicum of caution is recommended because MSNA measured by peroneal microneurography assesses sympathetic nerve activity at a single location. However, it is likely that sympathetic activity is widely increased during orthostasis. Thus, for example, vascular resistance is typically increased in the splanchnic and renal circulations. On the other hand, sympathetic vasoconstriction is generally blunted in the coronary and cerebral circulations due to autoregulation.

In addition the data obtained has not been compared to data from other experiments in which baroreflex function was assessed by lower body negative pressure (LBNP; el Bedawi and Hainsworth, 1994; Goldstein et al., 2002), although some LBNP data are used for qualitative comparison (Cooper and Hainsworth, 2001; Carter et al., 2009).

An additional limitation of the data is that the testing was not standardized to one particular phase of the menstrual cycle.

Baroreflex function may be influenced by menstrual phase (Carter et al., 2009). However, there remains controversy with other investigators finding no effect of menstrual phase on baroreflex (Fu et al., 2009).

Performing only one modified Oxford trial rather than two or more averaged trials is a potential limitation because of the inherent variability in modified Oxford trials within a subject. However, this was necessitated by the time limits during upright tilt testing.

Finally, averaged OPs do not fall on averaged baroreflex curves. These are fit curves and non-linear least squares methods can introduce more error than linear methods. However, the fact that least squared fitting was used practically assures that the fitted curves do not go precisely through the resting OP but instead represent averages in a least squares sense of all points generated by the Oxford maneuver. On the other hand an average of the OPs is arithmetic (sum/number of values) which yields a somewhat different value.

IN SUMMARY

Arterial baroreflexes remain effective in the regulation and maintenance of blood pressure during orthostasis because the relationships between arterial blood pressure, heart rate, respiration, and sympathetic nerve activity shift or reset when upright. These shifts prevent hypotension through sympathetic activation and improve CO by an increase in heart rate and engagement of the respiratory-abdominal pump.

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Have we missed that neural vasodilator mechanisms may contribute to exercise hyperemia at onset of voluntary exercise?

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Whether neurally-mediated vasodilatation may contribute to exercise hyperemia has not been completely understood. Bülbring and Burn (1935) found for the first time the existence of sympathetic cholinergic nerve to skeletal muscle contributing to vasodilatation in animals. Blair et al. (1959) reported that atropine-sensitive vasodilatation in skeletal muscle appeared during arousal behavior or mental stress in humans. However, such sympathetic vasodilator mechanism for muscle vascular bed in humans is generally denied at present, because surgical sympathectomy, autonomic blockade, and local anesthesia of sympathetic nerves cause no substantial influence on vasodilatation in muscle not only during mental stress but also during exercise. On the other hand, neural mechanisms may play an important role in regulating blood flow to non-contracting muscle. Careful consideration of the neural mechanisms may lead us to an insight about a possible neural mechanism responsible for exercise hyperemia in contracting muscle. Referring to our recent study measuring muscle tissue blood flow with higher time resolution, this review has focused on whether or not central command may transmit vasodilator signal to skeletal muscle especially at the onset of voluntary exercise.

Keywords: central command, exercise, vasodilatation, skeletal muscle, near infrared spectroscopy

INTRODUCTION

Bülbring and Burn (1935) reported for the first time the existence of sympathetic cholinergic nerve to skeletal muscle contributing to vasodilatation in the cat and dog. It is histologically verified that sympathetic cholinergic nerves innervate blood vessels of skeletal muscle in several species such as the cat, dog, sheep, etc., while muscle vasculature in the rat and mouse lacks sympathetic cholinergic innervation (Bolme and Fuxe, 1970; Burnstock, 1980; Guidry and Landis, 2000). Moreover, there is no histological evidence for the existence of sympathetic cholinergic nerves to skeletal muscle in the monkey and man (Bolme and Fuxe, 1970). Atropine-sensitive vasodilatation in skeletal muscle occurs during fighting or arousal behavior in cats and dogs (Ellison and Zanchetti, 1973; Just et al., 2000). Although Blair et al. (1959) reported that the forearm vasodilator response to mental stress in humans was atropine-sensitive and became absent after surgical sympathectomy, no electrophysiological evidence for sympathetic cholinergic nerves has been demonstrated in humans (Wallin and Sundlöf, 1982; Saito et al., 1993; Callister et al., 1994). Joyner and Dietz (2003) suggested that circulating epinephrine and locally released acetylcholine, but not sympathetic dilator nerve, play a role in producing the vasodilator response to mental stress in humans. Even in the dog possessing the sympathetic cholinergic system, exercise hyperemia of hindlimb contracting muscle is not significantly influenced by surgical sympathectomy or ganglionic or muscarinic blockade (Donald et al., 1970; Buckwalter et al., 1997; Buckwalter and Clifford, 1999). Taken together, it has been currently thought that the sympathetic nervous system is not responsible for exercise hyperemia as well as vasodilation during mental stress, although sympathetic vasoconstriction restrains blood flow to active muscle during exercise (Joyner et al., 1992; Joyner and Halliwill, 2000; Clifford and Hellsten, 2004; Joyner and Wilkins, 2007; Shoemaker, 2012). Since most previous findings were obtained with measurements of limb blood flow via venous occlusion plethysmography and Doppler ultrasound at relatively low time resolution, a possible contribution of neurally-mediated vasodilatation to exercise hyperemia will be discussed referring to a recent study which measured muscle tissue blood flow with near-infrared spectroscopy (NIRS) at higher time resolution (Ishii et al., 2012).

SYMPATHETIC VASODILATATION IN SKELETAL MUSCLE DURING EXERCISE IN ANIMALS

Sympathetic cholinergic nerve can be activated when the localized areas in the hypothalamus, the midbrain periaqueductal gray matter, and the midbrain ventral tegmental area (VTA) are stimulated in the cat (Eliasson et al., 1951; Abrahams et al., 1960; Hilton et al., 1983; Bandler and Carrive, 1988; Matsukawa et al., 1993, 2011). It is of interest that electrical and chemical stimulation of neurons in the VTA increased blood flow and vascular conductance of the rat triceps surae muscle (Matsukawa et al., 2011; Nakamoto et al., 2011), although its muscular vasculature lacks sympathetic cholinergic innervation (Guidry and Landis, 2000). The vasodilation may be mediated by nitrosyl factors released from sympathetic postganglionic fibers and/or the vascular endothelium (Davisson et al., 1994). When visualizing using

an X-ray angiography the vascular responses of small arteries (internal diameter, 100–500 μm) in the cat triceps surae muscle, stimulation of the hypothalamic defense area caused tremendous increases in internal diameter and cross sectional area of the small arteries, which were abolished by muscarinic blockade or section of the sciatic nerve (Matsukawa et al., 1997). Although the vasodilation is mediated by activating sympathetic cholinergic nerve, whether the sympathetic cholinergic system is functionally operating during exercise is controversial.

Atropine-sensitive vasodilatation in skeletal muscle occurs during fighting or arousal behavior or classical conditioning of limb flexion with conditioned audio-tone stimulus and unconditioned electrical stimulation of a paw in animals (Ellison and Zanchetti, 1973; Just et al., 2000). However, the increase in femoral blood flow during treadmill exercise is unaffected by surgical sympathectomy or muscarinic blockade (Donald et al., 1970; Buckwalter et al., 1997; Buckwalter and Clifford, 1999). A reason responsible for the discrepancy may be that the sympathetic cholinergic vasodilatation, if any, may be masked by other mechanisms such as metabolic or flow-mediated vasodilatation and may appear during a voluntary type of exercise with a smaller muscle mass, rather than automatic rhythmic movement with whole body mass. Komine et al. (2008) found that brachial blood flow of the exercising forelimb, heart rate (HR), arterial blood pressure (AP) increases at the onset of voluntary static exercise in cats and intravenous injection of atropine blunts the increases in brachial blood flow and vascular conductance (**Figure 1**). It is likely that the sympathetic cholinergic mechanism is capable of evoking muscle vasodilatation at the onset of a voluntary type of exercise in conscious animals as well as fighting or arousal behavior.

MUSCLE SYMPATHETIC NERVE ACTIVITY DURING EXERCISE IN HUMANS

Sympathetic mechanisms responsible for muscle vasodilatation, if any, can be explained by withdrawal of sympathetic adrenergic vasoconstrictor activity and/or facilitation of sympathetic cholinergic or nitroxidergic vasodilator activity. Saito et al. (1993) and Callister et al. (1994) reported that muscle sympathetic nerve activity (MSNA) to a resting leg or arm decreases during anticipation and initiation of cycling exercise and then increases during the later period of exercise, suggesting sympathetic withdrawal prior to and at the start of exercise. However, the response of MSNA to non-contracting muscle at the onset of one-legged cycling is still controversial [increased (Herr et al., 1999), decreased (Saito and Mano, 1991), and unchanged (Ray et al., 1993)]. Fisher et al. (2005) reported that vascular conductance in the resting leg transiently increases at the onset of contralateral isometric exercise, whereas MSNA to the leg is unchanged, suggesting that the transient increase in vascular conductance at the onset of exercise is unrelated to the changes in MSNA. Thus, withdrawal of muscle sympathetic vasoconstrictor activity if any cannot explain the initial vasodilatation during exercise.

Regarding sympathetic cholinergic nerve in animals, only a few studies have reported activity of presumable muscle sympathetic cholinergic fibers in the cat (Horeyseck et al., 1976; Lopes and Palmer, 1977; Dean and Coote, 1986). These studies revealed that postganglionic cholinergic fibers have quite different characteristics from sympathetic adrenergic vasoconstrictor fibers and they are spontaneously inactive and excited in association with atropine-sensitive vasodilatation during hypothalamic stimulation. In contrast, a microneurographical attempt to record sympathetic cholinergic vasodilator activity has been failed in humans (Wallin and Sundlöf, 1982; Saito et al., 1993; Callister et al., 1994). This may be attributed to no anatomical innervation of sympathetic cholinergic nerve for skeletal muscle (Bolme and Fuxe, 1970) or to a reason that it is difficult to measure sympathetic cholinergic activity with a conventional microelectrode. Thus, direct electrophysiological evidence for sympathetic cholinergic nerves is lacking in humans.

CAN NEURAL MECHANISMS CONTRIBUTE TO EXERCISE HYPEREMIA IN SKELETAL MUSCLE AT THE ONSET OF EXERCISE IN HUMANS?

Neural mechanisms may play an important role in regulating blood flow in non-contracting muscle. Accordingly, before considering neural mechanisms responsible for exercise hyperemia in contracting muscle, neural control of blood flow in non-contracting muscle during contralateral limb exercise should be taken into account. As candidates for this, it is considered that sympathetic cholinergic and/or β-adrenergic mechanisms may cause vasodilation in blood vessels of in non-contracting muscle, while a sympathetic α-adrenergic mechanism may cause vasoconstriction (Eklund and Kaijser, 1976; Sanders et al., 1989; Reed et al., 2000). These mechanisms affect in concert blood flow to non-contracting muscle, depending on the time course, modality, and intensity of exercise, to what extent muscle mass is engaged during exercise, of which limb blood flow is measured, and age (Duprez et al., 1989; Taylor et al., 1989, 1992). For example, the vascular response of non-contracting muscle changes along the time course of exercise (Taylor et al., 1989). However, as long as the initial transient phase of exercise is targeted, blood flow in a resting limb may increase during static or dynamic exercise of the contralateral limb (Eklund et al., 1974; Eklund and Kaijser, 1976; Rusch et al., 1981; Taylor et al., 1989; Jacobsen et al., 1994). Eklund and Kaijser (1976) suggested that vasodilatation of the resting forearm during contralateral handgrip is mediated by β-adrenergic mechanisms and, if the contraction is prolonged, α-adrenergic vasoconstriction takes place. Reed et al. (2000) suggested that β-adrenergic mechanisms due to circulating catecholamines and locally-released nitric oxide contribute to the vasodilatation, while sympathetic dilator nerves are not responsible for the limb vasodilatation seen after stellate block. In contrast, Sanders et al. (1989) reported that the initial vasodilatation in the resting limb was blocked by atropine but not by propranolol, suggesting that sympathetic cholinergic nerves may play a role in causing vasodilatation. The discrepancy among the previous findings may be partly attributed to technical limitation of blood flow measurement with venous occlusion plethysmography, which is a useful technique but does not provide the rapid dynamic changes in muscle tissue blood flow (Casey et al., 2008). Recently, Ishii et al. (2012) examined

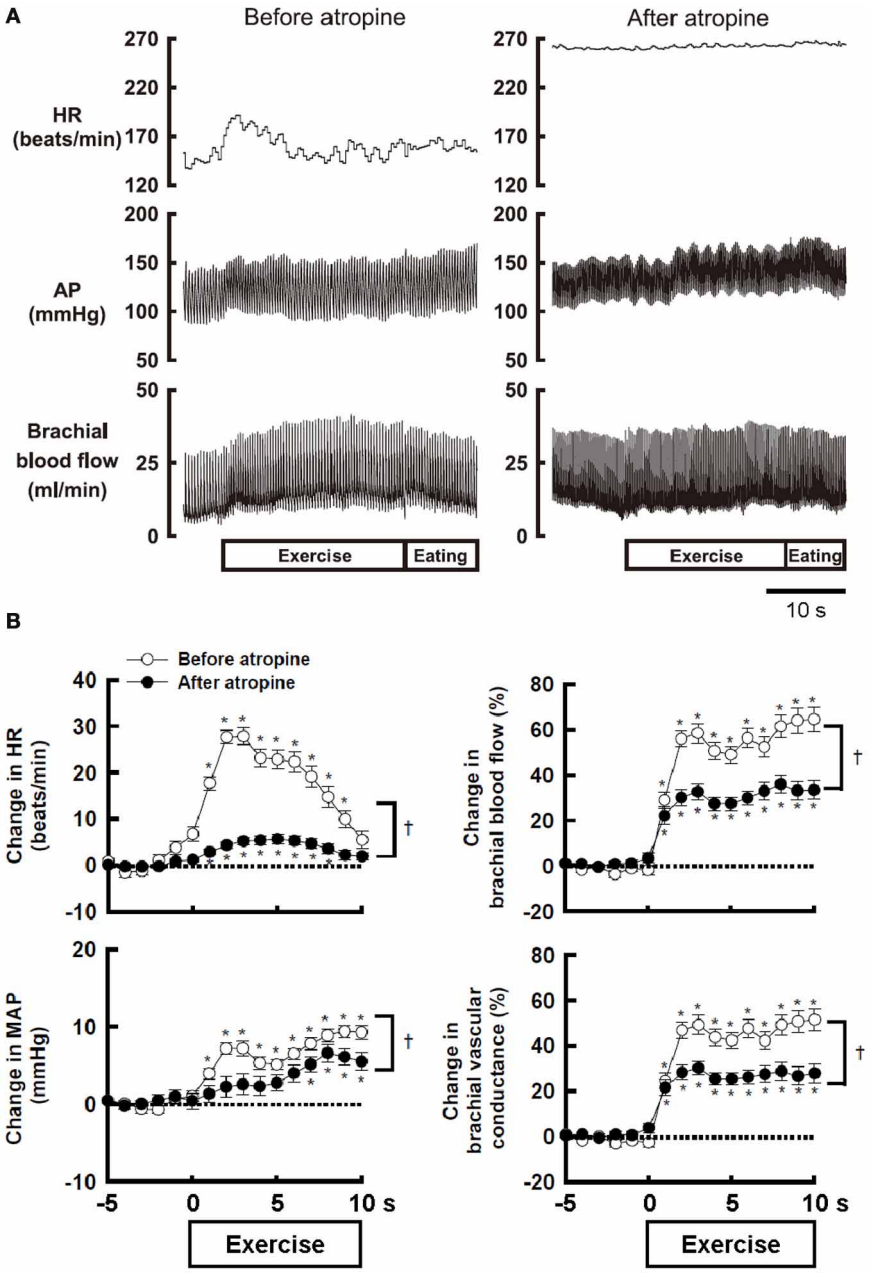

FIGURE 1 | (A) The effect of atropine on the responses in heart rate (HR), arterial blood pressure (AP), and brachial blood flow during voluntary static exercise in a conscious cat. In the presence of atropine, the increase in brachial blood flow was blunted, although the baseline blood flow was not altered. **(B)** The effect of atropine on the average responses in HR, mean arterial blood pressure (MAP), brachial blood flow, and brachial vascular conductance during static exercise. All cardiovascular responses at the onset of exercise were blunted by atropine. Especially, the peak increases in brachial blood flow and vascular conductance were decreased by atropine to 54–55% of the control responses. †Significant difference ($P < 0.05$) before and after atropine. *Significant difference ($P < 0.05$) at a given time from the preexercise level. Adopted from Komine et al. (2008) with permission.

the dynamic changes in concentration of oxygenated-hemoglobin (Oxy-Hb) of the non-contracting vastus lateralis (VL) muscle with NIRS as an index of muscle tissue blood flow. The Oxy-Hb in the non-contracting VL rapidly increased at the start of contralateral one-legged exercise (**Figure 2A**) but failed to increase at the start period of passive one-legged exercise. Since the Oxy-Hb also increased during mental imagery of the exercise, central command may contribute to increasing tissue blood flow in the non-contracting muscle at the start of contralateral exercise. On the other hand, Fisher and White (2003) reported that both voluntary and electrically-evoked isometric plantar flexion caused an initial increase in calf vascular conductance of the contralateral resting leg and Wray et al. (2005) and Trinity et al. (2010, 2011) reported that passive knee movement increased blood

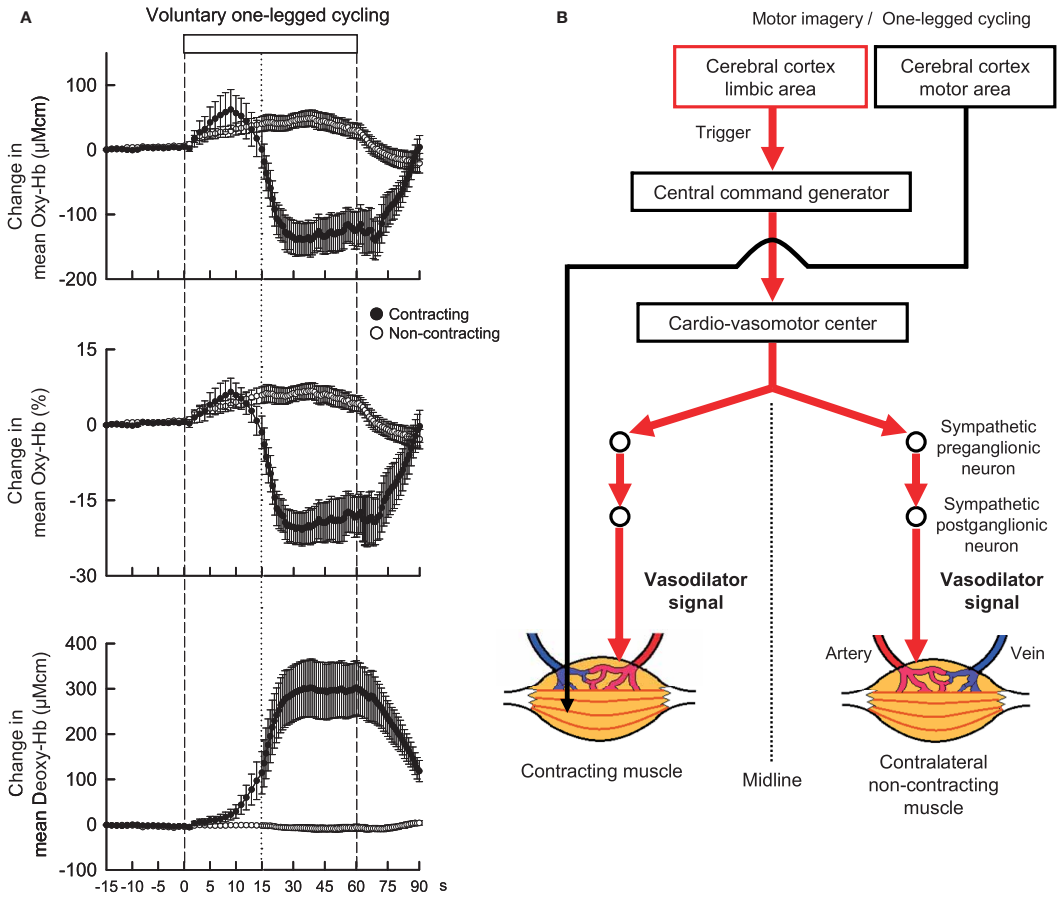

FIGURE 2 | (A) The time courses of the relative changes in oxygenated-hemoglobin (Oxy-Hb) and deoxygenated-hemoglobin (Deoxy-Hb) of the contracting (•) and non-contracting (○) vastus lateralis (VL) muscles during voluntary one-legged cycling exercise in humans. Vertical dashed lines indicate the start and end of one-legged cycling. A vertical dotted line is placed at 15 s from the exercise onset. At the start of voluntary one-legged exercise, Oxy-Hb in the contracting and non-contracting VL muscles increased with the same time course and magnitude. There was no significant difference ($P > 0.05$) in the Oxy-Hb response between the two muscles at the initial 15 s period of exercise. Subsequently, Oxy-Hb decreased and Deoxy-Hb increased in the contracting VL as long as exercise was continued. Adopted from Ishii et al. (2012) with permission. **(B)** A hypothesis that centrally-induced vasodilator signal is equally transmitted to bilateral VL muscles at the start of voluntary one-legged exercise in humans.

flow to the contralateral leg. These studies suggest that exercise pressor reflex, probably muscle mechanoreflex, may play a role in inducing the contralateral vasodilatation as well. In addition, the hyperemic response may result in part from a central hemodynamic mechanism, i.e., an increase in cardiac output resultant from mechanoreflexly evoked tachycardia (Trinity et al., 2010, 2011).

Control of increased blood flow to active muscles is much more complicated and is mainly the result of the interplay of neural vasoconstrictor activity, locally derived vasoactive substances (released from contracting muscles, vascular endothelium, or red blood cells), and mechanical factors (Rådegran and Saltin, 1998; Saltin et al., 1998; Clifford and Hellsten, 2004; Wray et al., 2005; Trinity et al., 2010, 2011, 2012). On the other hand, it has been thought that the sympathetic nervous system does not appear to be responsible for vasodilatation, although sympathetic α-adrenergic vasoconstrictor restrains blood flow to active muscles during exercise (Williams et al., 1985; Joyner et al., 1992; Clifford and Hellsten, 2004; Joyner and Wilkins, 2007). Instead, locally derived vasoactive substances and mechanical factors should determine the increase in blood flow to active muscle. Nevertheless, since the increase in Oxy-Hb of the contracting muscle had the almost same time course and magnitude as the increase in Oxy-Hb of the non-contracting muscle at the initial 15-s period of one-legged cycling (**Figure 2A**; Ishii et al., 2012), this finding leads to an idea that centrally-induced vasodilator signal may be transmitted to active muscle at least partly at the start of exercise (**Figure 2B**). However, a more comprehensive study including autonomic blockade will be necessary to test this hypothesis.

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α-Adrenergic blockade unmasks a greater compensatory vasodilation in hypoperfused contracting muscle

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We previously demonstrated that acute hypoperfusion in exercising human muscle causes an immediate increase in vascular resistance that is followed by a partial restoration (less than 100% recovery) of flow. In the current study we examined the contribution of α-adrenergic vasoconstriction in the initial changes in vascular resistance at the onset of hypoperfusion as well as in the recovery of flow over time. Nine healthy male subjects (29 ± 2) performed rhythmic forearm exercise (20% of maximum) during hypoperfusion evoked by intra-arterial balloon inflation. Each trial included; baseline, exercise prior to inflation, exercise with inflation, and exercise after deflation (3 min each). Forearm blood flow (FBF; ultrasound), local (brachial artery), and systemic arterial pressure (MAP; Finometer) were measured. The trial was repeated during phentolamine infusion (α-adrenergic receptor blockade). Forearm vascular conductance (FVC; ml min^{-1} 100 mmHg^{-1}) and resistance (mmHg ml min^{-1}) was calculated from BF (ml min^{-1}) and local MAP (mmHg). Recovery of FBF and FVC (steady state inflation plus exercise value − nadir)/[steady state exercise (control) value − nadir] with phentolamine was enhanced compared with the respective control (no drug) trial (FBF $= 97 \pm 5$% vs. 81 ± 6%, $P < 0.05$; FVC $= 126 \pm 9$% vs. 91 ± 5%, $P < 0.01$). However, the absolute (0.05 ± 0.01 vs. 0.06 ± 0.01 mmHg ml min^{-1}; $P = 0.17$) and relative (35 ± 5% vs. 31 ± 2%; $P = 0.41$) increase in vascular resistance at the onset of balloon inflation was not different between the α-adrenergic receptor inhibition and control (no drug) trials. Therefore, our data indicate that α-adrenergic mediated vasoconstriction restricts compensatory vasodilation during forearm exercise with hypoperfusion, but is not responsible for the initial increase in vascular resistance at the onset of hypoperfusion.

Keywords: alpha-adrenergic receptors, vascular resistance, blood flow, exercise, hypoperfusion

INTRODUCTION

In animals, when blood flow is restricted and/or perfusion pressure is reduced, the active muscle is capable of autoregulating its blood flow (Stainsby, 1962; Jones and Berne, 1964; Britton et al., 1985; Metting et al., 1986) via intrinsic control mechanisms (Jones and Berne, 1964; Britton et al., 1985). Additionally, a reflex pressor response contributes to the restoration of blood flow to under perfused exercising muscle in dogs (Wyss et al., 1983; Sheriff et al., 1987; Mittelstadt et al., 1994; O'leary and Sheriff, 1995; Laprad et al., 1999). Using a novel balloon catheter model in the brachial artery to reduce blood flow to contracting forearm muscles, we previously demonstrated that local vasodilator and/or myogenic mechanisms, rather than a pressor response, are responsible for a substantial portion of the restoration of flow to hypoperfused exercising human muscle (Casey and Joyner, 2009b). In subsequent studies we found that nitric oxide- and adenosine-, but not prostaglandin-, mediated vasodilation play a substantial role in the compensatory flow response to hypoperfusion (Casey and Joyner, 2009a, 2011a,c).

Collectively, our series of studies (Casey and Joyner, 2009a,b, 2011a,c) demonstrated that there is only partial compensation of flow (<100% recovery) in response to local reductions in oxygen availability via hypoperfusion. This is in contrast to other conditions in which oxygen availability is decreased (e.g., hypoxia and anemia). Under these conditions there is a compensatory vasodilation that mirrors the decrease in oxygen content (Gonzalez-Alonso et al., 2006; Wilkins et al., 2006; Casey et al., 2010). Interestingly, our hypoperfusion studies also revealed an initial increase in vascular resistance in the forearm at the onset of balloon inflation that progressively decreases over the inflation period. The initial rise in vascular resistance in these studies is in contrast to the classic view of autoregulation (a decrease in perfusion pressure is followed by a reduction in resistance to blood flow through the muscle; Stainsby, 1962; Granger et al., 1976; Britton et al., 1985; Ping and Johnson, 1992). However, the initial increase in vascular resistance is not unprecedented in that an immediate increase in vascular resistance has also been reported to occur in isolated perfused skeletal muscle of dogs (Jones and Berne, 1964). The reason for the initial increase in vascular resistance at the onset of balloon inflation and the incomplete restoration over time is not fully understood. Of interest to the current study, Daley et al. (2003) demonstrated that an increase in muscle sympathetic nerve activity (MSNA) can blunt the restoration of flow to hypoperfused exercising muscle induced by external positive pressure. An increased sympathetic restraint in the microcirculation distal to a stenosis of a proximal artery may have clinical implications as it suggests that conditions with elevated MSNA might be more susceptible to hypoperfusion and ischemia during exercise. Therefore, the aim of this study was to

examine the contribution of α-adrenergic vasoconstriction in the initial changes in vascular resistance at the onset of hypoperfusion as well as in the recovery of flow over time.

MATERIALS AND METHODS

SUBJECTS

A total of nine young healthy male subjects volunteered to participate in the study. Subjects gave written informed consent and were non-obese, non-smokers, and were not taking any medications. Studies were performed after an overnight fast and after the subjects refrained from exercise and caffeine for at least 24 h. All study protocols were approved by Institutional Review Board and in accordance with the *Declaration of Helsinki*. Other data collected in this set of subjects has been previously reported by our group (Casey and Joyner, 2009b).

HEART RATE AND SYSTEMIC BLOOD PRESSURE

Heart rate (HR) was measured by three-lead electrocardiography (ECG). Systemic blood pressure was assessed (beat-to-beat) with a finger plethysmograph (Finometer) on the non-exercising hand and verified with an automated cuff on the same arm. The systemic pressure was used as an index of pressure proximal (upstream) from the balloon. Cardiac output (CO) was estimated using the Modelflow technique which has been validated against other techniques and used in exercise studies (Wesseling et al., 1993; Ogoh et al., 2003).

ARTERIAL CATHETERIZATION AND BALLOON PLACEMENT

Brachial catheter placement and balloon insertion has been described in detail previously (Casey and Joyner, 2009b). Briefly, a 20-gage, 5 cm catheter was placed in the brachial artery in the experimental arm using ultrasound guidance under aseptic conditions after local anesthesia (2% lidocaine). A guide wire was then placed in the artery which was then cannulated with a four-French introducer (Cook Inc., Bloomington, IN, USA) that permitted insertion of a two-French Fogarty balloon catheter into the brachial artery. A port and stopcock system allowed the measurement of arterial pressure, administration of study drugs and drawing of arterial blood samples. The system was continuously flushed (3 ml h^{-1}) with heparinized saline. The configuration of the balloon upstream from the lumen of the introducer allowed measurement of the arterial pressure distal to the balloon that was perfusing the contracting forearm muscles.

FOREARM BLOOD FLOW

Brachial artery mean blood velocity (MBV) and brachial artery diameter were determined with a 12 MHz linear-array Doppler probe (Model M12L, Vivid 7, General Electric, Milwaukee, WI, USA). Brachial artery blood velocity was measured throughout each condition with a probe insonation angle previously calibrated to 60°. Brachial artery and balloon diameter measurements were obtained at end diastole and between contractions during steady-state conditions. Diameter measurement typically results in the loss of the pulse wave signal for 15–20 s. Therefore, brachial artery diameters were not obtained for the target balloon inflation (nadir) and the first 10 s immediately following balloon deflation (acute) portions of the trial. The diameters obtained immediately prior to each of these time points were used to calculate blood flow. It should be noted that we have previously demonstrated that the brachial artery diameter does not change in response to inflation and deflation of the balloon at rest and during exercise (Casey and Joyner, 2009b). Velocity and diameter measurements were made 2–3 cm proximal to the balloon. FBF was calculated as the product of MBV (cm s^{-1}) and brachial artery cross-sectional area (cm^2) and multiplied by 60 to present as milliliters per minute.

FOREARM EXERCISE

Rhythmic forearm exercise was performed with a hand grip device by the non-dominant arm at 20% of each subject's maximal voluntary contraction (MVC, mean 49 ± 2 kg, range 43–58 kg). The weight was lifted 4–5 cm over a pulley at a duty cycle of 1 s contraction/and 2 s relaxation (20 contractions per min) using a metronome to insure correct timing. The average weight used for forearm exercise was 9.9 ± 0.4 kg.

BRACHIAL ARTERY BALLOON INFLATION

To reduce FBF the brachial artery was partially occluded via inflation of the Fogarty balloon catheter with saline using a calibrated microsyringe for tight control of balloon volume. Balloon inflations were targeted to reduce MBV by 40–50%.

PHARMACOLOGICAL INFUSIONS

Phentolamine, a non-selective α-adrenergic antagonist, was administered to the exercising forearm via brachial artery catheter as a loading dose [10 μg (dl forearm volume)$^{-1}$ min^{-1} for 5 min] followed by a continuous maintenance dose (50 μg min^{-1}). To confirm α-adrenergic receptor blockade, tyramine was administered [12 μg (dl forearm volume)$^{-1}$ min^{-1} for 3 min] to evoke endogenous norepinephrine release and stimulate both α_1- and α_2-adrenergic receptors before and after phentolamine (Frewin and Whelan, 1968; Dinenno et al., 2002a,b).

EXPERIMENTAL PROTOCOL

A schematic of the general experimental design is illustrated in **Figure 1**. Each subject completed a control (no drug) and an α-adrenergic blockade (phentolamine) trial. Each trial consisted of 3 min of rest, exercise, exercise with balloon inflation, exercise following balloon deflation, and recovery (15 min total; 9 min of total exercise). The phentolamine trials were always performed last due to the drug's half-life. Each trial was separated by 20 min of rest to allow FBF to return to baseline.

DATA ANALYSIS AND STATISTICS

Data were collected at 200 Hz, stored on a computer and analyzed off-line with signal processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Local mean arterial pressure (BAP) was determined from the brachial artery pressure waveform measured distal to the balloon, systemic MAP (e.g., pressure proximal to the balloon) was derived from the Finometer pressure waveform, and HR was determined from the electrocardiogram. FBF, BAP, MAP, CO, and HR were determined by averaging values during the last 30 s of rest, exercise, exercise with inflation, exercise following deflation, and recovery. In addition, all values were analyzed and averaged during the first 10 s of target balloon inflation (nadir)

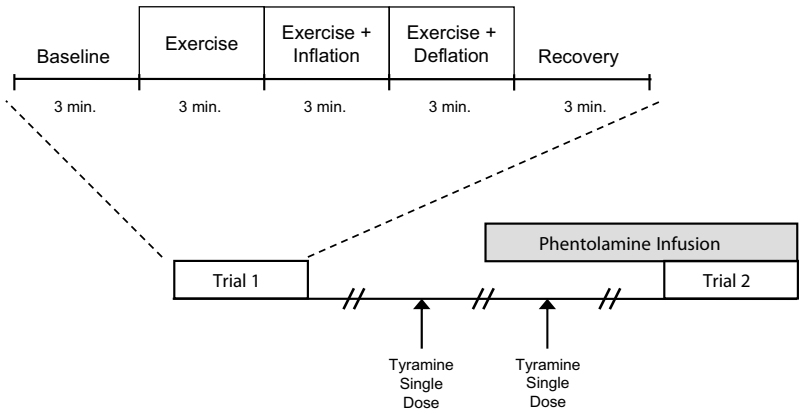

FIGURE 1 | Schematic diagram of experimental protocol. Subjects completed two trials. Each trial consisted of baseline, exercise (control), exercise during inflation, exercise following deflation, and recovery measurements (3 min each). Trials were performed during control (no drug) and α-adrenergic receptor blockade (phentolamine). Each trial was separated by at least 20 min of rest to allow FBF to return to baseline values. Tyramine was infused with and without phentolamine to confirm the effectiveness of α-adrenergic blockade.

and the first 10 s immediately following balloon deflation. FVC was calculated as (FBF/BAP) × 100 and expressed as ml min^{-1} (100 mmHg)$^{-1}$.

All values are expressed as means ± SE. Within a given protocol, the FBF, FVC, BAP, systemic MAP, HR, and CO during rest, exercise, the nadir after balloon inflation, exercise at the end of the balloon inflation, exercise following deflation, and recovery were analyzed by repeated measures analysis of variance (ANOVA). When significance was detected, Tukey's *post hoc* test was used to identify individual differences and adjust P values to account for multiple comparisons, to preserve an overall type I error rate of 0.05.

Percent recovery in FBF and FVC were calculated (steady state inflation plus exercise value − nadir)/[steady state exercise (control) value − nadir]. To investigate the contribution of α-adrenergic vasoconstriction on percentage recovery of blood flow and conductance, paired t-tests were performed between drug conditions (with and without phentolamine). To further explore the contribution of local vasodilation to any restoration of flow, we analyzed balloon resistance and forearm vascular resistance and considered them individually and in series (O'leary and Sheriff, 1995; Casey and Joyner, 2009a,b). Using systemic arterial pressure (SAP; Finometer), brachial artery pressure distal to the balloon (BAP; catheter) and brachial artery blood flow, we calculated the resistance of the balloon (SAP − BAP/flow) and vascular resistance (BAP/flow). The total resistance was calculated as the sum of these two resistors. Changes in vascular and balloon resistance were analyzed from the onset of balloon inflation (nadir) until the end of the inflation period and expressed as a percentage change. One way repeated measures ANOVA were used to compare the percentage change in resistance between drug conditions. Statistical significance was set *a priori* at $P < 0.05$. Statistical analyses were performed with SigmaStat 2.03 (SPSS, Inc.).

RESULTS

Eight of the nine subjects completed both of the exercise trials. One subject did not complete the entire protocol due to technical difficulties associated with the balloon during the phentolamine trial and was excluded from the analysis. Those subjects included in the group analysis were 29 ± 2 years of age, 180 ± 2 cm in height, and weighed 82 ± 4 kg (BMI: 25 ± 1 kg m^{-2}).

FOREARM BLOOD FLOW AND VASODILATION DURING EXERCISE WITH BALLOON INFLATION

Group mean data for FBF and FVC responses are presented in **Table 1**. As expected, exercise increased FBF and FVC in both exercise trials ($P < 0.01$). Balloon inflation (nadir) during the exercise trial with no drug acutely reduced FBF by 38% and FVC by 26% ($P < 0.001$). FBF and FVC at the end of inflation were partially restored to exercise (control) levels, which were substantially higher than their respective nadir values ($P < 0.001$). The percentage recovery of FBF and FVC during the exercise trials are presented in **Figures 2A,B**.

IMPACT OF α-ADRENERGIC RECEPTOR BLOCKADE ON BLOOD FLOW RECOVERY DURING HYPOPERFUSION

Infusion of phentolamine increased FBF and FVC throughout the entire exercise trial compared to the control (no drug) trial ($P < 0.01$–0.05; **Table 1**). Balloon inflation (nadir) during the exercise trial with phentolamine acutely reduced FBF by 45% and FVC by 28% ($P < 0.001$). The FBF and FVC at the end of inflation under α-adrenergic receptor blockade were greater than the values observed during the no drug trial ($P < 0.01$; **Table 1**). Consequently, the percentage recovery of FBF and FVC during the trial with phentolamine were substantially greater than the percentage recovery values observed during the no drug trial (**Figures 2A,B**). The time to reach steady state FBF during balloon inflation was similar between the no drug and phentolamine trials (49 ± 5 vs. 47 ± 4 s; $P = 0.48$).

Acute balloon inflation caused similar increases in absolute (0.06 ± 0.01 vs. 0.05 ± 0.01 mmHg ml min^{-1}; $P = 0.17$) and relative (31 ± 2% vs. 35 ± 5%; $P = 0.41$) vascular resistance between the no drug and phentolamine trials. Vascular resistance during balloon inflation (from nadir to

Table 1 | Forearm blood flow and vasodilation during exercise with balloon inflation.

	Baseline	Exercise (control)	Inflation (nadir)	Inflation (steady state)	Deflation (acute)	Deflation (steady state)
20% MVC (NO DRUG)						
FBF (ml min^{-1})	95 ± 25	468 ± 33*	298 ± 31*†	436 ± 37*‡	509 ± 43*‡§	501 ± 37*‡§
FVC [ml min^{-1} (100 mmHg)$^{-1}$]	107 ± 27	520 ± 42*	385 ± 28*†	510 ± 44*‡	558 ± 56*‡	558 ± 47*‡
20% MVC (PHENTOLAMINE)						
FBF (ml min^{-1})	328 ± 36^{a}	657 ± 70*^{a}	383 ± 49$^{\dagger b}$	642 ± 70*$^{\ddagger a}$	755 ± 88*$^{\ddagger\S a}$	685 ± 75*$^{\ddagger a}$
FVC [ml min^{-1} (100 mmHg)$^{-1}$]	379 ± 45^{a}	737 ± 85*^{a}	544 ± 62*$^{\dagger a}$	782 ± 91*$^{\ddagger a}$	839 ± 101*$^{\ddagger a}$	744 ± 88*$^{\ddagger a}$

*Values are means ± SE. FBF, forearm blood flow; FVC, forearm vascular conductance; MVC, maximal voluntary contraction. *$P < 0.01$ vs. baseline; $^{\dagger}P < 0.001$ vs. exercise (control); $^{\ddagger}P < 0.001$ vs. nadir; $^{\S}P < 0.05$ vs. inflation (steady state); $^{a}P < 0.01$ vs. no drug trial; $^{b}P < 0.05$ vs. no drug trial.*

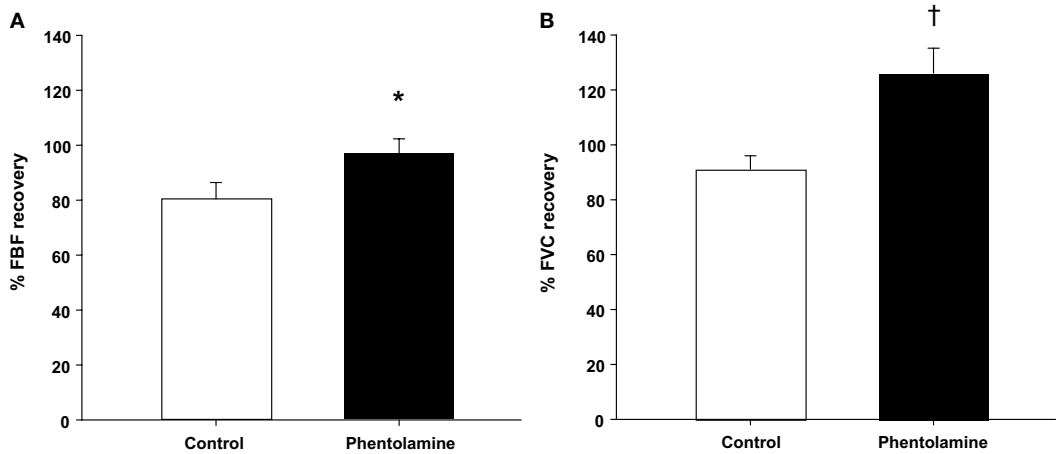

FIGURE 2 | Percentage recovery in forearm blood flow [FBF; (A)] and forearm vascular conductance [FVC; (B)] during balloon inflation. The percentage recovery in FBF and FVC was enhanced under local α-adrenergic blockade during exercise with hypoperfusion. *$P < 0.05$ vs. control (no drug); $^{\dagger}P < 0.01$ vs. control (no drug).

end of inflation) decreased during the no drug (0.27 ± 0.02 vs. 0.21 ± 0.02 mmHg ml min^{-1}; $P < 0.001$), and phentolamine (0.20 ± 0.02 vs. 0.14 ± 0.02 mmHg ml min^{-1}; $P < 0.01$) trials. Although the absolute reduction in vascular resistance was similar between trials ($P = 0.76$), the percentage reduction in vascular resistance was greater with phentolamine (−31 ± 2% vs. −24 ± 2%; $P < 0.01$ vs. no drug trial). Balloon resistance decreased (from nadir to end of inflation) in the no drug (0.05 ± 0.01 vs. 0.02 ± 0.01 mmHg ml min^{-1}; $P < 0.01$) and phentolamine (0.05 ± 0.01 vs. 0.03 ± 0.01 mmHg ml min^{-1}; $P < 0.01$) trails. However the absolute (−0.03 ± 0.01 vs. −0.03 ± 0.01; $P = 0.93$) and relative (−50 ± 8% vs. −49 ± 4%; $P = 0.91$) changes in balloon resistance were not different between drug conditions.

EFFECT OF α-ADRENERGIC RECEPTOR BLOCKADE ON VASCULAR RESPONSES TO EXOGENOUS TYRAMINE

Tyramine caused a substantial reduction in FVC (87 ± 26 vs. 124 ± 25 ml min^{-1} (100 mmHg)$^{-1}$; $P < 0.001$). α-Adrenergic receptor blockade with phentolamine prevented a significant vasoconstrictor response to tyramine (300 ± 33 vs. 318 ± 34 ml min^{-1} (100 mmHg)$^{-1}$; $P = 0.16$). The relative tyramine-induced reductions in FVC (with and without phentolamine) are presented in **Figure 3**.

VASOCONSTRICTOR RESPONSIVENESS AND BLOOD FLOW RECOVERY DURING HYPOPERFUSION

Figure 4 illustrates a strong relationship between the vasoconstrictor responsiveness to endogenous norepinephrine release via infusion of tyramine and the percentage FBF recovery during forearm exercise with hypoperfusion. Subjects with greater vasoconstrictor responsiveness to tyramine (i.e., greater reduction in FBF) demonstrated a lower percentage FBF recovery.

HEMODYNAMIC CHANGES

Systemic hemodynamic responses during exercise are presented in **Table 2**. Exercise resulted in an increase in MAP in both control (no drug) and phentolamine trials ($P < 0.05$). MAP remained elevated above baseline values throughout each trial ($P < 0.05$). Estimated CO did not change with exercise (control) compared to baseline in either trail, despite a slight increase in HR during the control (no drug) trial. Compared to baseline values CO was elevated during balloon inflation and deflation in the control (no drug trial), whereas it was only elevated during the deflation period in

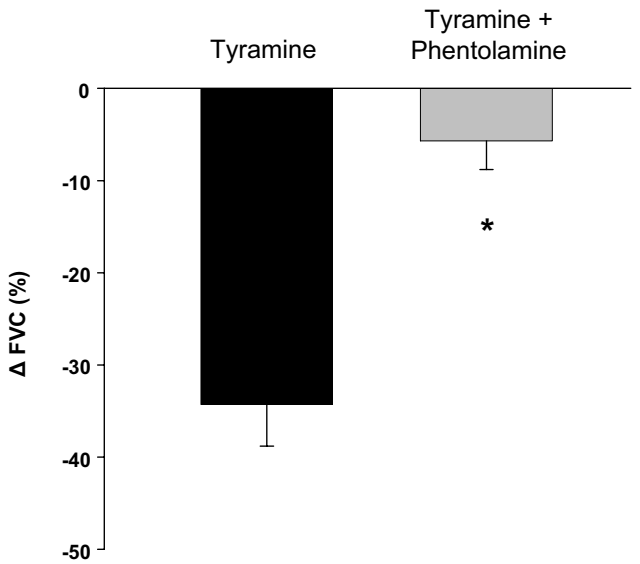

FIGURE 3 | Effectiveness of phentolamine in blocking α-adrenergic vasoconstriction. α-Adrenergic blockade via phentolamine substantially reduced the vasoconstrictor responsiveness (Δ forearm vascular conductance; FVC) to tyramine. * $P < 0.001$ vs. control (no drug).

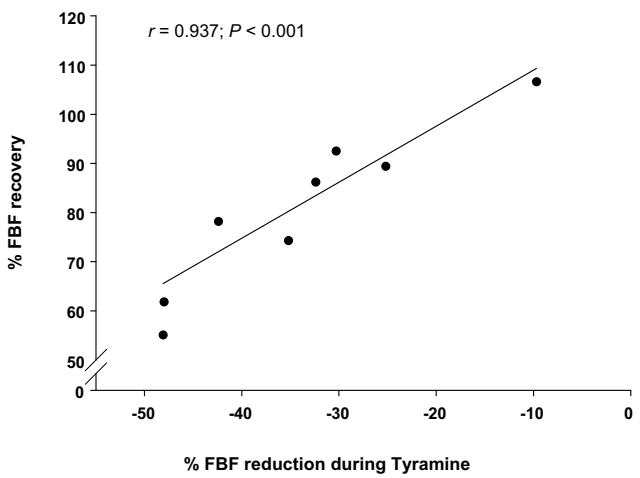

FIGURE 4 | Relationship between vasoconstrictor responsiveness and percentage recovery in forearm blood flow (FBF) during hypoperfusion. Vasoconstrictor responsiveness to endogenous norepinephrine release via tyramine (percentage reduction in forearm blood flow; FBF) is related to percentage recovery of FBF during balloon induced hypoperfusion under control (no drug) conditions.

the phentolamine trial ($P < 0.05$). Important to the present study, MAP, HR, and CO did not change with balloon inflation compared to exercise (control) values in either trail.

DISCUSSION

The novel findings of our study are (1) α-adrenergic blockade unmasks a greater compensatory vasodilation and flow recovery to hypoperfused contracting muscle, (2) the initial increase in vascular resistance at the onset of balloon inflation does not appear to be α-adrenergic mediated, and (3) vasoconstrictor responsiveness to endogenous norepinephrine is related to a lower flow recovery during exercise with hypoperfusion.

Stimulation of chemosensitive afferents in contracting muscle can elicit marked increases in MSNA to resting and active muscle (Mark et al., 1985; Savard et al., 1987; Victor et al., 1987, 1988). The increase in MSNA is further enhanced in hypoperfused and/or partially ischemic contracting human muscle (Michikami et al., 2000; Daley et al., 2003). Theoretically, the enhanced sympathetic drive to the muscle and subsequent vasoconstriction could limit blood flow to the active tissue. In the present study we examined whether α-adrenergic vasoconstriction restrained flow during exercise with hypoperfusion and possibly explained the incomplete compensatory flow response (<100% recovery) commonly observed in our studies (Casey and Joyner, 2009a,b, 2011a,c). Our data demonstrate that α-adrenergic blockade unmasks a substantial vasodilation and restores blood flow to pre-inflation levels when compared to control trials (97% vs. 80% recovery, respectively; **Figure 2**). These findings suggest that sympathetic outflow during concurrent exercise and hypoperfusion partially limits the compensatory vasodilation in the human forearm.

Interestingly, there is an absence of a significant pressor response (above exercise alone) in our model of hypoperfusion (**Table 2**), thus suggesting that balloon inflation and subsequent hypoperfusion likely did not result in greater sympathetic outflow when compared to free flow exercise conditions. Therefore, it may be possible that the α-adrenergic restraint observed with hypoperfusion may be due to an exaggerated α-adrenergic receptor responsiveness in the partially ischemic tissue. Along these lines, the relative vasoconstriction produced by α-adrenergic activity in the coronary circulation of dogs during exercise is greater during hypoperfusion (Laxson et al., 1989) than during normal arterial inflow (Bache et al., 1988). It is also possible that an attenuated functional sympatholysis might exist in the contracting muscle when blood flow and oxygen delivery are compromised. However, previous data form our group did not provide any evidence for reduced vasoconstrictor responsiveness (augmented functional sympatholysis) during hypoxic exercise (Wilkins et al., 2006).

Similar to our previous studies, vascular resistance increased at the onset of balloon inflation. The findings of the present study suggest that an enhanced α-adrenergic vasoconstriction at the onset of balloon inflation does not explain the initial increase in vascular resistance. In this context, α-adrenergic blockade did not prevent the initial rise in vascular resistance when expressed as absolute or relative changes. Although the reasons for the acute increase in vascular resistance at the onset of balloon inflation in our model of hypoperfusion are not completely clear it may be related to dampening of pulsatile flow by balloon inflation. Along these lines, pulsatile flow has been suggested to be a critical component for the release of endothelium-derived vasodilators (Rubanyi et al., 1986). Another possible explanation is that a sudden drop in perfusion pressure causes resistance vessels to recoil before autoregulatory vasodilation, a response that has been observed during mild exercise in dogs (Koch et al., 1991). Lastly, alterations in vascular bed compliance at the onset of balloon inflation may

Table 2 | Systemic hemodynamic responses ($n = 8$).

	Baseline	Exercise (control)	Inflation (nadir)	Inflation (steady state)	Deflation (acute)	Deflation (steady state)
20% (CONTROL)						
Mean arterial pressure (mmHg)	93 ± 3	98 ± 4*	99 ± 4*	100 ± 4*	100 ± 4*	99 ± 4*
Brachial artery pressure (mmHg)	90 ± 2	91 ± 3	79 ± 3*‡	86 ± 2†‡	91 ± 2†	89 ± 2†
Heart rate (beats min^{-1})	66 ± 2	68 ± 2*	68 ± 2*	68 ± 2*	68 ± 2*	68 ± 2*
Cardiac output (l min^{-1})	4.8 ± 0.3	5.2 ± 0.4	5.3 ± 0.4*	5.3 ± 0.3*	5.4 ± 0.4*	5.5 ± 0.4*
20% (PHENTOLAMINE)						
Mean arterial pressure (mmHg)	93 ± 4	97 ± 3*	98 ± 3*	100 ± 3*	99 ± 3*	101 ± 3*
Brachial artery pressure (mmHg)	88 ± 2	90 ± 2	70 ± 2*‡	78 ± 3*†‡	90 ± 2†	91 ± 2†
Heart rate (beats min^{-1})	67 ± 2	68 ± 2	68 ± 2	68 ± 2	68 ± 2	68 ± 2
Cardiac output (l min^{-1})	5.1 ± 0.3	5.4 ± 0.4	5.5 ± 0.4	5.5 ± 0.5	5.6 ± 0.5*	5.7 ± 0.5*

*Values are means ± SE; *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. nadir; ‡$P < 0.05$ vs. exercise (control).*

also contribute to the acute increase in vascular resistance observed in our model of hypoperfusion (Zamir et al., 2007).

In the current study we observed an extremely strong relationship between the vasoconstrictor responsiveness to endogenous norepinephrine release (via tyramine) and ability to restore blood flow to the contracting muscle during exercise with hypoperfusion (**Figure 4**). That is an individual with greater vasoconstrictor responses to endogenous norepinephrine release demonstrated a blunted recovery of flow during the period of exercise with balloon inflation. This relationship suggests that the α-adrenergic tone of the resistance vasculature in the forearm may play an important role in the ability to compensate and restore blood flow to underperfused skeletal muscle during exercise.

EXPERIMENTAL CONSIDERATIONS

The use of a non-specific α-adrenergic antagonist (phentolamine) in the current study did not allow us to discern the relative roles of α_1- and α_2-adrenergic receptors in the restriction of compensatory vasodilation during forearm exercise with hypoperfusion. In young healthy men α_2-adrenergic receptors have a greater contribution to basal forearm vascular tone compared to α_1-adrenergic receptors (Dinenno et al., 2002b). However, in the coronary circulation of dogs, α_1- but not α_2-adrenergic mediated vasoconstriction limits blood flow distal to a coronary artery stenosis (Laxson et al., 1989). Therefore, it is unclear whether α_1 and α_2-adrenergic mechanisms contribute differently to the vasoconstrictor restraint of flow during exercise with hypoperfusion in humans.

Administration of phentolamine altered baseline blood flow and the absolute blood flow responses to the exercise. It could be argued that these changes in flow before balloon inflation may explain the enhanced flow recovery following α-adrenergic blockade. However, the use of percent recovery (steady state inflation plus exercise value − nadir)/[steady state exercise (control) value − nadir] in the comparison between drug trials clearly accounts for the differences in flow before balloon inflation. Additionally, the reduction in flow caused by balloon inflation still evoked compensatory vasodilation suggesting that a metabolic error signal was present in spite of the higher flow.

Our series of experiments (Casey and Joyner, 2009a,b, 2011a,c) have consistently demonstrated that there is only a partial recovery of FBF (<100%) during exercise with acute hypoperfusion and this response is variable between subjects (see Figure 4 from Casey and Joyner, 2011b). However, the percentage FVC recovery tends to be greater than the percentage FBF recovery and in some cases reaches and/or exceeds 100% (Casey and Joyner, 2011a). In the current study the FVC recovery during the control trial was 91%, which was less than previously reported (Casey and Joyner, 2011a,c). The discrepancies in percentage FVC recovery between studies might be related to the extent of collateral channels in the forearm and magnitude of the restoration of distal perfusion pressure in the subjects of each study. Despite the somewhat lower percentage FVC recovery under control conditions in the current study there was a substantial improvement during phentolamine administration and supports the notion that α-adrenergic vasoconstriction limits compensatory vasodilation and flow recovery to hypoperfused contracting muscle.

CONCLUSION

This study demonstrates that α-adrenergic mediated vasoconstriction restricts compensatory vasodilation and flow during forearm exercise with hypoperfusion. However, the initial rise in vascular resistance at the onset of hypoperfusion via balloon inflation is not explained by an enhanced α-adrenergic vasoconstriction. Taken together our findings suggest that α-adrenergic mediated vasoconstriction plays an important role in the "less than perfect" compensatory flow response. Moreover, our data raise the concern that patients with enhanced α-adrenergic vasoconstrictor responsiveness might be more at risk to ischemia during exercise, especially in vascular regions distal to a stenosis.

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Persistence of functional sympatholysis post-exercise in human skeletal muscle

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Blunting of sympathetic vasoconstriction in exercising muscle is well-established. Whether it persists during the early post-exercise period is unknown. This study tested the hypothesis that it persists in human skeletal muscle during the first 10 min of recovery from exercise. Eight healthy young males (21.4 ± 0.8 yrs, SE) performed 7 min of forearm rhythmic isometric handgrip exercise at 15% below forearm critical force (fCF). In separate trials, a cold pressor test (CPT) of 2 min duration was used to evoke forearm sympathetic vasoconstriction in each of Rest (R), Steady State Exercise (Ex), 2–4 min Post-Exercise (PE_{early}), and 8–10 min Post-Exercise (PE_{late}). A 7 min control exercise trial with no CPT was also performed. Exercising forearm brachial artery blood flow, arterial blood pressure, cardiac output (CO), heart rate (HR), forearm deep venous catecholamine concentration, and arterialized venous catecholamine concentration were obtained immediately prior to and following the CPT in each trial. CPT resulted in a significant increase in forearm venous plasma norepinephrine concentration in all trials ($P = 0.007$), but no change in arterialized plasma norepinephrine ($P = 0.32$). CPT did not change forearm venous plasma epinephrine ($P = 0.596$) or arterialized plasma epinephrine concentration ($P = 0.15$). As assessed by the %reduction in forearm vascular conductance (FVC) the CPT evoked a robust vasoconstriction at rest that was severely blunted in exercise (R = −39.9 ± 4.6% vs. Ex = 5.5 ± 7.4%, $P < 0.001$). This blunting of vasoconstriction persisted at PE_{early} (−12.3 ± 10.1%, $P = 0.02$) and PE_{late} (−18.1 ± 8.2%, $P = 0.03$) post-exercise. In conclusion, functional sympatholysis remains evident in human skeletal muscle as much as 10 min after the end of a bout of forearm exercise. Persistence of functional sympatholysis may have important implications for blood pressure regulation in the face of a challenge to blood pressure following exercise.

Keywords: functional sympatholysis, skeletal muscle blood flow, sympathetic vasoconstriction, exercise, cold pressor test

INTRODUCTION

During skeletal muscle exercise, increases in exercising skeletal muscle vascular conductance paired with elevations in cardiac output (CO) act to increase muscle oxygen delivery in order to meet metabolic demand (Andersen and Saltin, 1985; Koskolou et al., 1997). Although exercising skeletal muscle vascular conductance increases, the magnitude of this increase appears to be restrained by increases in sympathetic vasoconstrictor influence, regardless of whether the exercise involves a small muscle mass (Joyner et al., 1992) or a large muscle mass (Rowell, 1997). Both baroreflex (Keller et al., 2004) and metaboreflex-mediated (Seals and Victor, 1991) increases in muscle sympathetic nerve activity (MSNA) can contribute to this sympathetic restraint. In the case of large muscle mass exercise at higher intensities, this restraint is believed to be essential for preventing skeletal muscle vasodilation from exceeding CO and threatening arterial blood pressure during exercise (Rowell, 1997; O'Leary et al., 1997).

While MSNA in exercising muscle maintains its effectiveness as part of blood pressure regulation (Keller et al., 2004), the responsiveness of resistance vessels to it is blunted. This blunting was first identified by Remensnyder et al. (1962) in an *in situ* animal model and termed functional sympatholysis. Over the past ∼15 years, it has been clearly established that functional sympatholysis occurs in exercising human skeletal muscle of both the arm and leg (Hansen et al., 2000; Tschakovsky et al., 2002; Dinenno et al., 2005; Watanabe et al., 2007; Fadel et al., 2012; Mortensen et al., 2012; Saltin and Mortensen, 2012). Its magnitude increases with exercise intensity, at least in the forearm (Tschakovsky et al., 2002; Watanabe et al., 2007).

Whether such blunting of sympathetic vasoconstriction persists following exercise, and if so for how long, has received less attention. DiCarlo's group has explored this in rabbit and rodent exercise studies and identified that between 10 and 26 min post-exercise blunted vasoconstriction in response to the α_1-receptor agonist phenylephrine remained in evidence (Howard and DiCarlo, 1992; Patil et al., 1993). In humans, Halliwill and colleagues have investigated the role of sympathetic vasomotor control in explaining the post-exercise hypotension and increased resting vasodilation in the exercised muscles that persists for a number of hours following cycling exercise (Halliwill et al., 1996, 2003). Their first study identified a decrease in MSNA at rest and in response to a hypotensive challenge (sodium nitroprusside

bolus infusion) ∼60 min post-exercise, as well as a reduced calf vasoconstrictor response per unit MSNA increase evoked by ischemic forearm exercise ∼90 min post-exercise (Halliwill et al., 1996). Their second study identified intact sympathetic vasoconstriction in the exercised legs in response to infusion of selective α_1 and α_2 agonists ∼60 min following cycling exercise (Halliwill et al., 2003).

To our knowledge, these are the only studies to date which have investigated impaired post-exercise sympathetic vasoconstriction in humans. The current interpretation of their findings is that in humans the impaired transduction of MSNA to vasoconstriction an hour or more after exercise is due to pre-junctional not post-junctional (α receptor response) mechanism(s). Since functional sympatholysis during exercise has repeatedly been demonstrated to be a post-junctional phenomenon in humans (Rosenmeier et al., 2003; Dinenno et al., 2005; Kirby et al., 2008) blunted transduction of MSNA to vasoconstriction so long after exercise cessation may represent a delayed alternate "sympatholytic" mechanism.

To date, blunting of sympathetic vasoconstriction during the very early post-exercise recovery period (0–10 min) has not been investigated in humans. This is likely because quantification of sympathetic vasoconstriction during the non-steady state of exercise recovery is problematic. Potential mechanisms of functional sympatholysis include NO and Prostaglandins (Thomas and Victor, 1998; Chavoshan et al., 2002; Dinenno and Joyner, 2004), ATP (Rosenmeier et al., 2004; Kirby et al., 2008) and most recently metabolic acidosis (Ives et al., 2012). Of these, only NO's role in elevated skeletal muscle blood flow immediately post-exercise (Shoemaker et al., 1997; Radegran and Saltin, 1999) has been explored, with findings suggesting that it is a contributor. However, elevated plasma [ATP] during (Gonzalez-Alonso et al., 2002) and acutely following (Yegutkin et al., 2007) exercise, as well as a sustained reduction in venous effluent pH for a number of minutes following intense exercise have also been observed (Wiltshire et al., 2010). This would suggest that persistence of functional sympatholysis during the immediate post-exercise period is plausible in humans. The inevitable decline in sympatholytic molecules might dictate the time course of diminishing functional sympatholytic potency.

Therefore, we developed and validated a curve-fitting approach to allow quantification of sympathetic vasoconstriction in the immediate post-exercise non-steady state. We used this to test the hypothesis that functional sympatholysis persists in human skeletal muscle during the first 10 min of recovery from exercise.

MATERIALS AND METHODS

GENERAL METHODS

Subjects

Originally, 10 healthy males [age = 21.4 ± 0.8 years (mean ± SE)] volunteered to participate in this study. Data from two had to be omitted from final analysis due to issues discussed later. Therefore, data analysis and interpretation was based on an $n = 8$. They were non-obese, non-smokers, normotensive, and had similar physical activity levels as they commonly engaged in moderate bouts of walking, intramural sports, and occasional full body workouts (**Table 2**). This experiment was approved by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board, in compliance with the terms of the Declaration of Helsinki. After receiving a complete verbal and written description of the experimental protocol and associated potential risks, each subject provided written, informed consent to participate in the outlined experiment.

Maximum voluntary contraction (MVC)

Maximum voluntary contraction (MVC) strength for each subject was determined as the peak force in kg of three 1–2 s maximal isometric handgrip contractions while laying supine with the arm extended to the side at heart level. Each contraction was followed by 1 min of recovery prior to the next contraction. Subjects were instructed to recruit only the forearm muscles during the voluntary contraction, so that the MVC performed would be representative of forearm handgrip strength in the position that was used for the experimental exercise protocol.

Forearm critical force (fCF) exercise test

Each subject underwent a single bout forearm critical force (fCF) exercise test in order to determine the work rate they would exercise at during the forearm exercise protocol. The fCF exercise task consisted of 10 min of rhythmic isometric maximal forearm handgrip contractions at a duty cycle of 1:2 s (1 s contraction, 2 s relaxation) with the handgrip dynamometer. The force output from the dynamometer and the contraction duty cycle were displayed to the subject throughout the exercise test. The subject was instructed to attempt to reach their MVC force output on each contraction over the 10 min. Each participant's fCF was calculated as the average power output of the last 30 s of the exercise test(Kellawan et al., 2010).

The fCF is the highest exercise intensity at which a metabolic steady state (plateau of VO_2 and high energy phosphates) still occurs (Poole et al., 1988; Jones et al., 2008). As such, it represents a "functional peak aerobic power," as it is the highest exercise intensity that can be maintained via aerobic ATP production without requiring supplemental ATP production via substrate level phosphorylation. It is commonly described as the boundary between heavy and severe exercise (Vanhatalo et al., 2007).

Forearm exercise at 15% below forearm critical force

Forearm exercise designed to evoke forearm functional sympatholysis consisted of 7 min of rhythmic isometric forearm handgrip contractions at a duty cycle of 1.2 s at 15% below the subject's fCF. This exercise intensity was chosen to maximize the potential for functional sympatholysis since it is exercise intensity-dependent (Tschakovsky et al., 2002), while still achieving a steady state exercise response (Moritani et al., 1981; Jones et al., 2008). As the active muscle mass is small, a central cardiovascular challenge was not expected while investigating the local vascular phenomenon of functional sympatholysis. The target force of 15% below fCF was displayed on the feedback monitor during the task. The exercise task was followed by 15 min of supine recovery.

Cold pressor test (CPT)

In this study, activation of sympathetic vasoconstriction with the subject laying supine was evoked using a CPT. The CPT required

the subject to place a foot into a prepared ice bath (0–3°C) for 2 min. Subjects were instructed to rest their foot at the bottom of the ice bath, remain relaxed, avoid tensing their muscles, and concentrate on their breathing to maintain a normal breathing pattern rather than holding their breath at any time during the CPT.

Arterialized blood sampling for catecholamines

Subjects had their right hand heated with a standard 50 W heating pad (Sunbeam, Jarden Consumer Solutions, Canada) for ∼10 min. Following hand heating, a 1-mL venous blood sample was taken from a dorsal hand vein or superficial vein of the wrist with an appropriately positioned butterfly needle (retrograde) or 20-gauge intravenous catheter (antegrade). The use of venous blood samples obtained from a heated vein has been previously demonstrated to accurately reflect the arterial concentration of lactate (Zavorsky et al., 2005). Thus, it was expected that other arterial plasma constituents, such as catecholamines, would also be representative.

The collected blood sample was immediately analyzed (StatProfile M Blood Gas Analyzer; Nova Biomedical, Mississauga, ON) to determine the hemoglobin oxygen saturation (%SO_2). Adequate arterialization was defined as greater than 90% SO_2. If appropriate arterialization was not achieved, further hand heating and subsequent blood sampling was required until a sample with SO_2 of greater than 90% was obtained. When this was confirmed, a blood sample of 3 mL was initially drawn and discarded, followed by two 6 mL blood samples collected into EDTA vacutainer tubes.

Forearm deep venous blood sampling for catecholamines

A 20-guage catheter was inserted retrograde into an antecubital vein of the participant's right arm (non-experimental arm). Echo ultrasound was utilized prior to catheterization in order to ensure the selected vein drained forearm muscle (cephalic or median cubital vein) and was not a superficial vein.

Venous blood samples were drawn immediately pre- and post-CPT in order to assess the forearm catecholamine response to the CPT. Samples were withdrawn from the non-exercising arm as the sympathetic neural activation due to the CPT would be the same for both arms, and the right arm would not have the confound of different blood flows between rest, exercise and recovery. A blood sample of 3 mL was initially drawn and discarded, followed by two 6 mL blood samples collected into EDTA vacutainer tubes at each sample period.

Central hemodynamic monitoring

Heart rate (HR) was measured beat-by-beat with an electrocardiogram 3-lead placement. Arterial blood pressure for the determination of mean arterial pressure (MAP) was measured continuously throughout the study via finger photoplethysmography (Finometer MIDI, Finapres Medical Systems, Amsterdam, Netherlands) on a finger of the non-exercising hand. Stroke volume (SV) was estimated from the blood pressure waveform (Beatscope Easy ModelFlow™ method, Finapres Medical Systems, Amsterdam, Netherlands) and the same software calculated CO as HR × SV.

Brachial artery diameter and mean blood velocity (MBV)

For the left arm which was used to perform the exercise (experimental arm), the brachial artery was imaged through the use of a linear 10 MHz echo ultrasound probe, operating in two-dimensional B-mode (Vingmed System FiVe; GE Medical Systems, London, ON). MBV was measured with a 4 MHz pulsed Doppler ultrasound probe (Model 500V TCD; Multigon Industries, Mt. Vernon, NY), which was secured to the skin at a location ∼5–10 cm distal to the Echo probe and proximal to the antecubital fossa. This position was marked on the skin to ensure the same probe position for experimental trials occurring on separate days. MBV was measured and recorded continuously on a personal computer data acquisition system. The probe insonation angle when parallel to the artery was 57°. The insonation angle was corrected based on imaging the vessel orientation relative to the skin surface at the Pulsed Doppler probe site. This correction adjusts the mean blood velocity voltage output that was calibrated at the probe insonation angle. For details see Pyke et al. (2008).

SUBJECT SCREENING AND FAMILIARIZATION

Subjects were instructed to abstain from consuming alcohol and/or caffeine 12 h prior and any food 4 h prior to the screening trial. Each subject participated in an fCF test, and practiced the constant work rate forearm exercise to become familiar with the exercise and be able to perform consistent contractions. In addition, each subject participated in the following screening activities.

7-day physical activity recall questionnaire (7-PAR)

Each subject completed a 7-PAR during the initial screening and familiarization visit to the laboratory. The 7-PAR was used to characterize their average level of physical activity prior to completing the study. The use of the 7-PAR has previously been demonstrated as a reliable and valid method of quantifying physical activity (Taylor et al., 1984; Dishman and Steinhardt, 1988).

Echo and Doppler ultrasound screening

Initial echo and pulsed Doppler ultrasound screening of the subject's experimental arm was necessary in order to ensure that a clear artery image and a blood velocity signal which did not contain signal from veins adjacent to the brachial artery could be obtained at rest, during and after forearm exercise. Subjects were instructed to lay supine on the laboratory bed while the investigator obtained a brachial artery image and brachial artery blood velocity signal as previously described. The subject then performed submaximal intensity rhythmic forearm handgrip contractions for a few minutes during which signal quality was assessed. No prospective subjects were turned away due to a poor brachial artery image or blood velocity signal.

CPT screening

Since the assessment of functional sympatholysis requires an experimental manipulation that elevates sympathetic vasoconstriction, it was necessary to select subjects in which a sympathetic vasoconstrictor response to the CPT could be confirmed. Therefore, all interested subjects completed CPT screening in order to confirm that the pressor response to the CPT was

primarily due to increased TPR (which indicates sympathetic vasoconstrictor activation) rather than elevations of CO. The outlined CPT screening protocol was repeated approximately 3–4 months following the initial screening visit in order to determine the repeatability of each participant's response to the CPT. Of the 27 interested in participating, 24 returned to the laboratory for a follow-up screening. Of these 24 individuals, 10 demonstrated a clear, consistent TPR response to the CPT. These TPR responders were the final subjects selected to participate in the experiment. Of these 10, two were not included in data analysis; one due to poor quality Doppler velocity signal and the other due to lack of vasoconstrictor response to CPT. Therefore, an $n = 8$ was used for data analysis.

EXPERIMENTAL PROTOCOLS

Subjects came to the laboratory on three separate days for three separate experimental protocols having abstained from consuming alcohol and/or caffeine for at least 12 h and food for at least 4 h. They were instructed to lie supine on the laboratory bed with both arms supported to the side at heart level and instrumented for data collection while resting quietly. The experimental protocols were designed to assess functional sympatholysis during exercise (Ex), at 4 min post-exercise (PE_{early}), and at 10 min post-exercise (PE_{late}). Each of these protocols consisted of three different experimental trials (**Figure 1**). The first trial was always a resting CPT trial (**A** in **Figure 1**) which consisted of 2 min resting baseline, followed by 2 min of the CPT, and finally another 2 min post-CPT. The second trial was either a control exercise trial (**B** in **Figure 1**), consisting of 2 min resting baseline, followed by 7 min forearm exercise at 15% below fCF, and finally 10 min of post-exercise recovery, or a CPT trial (one of either exercise CPT, PE_{early} CPT or PE_{late} CPT (**C–E** in **Figure 1**). The order in which a subject performed trial B vs. either of C, D, or E was consistent across protocols within subjects, but counter-balanced between subjects. For example, one subject may have performed trials on the three different days in the order A, B, C and therefore the other days would be A, B, E and A, B, D. Another may have performed them in order A, C, B and therefore the other days would be A, D, B and A, E, B. Each experimental trial was separated by at least 10 min of recovery in order to allow variables to return to baseline values. As there were two CPT's within each experimental protocol, the subject alternated the foot that was placed in the ice bath.

In order to confirm that the vasoconstrictor response to a second CPT was not blunted, we performed additional time control experiments ($n = 7$) in which a CPT was administered twice under resting conditions in one laboratory visit. The time between the two CPT tests was equivalent to the time interval that would have occurred between the rest CPT and half way between the time of the PE_{early} and PE_{late} CPT's.

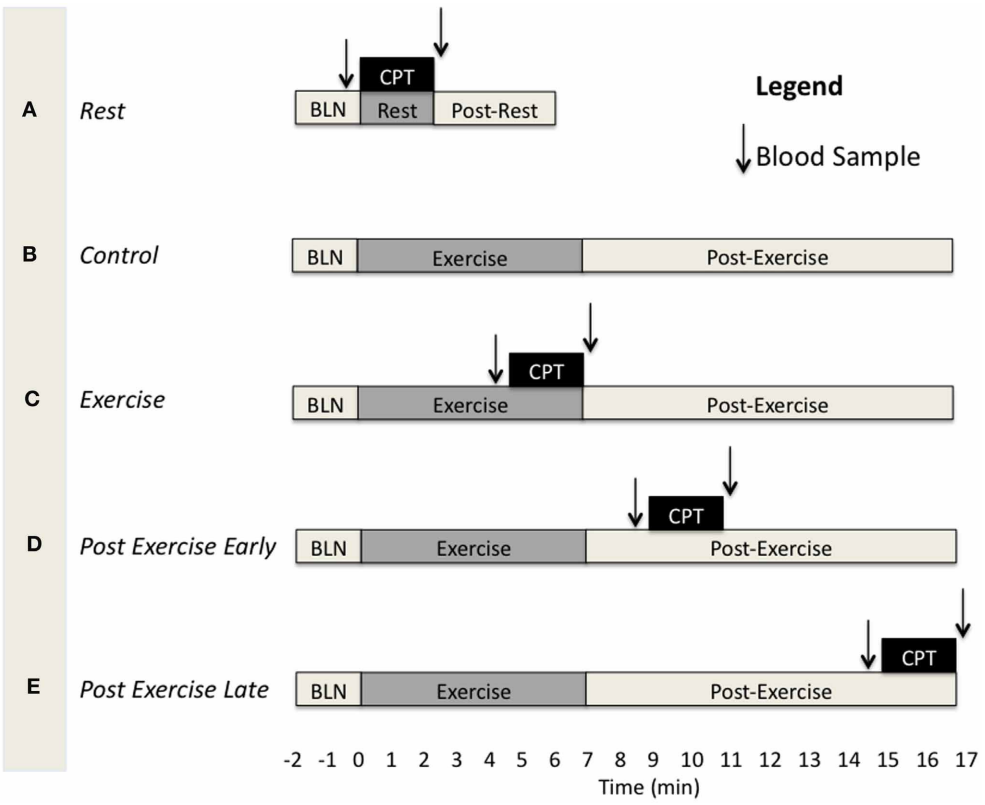

FIGURE 1 | Schematic depicting the different experimental trials and the timing of blood samples and the CPT for each. BLN, resting baseline. CPT, 2 min Cold pressor test. **(A)** CPT during rest. **(B)** Exercise trial without CPT. **(C)** CPT during exercise. **(D)** CPT at 2–4 min of the post-exercise period. **(E)** CPT at 8–10 min of the post-exercise period.

DATA ACQUISITION AND ANALYSIS

Central hemodynamic response to CPT

For all trials shown in **Figure 1**, central hemodynamic measurements were recorded continuously for the duration of each trial at 200 Hz using a Powerlab™ data acquisition system (ADInstruments, Sydney, Australia). To quantify the effect of the CPT on central hemodynamics and confirm that it was consistent across days, beat-by-beat measurements of mean arterial blood pressure (MAP), and calculated CO and TPR (MAP/CO) during the rest trial on the PE_{early} and PE_{late} days were averaged over 30 s at the following time points: baseline immediately prior to start of CPT, and 30, 60, 90, and 120 s of the CPT.

Forearm hemodynamic response to CPT

Brachial artery mean blood velocity (MBV). Brachial artery MBV was recorded continuously for the duration of each trial at 200 Hz using a Powerlab™ data acquisition system (ADInstruments, Sydney, Australia). Brachial artery MBV for the purpose of assessing the forearm hemodynamic response to CPT at rest and during exercise was the average MBV over the 30 s period immediately prior to the start of the CPT and the last 30 s of the CPT. For the post-exercise period the continuous measures of MBV were used in conjunction with brachial artery diameter (see below) to obtain a continuous FBF profile post-exercise.

Brachial artery diameter (BAD). Brachial artery images were recorded continuously in Digital Imaging and Communications in Medicine (DICOM) format and stored on a personal computer for offline analysis. BAD was analyzed via custom built automated edge-detection software (Woodman et al., 2001), which allows the user to identify a region of interest where the vessel walls are the clearest. The arterial walls are then tracked by detecting the greatest change in pixel brightness in the vertical axis. The edge-detection software collects one diameter measurement for every pixel column and uses the median diameter as the diameter for that frame.

With this approach, brachial artery diameter measurements for the purpose of assessing the forearm hemodynamic response to CPT at rest and during exercise were obtained at 5 s increments over the 30 s period immediately prior to the start of the CPT and the last 30 s of the CPT. These 5 s increment measurements were averaged to obtain a single diameter measurement that was used with the corresponding MBV to calculate FBF for rest and exercise pre- and end-CPT, respectively. For post-exercise, diameter measurements were obtained every 20 s. These were then curve fit (mono-exponential decay) with statistical software (SigmaPlot 11.0, Systat Software, Inc.) to provide a continuous diameter estimate and allow forearm blood flow calculations for the entire recovery period.

Forearm blood flow (FBF). FBF (ml/min) was calculated as follows: $FBF = [MBV \times 60 \times \pi \times (BAD/2)^2]$, where MBV is cm/s, 60 is s/min, BAD is cm.

In the case of the Rest and Exercise CPT experimental trials, where there is a steady state in the absence of CPT, FBF was derived from the 30 s averaged MBV and brachial artery diameter at the pre- and end-CPT time points. In this way, CPT-evoked sympathetic vasoconstriction is derived by comparing pre- and end-CPT time points. In the case of the Post-Exercise CPT experimental trials, the curve fit of the diameter measurements provided beat-by-beat diameter values corresponding to the beat-by-beat MBV, allowing beat-by-beat FBF calculation (see below for explanation of analysis required for quantifying CPT-evoked sympathetic vasoconstriction during the non-steady state post-exercise period).

Forearm vascular conductance (FVC). FVC (ml/min/100 mmHg) was calculated as follows: $FVC = FBF/MAP \times 100$, where MAP is mmHg and multiplying by 100 just allows FVC values to be in the same order of magnitude as FBF.

Sympatholysis during rest and exercise. In the case of the Rest and Exercise CPT experimental trials, where there is a steady state in the absence of CPT, FVC was derived from the 30 s averaged FBF and MAP at the pre- and end-CPT time points. The vasoconstrictor effect of the CPT in the experimental forearm in these trials was calculated as the %reduction in $FVC = [(FVC_{post\text{-}CPT} - FVC_{pre\text{-}CPT})/FVC_{pre\text{-}CPT}] \times 100$, where $FVC_{post\text{-}CPT}$ is the FVC averaged over the last 30 s of the CPT and $FVC_{pre\text{-}CPT}$ is the FVC averaged over the 30 s immediately prior to the CPT. A blunting of the %reduction in FVC in response to the CPT during exercise compared to rest was interpreted as representing functional sympatholysis (Tschakovsky et al., 2002).

Sympatholysis during post-exercise recovery. In the case of the Post-Exercise CPT experimental trials, beat-by-beat FBF obtained as described previously, was combined with beat-by-beat MAP to obtain beat-by-beat $FVC = FBF/MAP \times 100$ for the entire recovery period. There is no steady-state FBF, FVC, or MAP in the absence of CPT during the post-exercise recovery phase since these are changing over time as a function of normal recovery from exercise. Therefore, one cannot use a pre- vs. end-CPT comparison to quantify the CPT vasoconstrictor effect. To overcome this, we developed an analysis approach that allowed us to determine what the FVC would have been during the last 30 s of the CPT if the CPT had not occurred (i.e., $FVC_{predicted}$; what the normal recovery FVC at that time would have been). The difference between this and the actual FVC (FVC_{actual}) in the last 30 s of the CPT would therefore constitute the degree of vasoconstriction evoked by the CPT during recovery from exercise.

To obtain this $FVC_{predicted}$ the beat-by-beat post-exercise FVC data was averaged into 3 s bins and curve fit using a mono-exponential decay model (Sigmaplot 11.0, Systat Software Inc.), with the CPT data removed. This curve fit then provided an estimate of what FVC would have been during the time at which the last 30 s of the CPT was occurring. We validated this analysis approach as follows. We used the 3 s averaged post-exercise FVC from the control trials (no CPT administered), with a 2 min section of data corresponding to the timing of the CPT in the corresponding post-exercise CPT trials removed. We curve fit these data and compared the curve fit values averaged over the last 30 s of this 2 min period with the 30 s average of the actual FVC (FVC_{actual}) (see **Figure 2** and **Table 2**) to ensure that the curve fit approach provided valid estimates of FVC_{actual}.

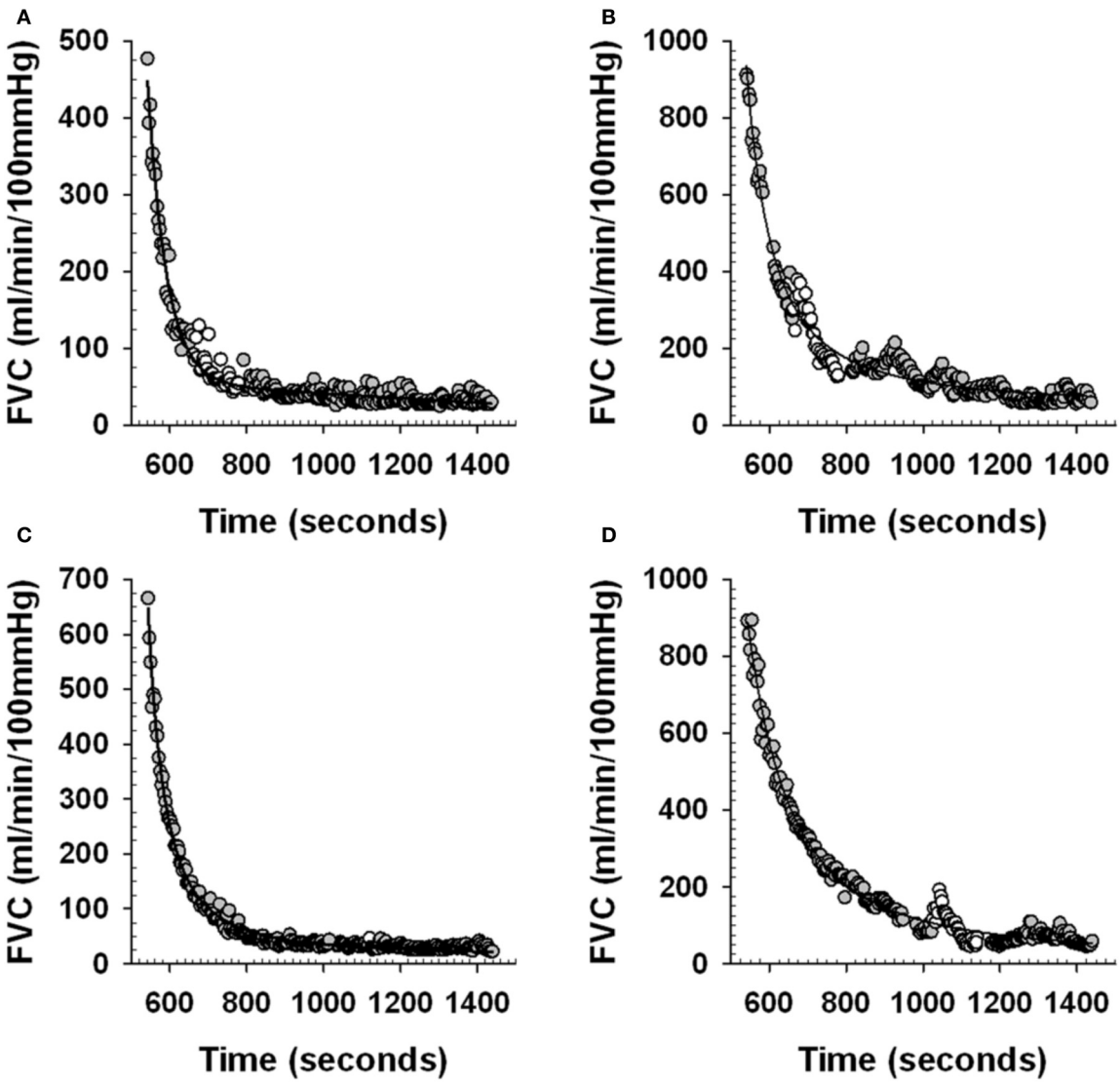

FIGURE 2 | Examples of curve fit estimation of post-exercise forearm vascular conductance (FVC). Panels **(A)** and **(C)** examples of individual subject beat-by-beat post-exercise FVC responses during Control trials (see **Figure 1**). White circles represent the beat-by-beat FVC data that is removed during the curve fitting to mimic the curve fitting procedure during an actual PE_{early} and PE_{late} CPT trial. Gray circles represent the beat-by-beat FVC data that is curve fit. Solid line is the curve fit. Panels **(B)** and **(D)** examples of individual subject beat-by-beat post-exercise FVC responses during a PE_{early} and PE_{late} trial respectively (see **Figure 1**). White circles represent the beat-by-beat FVC data that is removed during the curve fitting to allow curve fit estimation of what FVC would have been without the CPT. Gray circles represent the beat-by-beat FVC data that is curve fit. Solid line is the curve fit.

The degree of vasoconstriction due to the CPT in the PE_{early} and PE_{late} CPT trials was then calculated as %reduction in FVC = $[(FVC_{actual} - FVC_{predicted})/FVC_{predicted}] \times 100$, where FVC_{actual} was the average of the FVC during the last 30 s of the CPT and $FVC_{predicted}$ was the curve fit estimate of what FVC would have been at that time in the absence of the CPT.

CPT catecholamine response: blood sample acquisition

For each experimental trial in which a CPT was performed 6 mL venous blood samples were obtained from the non-exercising (right) arm for 10 s 1–2 min prior to the CPT and immediately after the end of the CPT. After being drawn, blood samples were immediately centrifuged at 1500 Gs for 10 min in a refrigerated centrifuge at 4°C. Plasma was separated from each sample and pipetted into separate 1.5 mL Eppendorf tubes. Plasma samples were immediately stored in a –80°C freezer for catecholamine analysis. Norepinephrine [NE] and Epinephrine [Epi] were analyzed at a later date with a 2-CAT ELISA—Fast Track (Rocky Mountain Diagnostics Inc., Colorado Springs, CO).

STATISTICAL ANALYSIS

The data collected from two subjects were omitted prior to statistical analysis. For one subject this was due to poor data quality (MBV and brachial artery diameter measurements) during data collection. For the other subject, analysis revealed that the CPT did not evoke any forearm vasoconstriction in the Rest CPT trial.

Since the CPT was used as a tool to evoke forearm sympathetic vasoconstriction in order to determine if this vasoconstriction was blunted under exercise and post-exercise conditions, failure to evoke vasoconstriction meant that we did not create the required experimental conditions, and justified removal of the subject. As a result, statistical analysis was performed on a total of 8 subjects. All values are reported as means ± SE and significance was set at $P < 0.05$ for all statistical analysis. For all multiple comparison analysis, significant interactions or main effects were analyzed with a Student-Newman-Keuls *post-hoc* test. All data analysis was completed with standard statistical software (SigmaPlot 12.3, Systat Software, Inc.).

$FVC_{predicted}$ vs. FVC_{actual}

To assess the agreement between the curve fit derived $FVC_{predicted}$ vs. the FVC_{actual} during the PE_{early} and PE_{late} time points in Control trials, we calculated a coefficient of variation between these for each subject within each of the PE_{early} and PE_{late} time points and an intraclass correlation coefficient. In addition, we plotted a linear regression of $FVC_{predicted}$ (x-axis) vs. FVC_{actual} (y-axis) in order to determine if the relationship between the two was on the line of identity, or if a correction factor for conversion of $FVC_{predicted}$ to FVC_{actual} was required.

CPT activation repeatability

To confirm that the CPT evoked the same systemic response across experimental days, a Two-Way repeated-measures ANOVA (Time within CPT test, Day of CPT test) was performed on MAP, CO, and TPR. Furthermore, to confirm the CPT evoked the same forearm vasoconstriction between two trials on the same day, as occurred on each experimental day, a Two-Way repeated-measures ANOVA (Time within CPT test, Trial of CPT test) was performed on FVC, and a One-Way repeated-measures ANOVA was performed on the %change in FVC between trials.

Catecholamine response

To test the hypothesis that the CPT elevated forearm venous plasma catecholamine concentration similarly in all experimental trials, a Two-Way repeated-measures ANOVA was used to determine the main effect of Trial (Rest, Exercise, PE_{early}, and PE_{late}) and measurement Time ("Pre-CPT" and "Post-CPT") and interaction effects of Trial × Time. A One-Way repeated-measures ANOVA was used to compare the TIME ("Pre-CPT" and "Post-CPT") measurements of NE and Epi from arterialized venous samples during the *Rest* trial to test the hypothesis that CPT did not alter arterial catecholamine concentration.

Sympathetic vasoconstriction response to CPT

To test the hypothesis that there was a main effect of trial on the reduction in FVC during the CPT, a One-Way repeated-measures ANOVA was utilized to compare the TRIAL (Rest, Exercise, PE_{early}, PE_{late}) measurements of %change in FVC from pre- to end-CPT.

RESULTS

Subject and 15% below foerearm critical force exercise characteristics are presented in **Table 1**.

Table 1 | Subject characteristics.

	***N* = 8**
Age (years)	21.4 ± 0.8
Height (cm)	179.8 ± 1.3
Weight (kg)	80.3 ± 3.3
Forearm circumference (cm)	25.9 ± 0.5
Forearm volume (mL)	1125.0 ± 49.3
Energy expenditure (METS)	261.0 ± 11.6
Maximal voluntary contraction (kg)	56.4 ± 4.4
Forearm critical force (kg)	29.4 ± 2.4
Exercise at 15% below forearm critical force (kg),	25.0 ± 2.1,
(%MVC)	44.9 ± 2.9

Values are means ± S.E. METS—metabolic equivalents. Exercise at 15% below forearm critical force is reported in kg and as a % of maximal voluntary contraction (MVC).

CURVE FITTING TECHNIQUE FOR ESTIMATING $FVC_{PREDICTED}$

Figures 2A,C provide an example from one subject of the curve fit approach applied to the control trial post-exercise period that was used to determine the ability for the curve fit to predict where FVC would have been during a normal recovery. The data during the time period where the 2 min CPT would have occurred in the PE_{early} and PE_{late} CPT trials were removed from the post-exercise period of the control trials for that day and then the curve fitting was done, as would be the case during the actual PE CPT trials.

Table 2 presents the agreement between the curve fit predicted FVC and the actual FVC during the last 30 s of this 2 min period. The validity of this curve fit approach for estimating what FVC would have been in the last 30 s of the 2 min CPT period is evidenced by the low coefficient of variation across subjects, the large ICC (0.99 for PE_{early} day, 0.95 for the PE_{late} day) and the lack of difference between the average of the FVC_{actual} and $FVC_{predicted}$.

Figure 3 shows that for the PE_{early} protocol control trials, a regression of the $FVC_{predicted}$ vs. FVC_{actual} values was virtually identical to the line of identity. For the PE_{late} control trials the $FVC_{predicted}$ vs. FVC_{actual} values, although very highly correlated, were represented by a regression that was different from the line of identity. However, on evaluation with a Cook's distance test to assess whether a data point has a disproportionately influential effect on the regression parameters (indicated by a Cook's *d*-value greater than 1), we identified one data point with a Cook's *d*-value of 1.6 (**Figure 3**). This data point was subsequently omitted from the regression, and the regression then was virtually identical to the line of identity, indicating that our approach provided valid $FVC_{predicted}$.

Figures 2B,D provide an example from one subject of the curve fit approach applied to the PE_{early} and PE_{late} CPT trials. The curve fit was performed on the data with the CPT section of data removed. The comparison between the curve fit and the actual FVC data in the last 30 s of the CPT was used to quantify the magnitude of vasoconstriction.

HEMODYNAMIC RESPONSE TO THE CPT

Table 3 presents the absolute values for HR, MAP, FBF, and FVC for each exercise and post-exercise trial and time point. **Figure 4**

Table 2 | FVC_{actual} and $FVC_{predicted}$ for control trials in PE_{early} and PE_{late} protocols.

Subject	Control trial PE_{early}			Control trial PE_{late}		
	FVC_{actual}	$FVC_{predicted}$	CV (%)	FVC_{actual}	$FVC_{predicted}$	CV (%)
	(ml/min/100 mmHg)			(ml/min/100 mmHg)		
B	430.6	422.2	1.4	91.7	114.9	15.9
I	131.7	96.5	21.8	102.1	100.8	0.9
K	288.5	249.6	10.2	46.2	49.4	4.6
L	64.7	60.3	5.0	72.0	69.7	2.3
T	103.1	114.2	7.2	67.2	52.7	17.1
V	30.1	36.2	13.1	23.5	22.3	3.6
W	213.6	217.3	1.2	56.0	61.7	6.9
AA	55.5	52.9	3.3	32.5	29.4	7.1
Mean	164.7 ± 48.8	156.2 ± 46.8	7.9 ±2.5	61.4 ± 9.7	62.6 ± 11.4	7.3 ±2.1

*Mean ± SE. Control trial PE_{early} and PE_{late}, refer to the time during the post-exercise period of the Control trial (**B** in **Figure 1**) at which the FVC_{actual} and the $FVC_{predicted}$ from the curve fitting procedure (see **Figures 2A,C**) were obtained. CV—within-subject coefficient of variation.*

presents the systemic hemodynamic response to the CPT at rest for both the PE_{early} and PE_{late} days. The CPT resulted in a progressive increase in MAP (main effect of time, $P < 0.001$) but there was no difference between days (main effect of day, $P = 0.727$). The same comparisons for TPR revealed a main effect of time ($P < 0.001$) such that TPR decreased during the first 30 s of CPT compared to pre-CPT and then progressively increased above this nadir over the course of the CPT, such that it was significantly greater than pre-CPT at 90 and 120 s (both $P = 0.02$). This response was consistent between days (main effect of day, $P = 0.801$). Lastly, the same comparisons for CO revealed main effect of time ($P < 0.001$). CO was elevated by 30 s of CPT followed by a steady decline over the rest of the CPT, although it was still significantly elevated compared to pre-CPT at the end of CPT ($P = 0.02$). Again, there was no main effect of day ($P = 0.595$).

CATECHOLAMINE RESPONSE TO THE CPT

The response of venous plasma [NE] and [Epi] concentration to the administered CPT is portrayed in **Figure 5**. The CPT resulted in a significant rise in forearm deep venous plasma [NE] for all trials (main effect Pre- to End-CPT $P = 0.001$) and there was no interaction effect ($P = 0.777$). One-Way repeated-measures ANOVA comparison of the increase in venous plasma [NE] between rest, exercise, PE_{early}, and PE_{late} indicated no difference ($P = 0.500$). There was no significant increase ($P = 0.32$) in the arterialized plasma [NE] response to the CPT assessed during the Rest trial. The arterialized plasma [Epi] concentration did not change with CPT ($P = 0.152$), nor did the forearm deep venous plasma [Epi] concentrations (Main effect of Pre- to End-CPT $P = 0.595$). However, there was an interaction between trial and Pre- to End-CPT such that the Pre-CPT during Exercise was greater than Rest ($P = 0.004$), PE_{early} (0.007), and PE_{late} ($P = 0.002$).

FOREARM VASOCONSTRICTOR RESPONSE TO THE CPT

Figure 6 presents the vasoconstrictor response to CPT as %change FVC. All subjects demonstrated a severely blunted to completely abolished vasoconstriction when CPT was administered during exercise. In contrast, 6 of 8 subjects in PE_{early} and 5 of 8 subjects in PE_{late} still demonstrated a blunted vasoconstriction. At the group level, a One-Way repeated-measures ANOVA and subsequent *post-hoc* analysis identified that all trials were significantly different from rest (Exercise: $P < 0.001$, PE_{early}: $P = 0.022$, PE_{late}: $P = 0.032$).

While *P*-values for the comparison of PE_{early} vs. Exercise and PE_{late} vs. Exercise were 0.073 and 0.052, respectively and therefore did not reach the a priori 0.05 threshold for accepting that the differences are not likely to be due to chance, the inadequate power of the study to detect these differences must be considered when interpreting the data.

TIME CONTROL EXPERIMENT FOREARM VASOCONSTRICTOR RESPONSE TO CPT

Figures 7A,B present the vasoconstrictor response to CPT at rest for two trials, used to confirm that the vasoconstrictor response to a second CPT trial on a given day was maintained. There was no main effect of trial (i.e., Pre- and End-CPT FVC was not different between trials, $P = 0.271$), but there was a main effect of time (i.e., End-CPT FVC was less than Pre-CPT, $P = 0.002$). Furthermore, the %reduction in FVC in CPT 1 vs. CPT 2 was not different ($P = 0.405$).

DISCUSSION

This study was the first to test the hypothesis that functional sympatholysis persists in human skeletal muscle in the immediate (10 min) post-exercise period. Functional sympatholysis is a blunting of sympathetic vasoconstriction due to mechanisms activated within skeletal muscle as a consequence of muscle contractions. The nature of these mechanisms means that they would not disappear immediately with cessation of muscle contractions as their presence is not contingent on the mechanical or muscle activation aspects of muscle contraction which disappear immediately at end exercise. We have therefore termed the blunted sympathetic vasoconstriction in the first 10 min of exercise as a

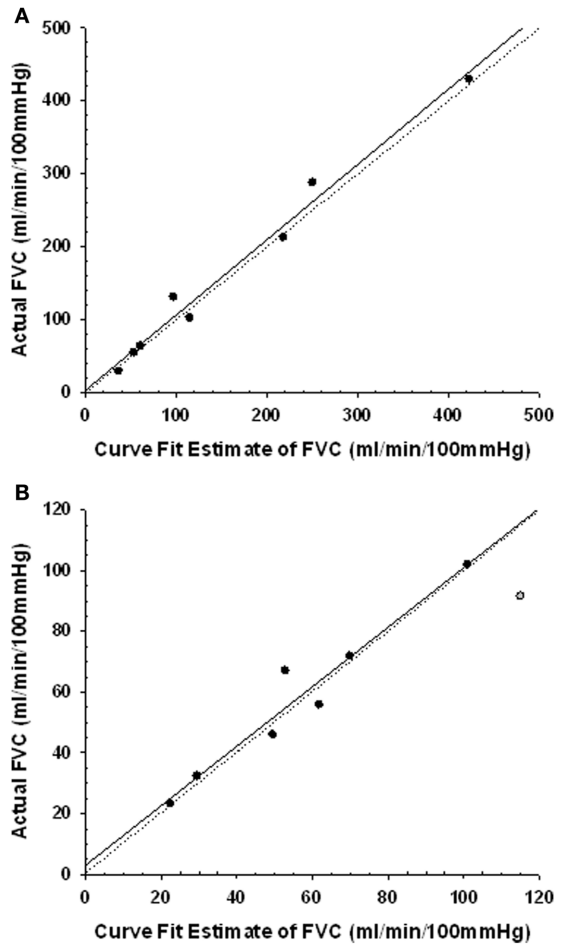

FIGURE 3 | Regression plots of the curve fit estimated forearm vascular conductance (FVC) and the actual FVC during the last 30 s of the 2 min period in the control post-exercise condition coincident with the timing of the CPT. Line of identity represented by dotted line. Regression represented by solid line. Panel **(A)** regression for the PE_{early} CPT timing. Panel **(B)** regression for the PE_{late} CPT timing. One subject data point (gray symbol) was excluded from the regression as it had a Cook's *d*-value of 1.6, indicating it had disproportionate impact on the regression parameters.

persistence of functional sympatholysis rather than referring to it as a separate sympatholytic phenomenon.

The primary findings of this study were as follows. First, assessment of the central hemodynamic response to CPT in the Rest trial on each of the two post-exercise testing days (PE_{early} and PE_{late}) confirmed the repeatability of the CPT effect between days. Namely, an initial CO-mediated pressor response transitioned into continued increases in MAP resulting from increasing TPR while CO was declining. When taken together with observations of a robust forearm vasoconstriction at rest, and elevated forearm venous plasma [NE] responses to the CPT in all trials, it is clear that the CPT evoked sympathetic forearm vasoconstriction. Second, in a separate time control experiment we confirmed that the forearm vasoconstrictor response to CPT does not diminish with a repeated CPT test. Third, robust functional sympatholysis was observed during exercise, confirming the activation of these mechanisms. Finally, and most importantly, the reduction in FVC in response to the CPT at 4 min (PE_{early}) and 10 min (PE_{late}) post-exercise was blunted compared to Rest. These observations support the concept of a lingering functional sympatholysis effect for as long as 10 min following human skeletal muscle exercise at 15% below critical force.

THE USE OF THE CPT TO EVOKE INCREASED SYMPATHETIC VASOCONSTRICTION

In this investigation, the CPT was a reliable methodological approach for elevating forearm sympathetic vasoconstriction and therefore allowing the assessment of functional sympatholysis. The administration of the CPT resulted in repeatable elevations of forearm deep venous plasma [NE] in all experimental conditions and a robust forearm vasoconstriction at rest. Previous evidence has also linked the use of the CPT with elevations in venous plasma [NE]. Winer and Carter (1977) observed significant increases in plasma [NE] during hand immersion in ice water that was linked to elevations in MAP and HR. Stratton et al. (1983) also observed significant elevations in [NE] after 6 min of CPT administration as compared to rest. These observations confirm the use of the CPT for elevating forearm vasoconstrictor activity.

FUNCTIONAL SYMPATHOLYSIS DURING 15% BELOW fCF EXERCISE

In the present study, the administration of the CPT during Rest resulted in a substantial reduction in FVC ($-39.9 \pm 6.1\%$), indicating that sympathetically-mediated vasoconstriction is robust at the skeletal muscle during resting periods. The mean percent change in FVC due to the CPT during Exercise indicated that the vasoconstrictor response appeared to be abolished at the skeletal muscle during exercise at 15% below fCF.

These primary findings are in accordance with a wealth of literature demonstrating the presence of functional sympatholysis in exercising muscle. This includes studies that specifically used CPT to elevate sympathetic vasoconstriction. For example, Wray et al. (2007) also utilized the CPT in their investigations into sympathetic vasoconstriction during exercise in cyclists and sedentary humans. Similar to the findings reported in this investigation, they demonstrated a significant reduction in FVC during CPT administration at rest ($-38 \pm 6\%$) which appeared abolished during 50% maximal voluntary contraction handgrip exercise ($13 \pm 11\%$). Differences between our study forearm exercise and theirs were the use of isometric rather than dynamic exercise contractions, a contraction force on average of ∼44% of MVC, and a 1 s contraction to 2 s relaxation duty cycle compared to their 1:1 duty cycle. The latter two characteristics indicate the exercise intensity in our study was therefore considerably less. In fact the moderate increase in arterial blood pressure from rest to exercise (see **Table 3**) and the lack of increase in forearm venous effluent NE (see **Figure 5**) is consistent with a sustainable exercise intensity we have used previously to investigate functional sympatholysis (Tschakovsky et al., 2002).

Despite blunting of exercising muscle vasoconstriction, the carotid baroreflex recruitment of exercising muscle sympathetic restraint for the purpose of blood pressure regulation can occur during exercise. This is evidenced by blood pressure elevation

Table 3 | Absolute hemodynamic responses at all time-points during Trial B–E (see Figure 1).

Time (min)	Exercise CPT protocol		PE_{early} CPT protocol		PE_{late} CPT protocol	
	Exercise control trial	Exercise CPT trial	Exercise control trial	PE_{early} CPT trial	Exercise control trial	PE_{late} CPT trial
HEART RATE (bpm)						
BLN	65.7 ± 4.0	69.9 ± 7.5	64.8 ± 2.2	61.9 ± 1.9	64.7 ± 5.5	66.7 ± 2.6
5	75.8 ± 4.9	85.5 ± 7.4	74.1 ± 3.2	75.6 ± 2.9	69.9 ± 3.3	73.9 ± 3.5
7	77.4 ± 5.8	75.8 ± 5.3	74.4 ± 3.4	79.3 ± 2.6	70.9 ± 3.2	74.1 ± 4.3
9	62.4 ± 5.8	64.9 ± 7.4	62.9 ± 1.7	61.6 ± 2.0	64.7 ± 7.1	63.8 ± 3.2
11	65.3 ± 7.8	66.4 ± 7.8	59.5 ± 2.6	62.8 ± 3.4	59.3 ± 4.0	62.2 ± 4.8
15	65.7 ± 6.5	63.7 ± 7.5	58.4 ± 3.4	59.3 ± 2.7	61.3 ± 4.8	59.7 ± 2.9
17	65.4 ± 5.4	64.8 ± 7.3	59.1 ± 2.1	59.2 ± 2.3	61.6 ± 5.6	63.4 ± 2.4
MEAN ARTERIAL PRESSURE (mmHg)						
BLN	92.9 ± 2.4	96.0 ± 2.9	95.0 ± 3.7	95.7 ± 3.0	91.4 ± 2.0	93.3 ± 2.9
5	102.2 ± 3.7	105.6 ± 4.1	108.2 ± 4.3	110.1 ± 3.2	105.1 ± 3.6	105.9 ± 4.0
7	104.6 ± 3.7	118.8 ± 4.2	113.9 ± 5.3	110.6 ± 3.1	106.1 ± 3.6	106.8 ± 3.9
9	91.3 ± 2.2	96.4 ± 3.1	96.5 ± 4.9	96.7 ± 2.2	93.6 ± 2.1	93.7 ± 3.1
11	91.5 ± 2.2	94.2 ± 2.9	95.1 ± 5.1	109.7 ± 3.1	90.5 ± 1.4	95.4 ± 2.3
15	92.2 ± 2.4	94.8 ± 3.1	95.9 ± 4.0	95.4 ± 2.8	91.3 ± 1.6	91.8 ± 2.7
17	92.2 ± 2.6	94.9 ± 3.3	94.6 ± 3.6	95.7 ± 2.2	90.7 ± 1.3	109.1 ± 3.3
FOREARM BLOOD FLOW (ml/min)						
BLN	30.7 ± 4.4	34.3 ± 5.3	39.2 ± 5.2	37.5 ± 6.1	32.3 ± 5.0	45.3 ± 12.0
5	376.6 ± 39.7	365.5 ± 28.9	471.0 ± 65.0	464.8 ± 59.3	451.8 ± 67.2	411.9 ± 62.4
7	427.4 ± 48.2	420.5 ± 27.3	520.7 ± 73.7	506.6 ± 71.0	471.9 ± 84.9	469.8 ± 64.6
9	169.4 ± 42.6	153.0 ± 37.9	213.0 ± 52.6	205.7 ± 33.9	222.2 ± 39.0	188.6 ± 37.7
11	112.2 ± 29.4	99.4 ± 24.9	146.2 ± 41.9	100.7 ± 17.5	126.0 ± 29.9	121.5 ± 23.0
15	78.4 ± 17.7	57.4 ± 10.5	87.0 ± 24.6	79.8 ± 11.2	78.0 ± 18.7	65.4 ± 8.6
17	67.0 ± 16.0	51.9 ± 9.8	75.6 ± 17.7	68.4 ± 12.1	59.9 ± 10.2	54.4 ± 6.5
FOREARM VASCULAR CONDUCTANCE (ml/min/100 mmHg)						
BLN	34.8 ± 5.3	38.2 ± 4.6	45.8 ± 7.7	41.0 ± 6.2	35.3 ± 6.1	50.1 ± 11.2
5	380.0 ± 36.1	363.8 ± 26.4	459.8 ± 76.1	443.8 ± 65.3	436.2 ± 65.4	405.1 ± 59.9
7	422.4 ± 45.0	370.1 ± 17.8	478.6 ± 77.5	478.9 ± 72.0	452.7 ± 78.7	456.5 ± 65.8
9	178.7 ± 43.8	154.6 ± 37.5	222.5 ± 55.1	217.6 ± 34.9	233.2 ± 38.1	202.6 ± 41.3
11	118.8 ± 30.0	99.7 ± 24.0	148.8 ± 43.4	94.1 ± 16.1	133.0 ± 29.9	121.4 ± 21.9
15	81.5 ± 17.2	55.0 ± 9.0	87.8 ± 24.8	82.9 ± 12.0	79.9 ± 19.1	69.4 ± 10.3
17	71.2 ± 16.0	50.3 ± 8.9	81.9 ± 17.6	73.7 ± 16.4	62.8 ± 9.8	50.8 ± 7.9

All data mean ± SE. BLN—Baseline rest prior to exercise onset. Exercise occurred from end BLN to end 7 min. Post-exercise period began at end of 7 min until 17 min. 5 min—pre-CPT in Exercise CPT Protocol; 7 min—post-CPT in Exercise CPT Protocol; 9 min—pre-CPT in PE_{early} CPT Protocol; 11 min—post-CPT in PE_{early} CPT Protocol; 15 min—pre-CPT in PE_{late} CPT Protocol; 17 min—post-CPT in PE_{late} CPT Protocol. Gray shaded values represent the pre- and post-CPT within the given CPT trial.

and exercising leg vasoconstriction in response to simulated carotid hypotension via neck compression (Keller et al., 2004). However, it must be noted that to date no studies have determined whether blood pressure regulation in response to an actual hypotensive disturbance during exercise is adequately preserved. In other words, whether functional sympatholysis can interfere with blood pressure regulation in the face of sudden hypotension. Furthermore, impairment in blood pressure regulation in response to a hypotensive disturbance might be even greater post-exercise, where the muscle pump is not supporting central venous filling pressure.

FUNCTIONAL SYMPATHOLYSIS POST-EXERCISE

We developed a new analysis approach to detect blunting of sympathetic vasoconstriction during a non-steady state period immediately following exercise. This technique has very good predictive validity (curve fit estimation of FVC during the post-exercise period closely matched actual FVC), and provides a new research tool for understanding sympathetic vasoconstriction in muscle post-exercise.

The most important finding of the current study was that sympathetic vasoconstriction remained significantly blunted in the human forearm at both 4 and 10 min post-exercise. The possibility also existed for us to be able to assess whether the potency of sympatholysis diminished over this time period. We would hypothesize that over time, sympatholytic molecules like ATP, NO, and H^+ would diminish, and if they were responsible for sympatholysis in the acute post-exercise period, the potency of sympatholysis would diminish as well. *Post-hoc* analysis identified a $P = 0.073$ for PE_{early} vs. Exercise, and $P = 0.052$ for PE_{late}

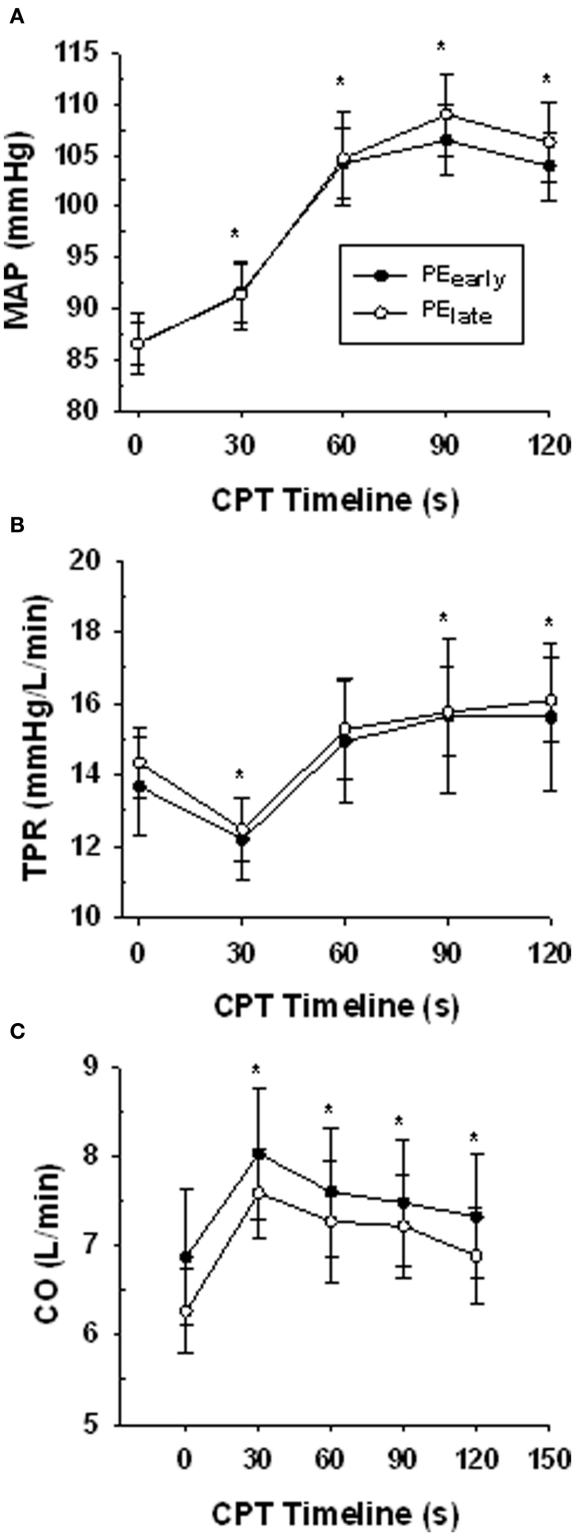

FIGURE 4 | Central hemodynamic responses over the course of the CPT during the resting trial on the PE_{early} and PE_{late} experimental days. Panel **(A)** mean arterial pressure (MAP). Panel **(B)** total peripheral resistance (TPR). Panel **(C)** cardiac output (CO). *indicates significantly different from time 0 (baseline) for both PE_{early} and PE_{late} $P < 0.05$.

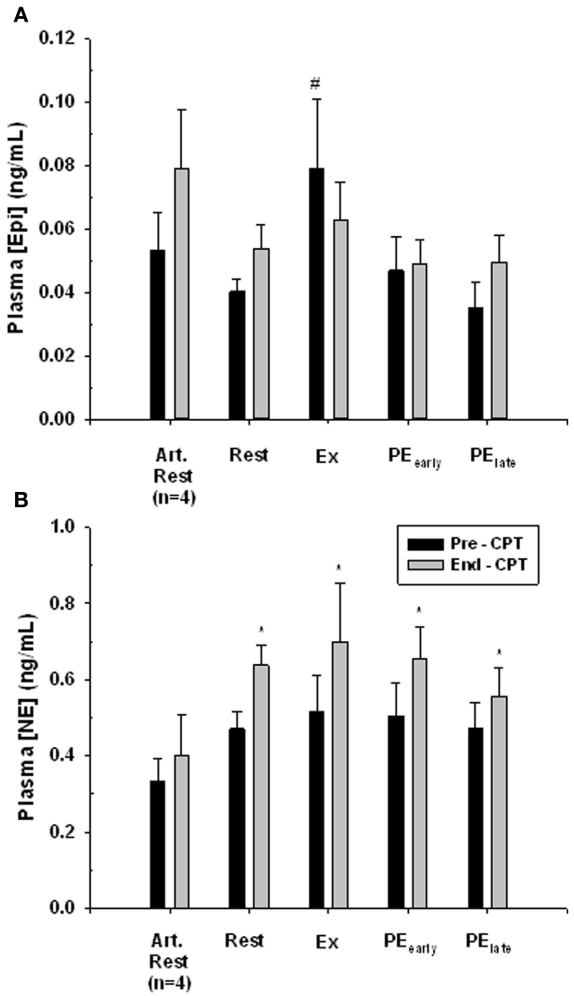

FIGURE 5 | Catecholamine response to CPT in arterialized venous blood at rest (Art. Rest), and deep forearm venous blood at rest, exercise (Ex) early post-exercise (PE_{early}) and late post-exercise (PE_{late}). Panel **(A)** plasma epinephrine concentration. # indicates significantly different from all other Pre-CPT forearm deep venous values, $P < 0.05$. Panel **(B)** plasma norepinephrine concentration. *indicates End-CPT significantly different from corresponding Pre-CPT, $P < 0.05$.

vs. Exercise. Strictly speaking, failure to achieve $P < 0.05$ that is set a priori requires we interpret the findings to suggest there was not a diminished functional sympatholysis as much as 10 min post-exercise. However, given the sample size and variability in the data, it is quite likely that this interpretation would represent an incorrect failure to reject the null hypothesis.

An additional important consideration in this regard is the possibility of individual differences in the time course of sympatholytic molecule, and therefore sympatholysis, disappearance. By 10 min, three of eight subjects appeared to have restored sympathetic vasoconstriction with CPT, while five maintained robust sympatholysis. Group level data interpretation may become problematic when there are apparent individual differences in the time course of functional sympatholysis decay. Follow-up investigations with a larger

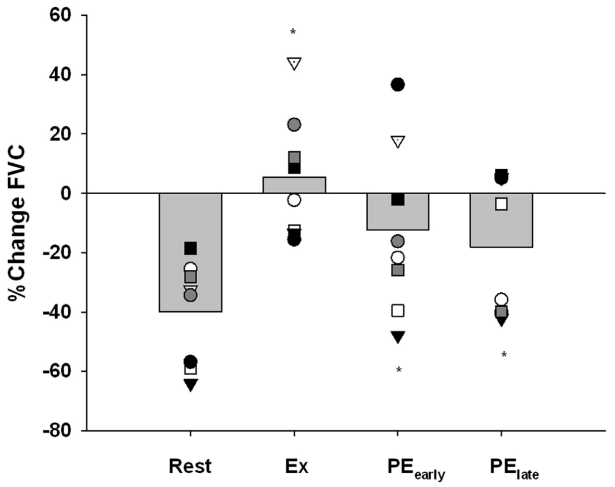

FIGURE 6 | Percent change in forearm vascular conductance (FVC) in response to CPT. The response of each subject is identified with a unique symbol. *indicates significantly different from Rest, $P < 0.05$.

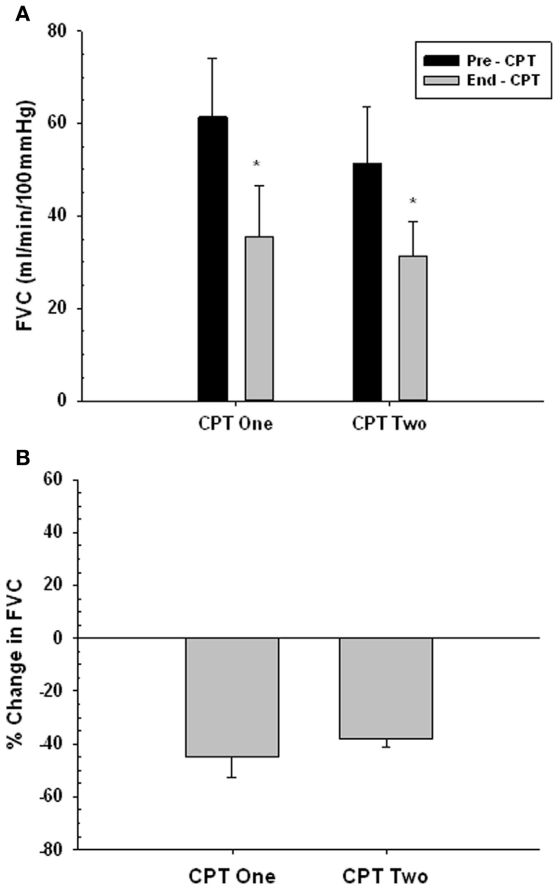

FIGURE 7 | Forearm vasoconstrictor response to CPT during repeated trials at rest. CPT 1—first CPT test. CPT 2—second CPT test. Panel **(A)** forearm vascular conductance (FVC) before (Pre-CPT) and at the end of (End-CPT) a CPT. Panel **(B)** The difference in Pre- vs. End-CPT FVC expressed as %change. *Main effect, significantly different from Pre-CPT, $P = 0.002$.

subject pool with repeated measures within each individual at multiple post-exercise assessment time points are necessary to better determine the exact timeline of functional sympatholysis decay post-exercise and its variation between individuals.

Our findings in humans are consistent with those of Howard and DiCarlo (1992) in rabbits, who observed a blunting of vasoconstriction in response to phenylephrine infusion 10 min post-exercise compared to rest. This finding was later replicated by the same group (Patil et al., 1993) who utilized phenylephrine infusion and bouts of treadmill exercise to examine alpha-adrenergic receptor-mediated vasoconstrictor response post-exercise in Sprague–Dawley rats. In the exercise trial, rats were infused with phenylephrine then ran on a treadmill at 12–18 m/min at a 10–18% incline until exhaustion (mean = 45 min). These investigators observed a blunted vasoconstrictor response to phenylephrine infusion at 6 min post-exercise. In addition, they examined the effect of nitric oxide synthase (NOS)blockade and found that both at rest and after exercise, this resulted in more robust vasoconstriction in response to phenylephrine.

Whether nitric oxide is responsible for post-exercise sympatholysis remains to be determined. Previous evidence suggests a role for NO in the regulation of vasomotor tone following skeletal muscle exercise. Radegran and Saltin (1999) investigated the impact of NOS inhibition (via L-NMMA infusion) on the femoral artery blood flow response following submaximal one-legged, dynamic knee-extensor exercise. Ten minutes post-exercise, these investigators observed a significant reduction of 66 ± 5% in femoral artery blood flow during exercise + L-NMMA infusion as compared to exercise alone. Shoemaker et al. (1997) found that brachial artery infusions of L-NMMA also reduced post-exercise forearm blood flow following handgrip exercise.

Advantages and potential limitations

A critical characteristic of the present study was the inclusion of time control experiments which confirm the reproducibility of the CPT vasoconstrictor response with repeated trials. Since the resting CPT trial was always performed before the exercise or post-exercise CPT trials, the possibility existed that blunted sympatho-excitation response to a second CPT could explain the reduced vasoconstriction compared to rest. The control experiments confirmed that this was not the case. We observed no difference in the vasoconstrictor effect of CPT across two trials that were separated by the amount of time that occurred between rest CPT and the experimental post-exercise CPT's.

Another strength of the current study is that participants were screened prior to recruitment in order to ensure that only participants that would demonstrate sympathetic vasoconstriction as the major component of their CPT response were included in the study. Remembering that the CPT was merely a tool to evoke sympatho-excitation and forearm vasoconstriction, it was important to ensure that this tool did indeed evoke

what it was intended to evoke. A lack of participant screening prior to investigation when utilizing the CPT could easily lead to confounded conclusions due to the non-homogeneous response to the CPT within a population (Ifuku et al., 2007; Moriyama and Ifuku, 2007, 2010). Furthermore, we accounted for normal changes in post-exercise blood flow by validation of a curve fit prediction technique that provided good estimates of what the normal FVC would have been during the CPT test.

A potential limitation of the study is the inability to conclusively confirm that underlying MSNA was the same across experimental conditions. In the current study, we wished to determine whether post-exercise functional sympatholysis persisted. For this reason, we chose a work rate that would maximize potential sympatholytic mechanisms during exercise so that detection of their persistence post-exercise would be enhanced. Additionally, in order to assess the effect of additional sympatho-excitation during exercise, it was necessary for the exercise intensity to result in a steady state. Given the problems with %MVC representing a given relative metabolic intensity, (Kent-Braun et al., 1993; Saugen et al., 1997) we identified an exercise intensity of 15% below fCF, which maximizes the exercise intensity while still safely ensuring the achievement of metabolic steady state in exercise. An important consideration when interpreting the responses to an imposed sympatho-excitation is the potential confound of underlying MSNA levels. In other words, is comparison of the effect of sympatho-excitation (1) at rest vs. during exercise at 15% below fCF confounded by different levels of underlying MSNA, and (2) at rest vs. post-exercise confounded by withdrawal of exercise-evoked MSNA during recovery?

In answer to the first issue: measurement of forearm venous effluent [NE] (see **Figure 5B**) confirmed that there was no increase in response to exercise. Yet there was a robust and virtually identical increase in forearm venous effluent [NE] in response to CPT at rest and during exercise. Finally, the exercise intensity in this present study resulted in similar FVC and MAP increases from rest to exercise as in our previously published work which established the exercise intensity dependence of functional sympatholysis in exercising human forearm (Tschakovsky et al., 2002). Taken together, these findings support our interpretation of robust functional sympatholysis during exercise at 15% below fCF in the present study.

In answer to the second issue: it might be argued that post-exercise withdrawal of any exercise-induced elevations in MSNA could serve as a "passive" dilatory signal, explaining the reduced vasoconstriction in response to CPT post-exercise. For this to be the case, the post-exercise withdrawal of MSNA would have to be occurring concomitant with the CPT stimulation of MSNA, resulting in a net blunting of the increase in MSNA. Arguing against this is the fact that already by the time the early post-exercise CPT is initiated, HR and MAP have returned to resting baseline levels, indicating a rapid return to baseline of systemic sympatho-excitation. Furthermore, there is no difference in the end-CPT forearm venous effluent [NE] response in the rest vs. the two post-exercise CPTs. For all *in vivo* studies of functional sympatholysis the basal MSNA upon which a sympatho-excitatory stimulus is superimposed is not known. Whether different basal MSNA affects the response to CPT MSNA elevation is also not known. Therefore, as in all such studies, the possibility that a difference in sympatho-excitation evoked vasoconstriction between conditions is due to underlying MSNA differences cannot be conclusively ruled out. In this regard the present study is not different from previous work in this area.

Finally, another potential limitation of the present study is the $n = 8$ due to the loss of two subjects, which decreases statistical power. In the case of the difference between resting and post-exercise %reduction in FVC, statistical significance was reached. However, it is possible that for the catecholamine analysis, specifically the epinephrine response, differences were not detected due to lack of statistical power. It is also possible that a failure to reject the null hypothesis in comparing functional sympatholysis potency between exercise and post-exercise conditions is due to a lack of statistical power.

Implications

This is the first study to confirm lingering functional sympatholysis in human forearm muscle post-exercise. The implications of these findings must be considered within the context of the subject selection criteria. These were young, healthy males who demonstrated a vasoconstrictor response to CPT sympatho-excitation. The data cannot be generalized to persons outside of these criteria. Since sympatholysis during exercise occurs in both upper (Tschakovsky et al., 2002; Wray et al., 2007) and lower limb (Wray et al., 2007) exercise in humans, it is likely that sympatholysis would linger post-lower limb exercise as well. The persistence of functional sympatholysis following exercise with large muscle mass holds significance for athletes and the modern day exercise-enthusiast. Following an endurance exercise task, an athlete may experience light-headedness. This is in part due to the relaxation of the muscle pump, which leads to a decline in venous return and an acute reduction in filling pressure of the heart. Combined with prolonged muscle vasodilation post-exercise, this could ultimately result in syncope. Prevention of syncope via baroreflex-mediated sympathetic vasoconstriction at a time when CO increase may be limited would clearly be difficult due to the prolonged blunting of vasoconstriction at the previously exercised skeletal muscle.

Factors which impair blood pressure regulation in the face of a hypotensive disturbance have serious implications for fighter pilots (Scott et al., 2007). Because they complete full body contractions in order to avoid gravity-induced loss of consciousness (GLOC), the persistence of functional sympatholysis in the large muscle mass used for this could be problematic during subsequent mild +Gz maneuvers following these muscular efforts where pilots may not engage in countermeasure contraction. In this context, further research into the duration, magnitude, and individual differences in post-exercise functional sympatholysis is warranted. This would include determining whether blood pressure regulation in response to +Gz is compromised post-exercise.

CONCLUSIONS

In conclusion, this study has demonstrated for the first time in humans the existence of a lingering functional sympatholysis in the first 10 min following a bout of forearm exercise. This may have important implications for blood pressure regulation in the face of a hypotensive challenge. In this context, differences between individuals in the duration that functional sympatholysis is maintained post-exercise may also need to be considered.

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Face cooling with mist water increases cerebral blood flow during exercise: effect of changes in facial skin blood flow

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Facial cooling (FC) increases cerebral blood flow (CBF) at rest and during exercise; however, the mechanism of this response remains unclear. The purpose of the present study was to test our hypothesis that FC causes facial vasoconstriction that diverts skin blood flow ($SkBF_{face}$) toward the middle cerebral artery (MCA V_{mean}) at rest and to a greater extent during exercise. Nine healthy young subjects (20 ± 2 years) underwent 3 min of FC by fanning and spraying the face with a mist of cold water (~4°C) at rest and during steady-state exercise [heart rate (HR) of 120 bpm]. We focused on the difference between the averaged data acquired from 1 min immediately before FC and last 1 min of FC. $SkBF_{face}$, MCA V_{mean}, and mean arterial blood pressure (MAP) were higher during exercise than at rest. As hypothesized, FC decreased $SkBF_{face}$ at rest ($-32 \pm 4\%$) and to a greater extent during exercise ($-64 \pm 10\%$, $P = 0.012$). Although MCA V_{mean} was increased by FC (Rest, $+1.4 \pm 0.5$ cm/s; Exercise, $+1.4 \pm 0.6$ cm/s), the amount of the FC-evoked changes in MCA V_{mean} at rest and during exercise differed among subjects. In addition, changes in MCA V_{mean} with FC did not correlate with concomitant changes in $SkBF_{face}$ ($r = 0.095$, $P = 0.709$). MAP was also increased by FC (Rest, $+6.2 \pm 1.4$ mmHg; Exercise, $+4.2 \pm 1.2$ mmHg). These findings suggest that the FC-induced increase in CBF during exercise could not be explained only by change in $SkBF_{face}$.

Keywords: middle cerebral artery blood velocity, external carotid artery, internal carotid artery, laser Doppler flowmetry, diving reflex

INTRODUCTION

Selective facial cooling (FC) increases middle cerebral artery mean blood velocity (MCA V_{mean}) at rest (Brown et al., 2003) and during exercise (Kjeld et al., 2009). Interestingly, FC is particularly effective in improving endurance exercise performance in hot environments, e.g., increasing exercise time to fatigue (Ansley et al., 2008) and lowering ratings of perceived exertion (RPE) (Mundel et al., 2007; Ansley et al., 2008). This exercise performance improvement may be related to a FC-induced increase in cerebral blood flow (CBF) which may support brain neuronal activity and metabolism and reduce central fatigue during exercise (Ide and Secher, 2000; Secher et al., 2008; Ogoh and Ainslie, 2009). Although FC elicits the diving reflex in which facial cold receptors stimulate cardiovascular changes, including bradycardia and peripheral vasoconstriction (Fagius and Sundlof, 1986; Foster and Sheel, 2005; Kinoshita et al., 2006) that may divert blood toward the brain, the underlying mechanism of a FC-induced increase in CBF remains unclear.

Anatomically, the internal carotid arteries (ICA) and external carotid arteries (ECA) branch from the common carotid artery and supply blood to brain and facial regions, respectively. Through graded dynamic exercise, ECA blood flow and facial skin blood flow ($SkBF_{face}$) gradually increases in an intensity-dependent manner (Sato et al., 2011). In addition, this increase in ECA blood flow is negatively correlated with the concomitant change in ICA blood flow (Sato et al., 2011), suggesting that an exercise-induced increase in ECA blood flow may restrict blood flow into the brain during exercise. Therefore, it is plausible that changes in ECA blood flow, i.e., $SkBF_{face}$, contribute to CBF regulation independently of the diving reflex.

Given this background, the aim of this study was to examine whether carotid artery blood flow distribution (e.g., toward the brain and face) contributes to a FC-induced increase in CBF during exercise. We hypothesized that FC elicits a greater attenuation of $SkBF_{face}$ during exercise, since a greater proportion of CBF may be directed to the ECA for thermoregulation, and thus we would measure a larger increase in MCA V_{mean} than at rest.

MATERIALS AND METHODS

SUBJECTS AND ETHICAL APPROVAL

Nine healthy men with a mean (±SD) age of 20 ± 2 years, height of 170 ± 5 cm, and body mass of 62 ± 9 kg voluntarily participated in this study. Each subject provided written informed consent after all potential risks and procedures were explained.

All experimental procedures and protocols conformed to the Declaration of Helsinki and were approved by the Institutional Review Boards of Faculty of Science Engineering, Toyo University (IRB # 2010-R-07). None of the subjects were taking any medication that may have influenced the hemodynamic responses to exercise. All subjects were familiarized with the equipment and procedures before any experimental sessions.

EXPERIMENTAL DESIGN

On the experimental day, all subjects arrived at the laboratory in the morning 2 h after a light breakfast. The subjects were requested to avoid caffeinated beverages, alcohol, and strenuous physical activity for at least 24 h before the experiment. After the subjects were instrumented, they were seated in a semi-recumbent position with a backrest and rested quietly to allow for cardiovascular stability. In the Rest trial, after 3 min data collection (i.e., baseline at rest) the face of subject was cooled for 3 min by spraying the face with a mist of cold water (∼4°C) and fanning with fan placed 50 cm from the subject's face and positioned facing upwards so as to cool only the head. In the Exercise trial conducted after securing sufficient time for the recovery from physiological changes induced by the Rest trial, subjects performed 4 min of an incremental warm up from the initial work load of 60 W at a pedal rate of 60 revolutions/min. The subjects were told to maintain the frequency of pedaling, and work load was increased 10–30 W every minute. With the target heart rate (HR: corresponding to 120 bpm) was achieved, workload was held constant (129 ± 12 W) for 5 min. After verification of steady-state—no increase in HR during the last 1 min of constant load cycling—each subject underwent 3 min of FC while continuing exercise. Subjects were instructed to breathe as normally as possible throughout the test. All the tests were carried out in a warm laboratory environment (27–28°C).

CARDIORESPIRATORY AND RPE MEASUREMENT

HR was monitored using a lead II electrocardiogram (ECG). Beat-to-beat mean arterial blood pressure (MAP) was measured using finger photoplethysmography (Finometer, Finapres Medical Systems BV, Netherlands). Stroke volume (SV) and cardiac output (Q) were estimated using the Modelflow method (Beat Scope 1.1, Finapres Medical Systems BV). This method provides a reliable estimate of changes in SV and Q in healthy humans from rest to submaximal exercise (Sugawara et al., 2003). Total peripheral resistance (TPR) was calculated as MAP divided by Q. Expired air was sampled breath-by-breath and end-tidal partial pressure of carbon dioxide (CO_2) ($P_{ET}CO_2$) was measured with a gas analyzer system (AE-310S, Minato medical science co., Osaka, Japan). In the Exercise trial, RPE were recorded using the 15-point Borg scale (Borg, 1982) immediately before FC and the end of FC.

CEREBRAL BLOOD FLOW MEASUREMENT

Mean blood flow velocity in the left middle cerebral artery (MCA V_{mean}) was obtained by transcranial Doppler ultrasonography (Multidop T, DWL, Sipplingen, Germany). A 2-MHz Doppler probe was placed over the left temporal window and fixed with an adjustable headband. Cerebrovascular resistance index (CVRi) was calculated as MAP divided by MCA V_{mean}. Relative change (%) in skin blood flow in the left forehead, approximately 3 cm from the midline and just above the supra-orbital ridge ($SkBF_{face}$), was measured by using laser Doppler flowmetry (ALF21, Advance, Japan).

TEMPERATURE MEASUREMENT

Skin temperature was measured using thermistors (LT-ST08-12, Gram Co., Japan) placed on the center of the forehead (T_{face}), the right side of the upper arm (T_{arm}), chest (T_{chest}), thigh (T_{thigh}), and leg (T_{leg}). Mean skin temperature (T_{sk}) was calculated from the body surface area distribution and thermal sensitivity of each skin area using the following formula, which was proposed by Ramanathan (1964):

$$T_{sk} = 0.3 \times (T_{arm} + T_{chest}) + 0.2 \times (T_{thigh} + T_{leg})$$

DATA ANALYSIS

All measurement data were sampled continuously at 1 kHz using analog-to-digital converter (PowerLab, AD Instruments, Milford, MA) interfaced with a computer. Baseline data were obtained by averaging across 1 min immediately before FC at rest (i.e., baseline at rest) and from 4th to 5th min of exercise at constant workload (i.e., baseline during exercise). Averaged data were also acquired from 2nd to 3rd min of facial cooling at rest (i.e., FC at rest) and during exercise (i.e., FC during exercise).

STATISTICS

Statistical analysis was performed using SigmaStat 3.5 software (Systat Software Inc., CA, USA). Following confirmation of distribution normality using Shapiro Wilk *W* tests, data were analyzed using a Two-Way (*Condition*: Baseline and FC) × (*State*: Rest and Exercise) repeated measures analysis of variance (ANOVA) with *post-hoc* Tukey's test. Student's paired *t*-tests were used to analyze influence of rest and exercise for the FC-induced changes of physiological parameters. Correlation coefficients were obtained to determine the relation between FC-induced changes in $SkBF_{face}$ and MCA V_{mean}. Data are expressed as mean ± S.E.M. with significance for all two-tailed tests set at $P < 0.05$.

RESULTS

Overall T_{sk} was significantly increased during exercise, but FC did not change T_{sk} at rest or during exercise. In contrast, T_{face} was decreased (Rest, −6.2 ± 0.3°C; Exercise, −6.9 ± 0.3°C), indicating that body parts other than face were not affected by FC (**Table 1**).

During exercise MCA V_{mean}, $SkBF_{face}$, MAP, HR, SV, and Q were increased, whereas TPR was decreased and CVRi was

Table 1 | Skin temperature responses.

	Rest		Exercise	
	Baseline	**FC**	**Baseline**	**FC**
T_{face}	34.6 ± 0.2	28.5 ± 0.4*	35.6 ± 0.2†	28.7 ± 0.3*
T_{sk}	33.9 ± 0.2	33.8 ± 0.2	34.8 ± 0.2†	34.7 ± 0.2†

*Values are means ± S.E.M.; T_{face}, forehead skin temperature; T_{sk}, mean skin temperature. * $P < 0.05$ different from baseline. † $P < 0.05$ different from Rest.*

Table 2 | Hemodynamic and ventilatory responses and RPE.

Parameter	Rest		Exercise	
	Baseline	FC	Baseline	FC
MCA V_{mean}, cm/s	53 ± 3	55 ± 3*	58 ± 4†	60 ± 4*†
$SkBF_{face}$, %	0 ± 0	−32 ± 4*	132 ± 43†	68 ± 36*†
MAP, mmHg	87 ± 3	93 ± 3*	101 ± 2†	105 ± 3*†
HR, bpm	64 ± 2	57 ± 2*	120 ± 3†	118 ± 3†
SV, %	0 ± 0	11 ± 3*	36 ± 6†	40 ± 7*†
Q, %	0 ± 0	−1 ± 2	157 ± 16†	161 ± 16†
TPR, mmHg/(L/min)	17.5 ± 1.1	18.8 ± 1.1*	7.9 ± 0.4†	8.0 ± 0.4†
CVRi, mmHg/(cm/s)	1.69 ± 0.10	1.77 ± 0.13*	1.78 ± 0.09	1.81 ± 0.10
$P_{ET}CO_2$, mmHg	44 ± 1	44 ± 1	49 ± 1†	49 ± 1†
RPE	–	–	11 ± 1	12 ± 1

*Values are means ± S.E.M.; MCA V_{mean}, middle cerebral artery mean blood velocity; $SkBF_{face}$, forehead skin blood flow; MAP, mean arterial pressure; HR, heart rate; SV, stroke volume; Q, cardiac output; TPR, total peripheral resistance; CVRi, cerebrovascular resistance index; $P_{ET}CO_2$, partial pressure of end tidal carbon dioxide; RPE, rating of perceived exertion. * $P < 0.05$ different from baseline. † $P < 0.05$ different from rest.*

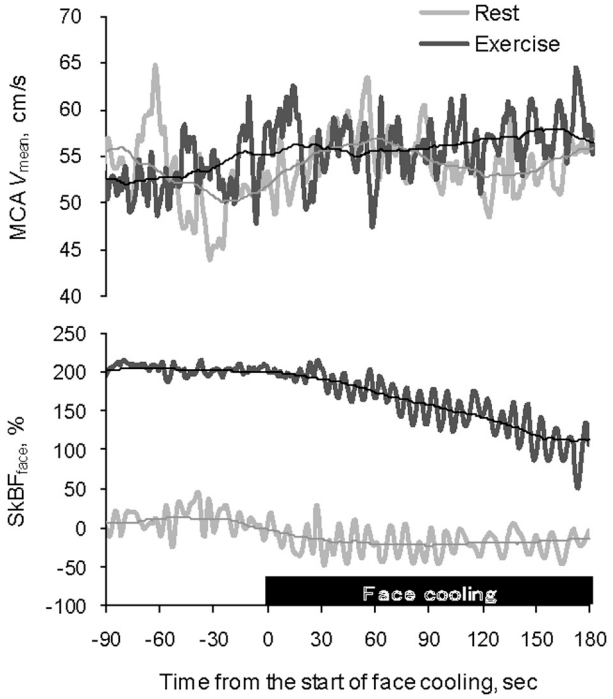

FIGURE 1 | Typical example of recordings of MCA V_{mean} and $SkBF_{face}$ at rest (gray) and during exercise (black) showing the effect of facial cooling. Each thin line represents a moving average for 1 min. MCA V_{mean}, middle cerebral artery mean blood velocity; $SkBF_{face}$, forehead skin blood flow.

unchanged (**Table 2**). In response to FC, $SkBF_{face}$ decreased and MCA V_{mean}, MAP, and SV increased at rest and during exercise. HR was decreased and TPR and CVRi were increased with FC at rest, but unchanged with FC during exercise. Q was unchanged with FC at rest and during exercise, but FC induced-change in Q tended to be higher during exercise ($P = 0.08$). $P_{ET}CO_2$ was significantly increased during exercise but was unchanged with FC at the both conditions. RPE was not affected by FC during exercise.

The average MCA V_{mean} was significantly increased by FC (Rest, +1.4 ± 0.5 cm/s; Exercise, +1.4 ± 0.6 cm/s: $P < 0.05$). However, the relation between the FC-evoked changes in MCA V_{mean} at rest and during exercise differed among subjects; two subjects showed similar changes, three subjects showed larger responses during exercise; and four subjects showed smaller responses during exercise. The FC-induced decrease in $SkBF_{face}$ was augmented during exercise compared with the resting condition ($SkBF_{face}$: Rest, −32 ± 4%; Exercise, −64 ± 10%, $P = 0.012$; **Figures 1** and **2**). MAP at rest and during exercise were increased by FC (Rest, +6.2 ± 1.4 mmHg; Exercise, +4.2 ± 1.2 mmHg, $P < 0.05$). However, the changes in MAP in response to FC were not different between at rest or during exercise ($P = 0.13$). The FC-induced decrease in HR and increase in SV were significantly attenuated during exercise compared with the resting condition (HR: Rest, −6.8 ± 1.5 bpm; Exercise, −1.8 ± 1.1 bpm, $P < 0.05$; SV: Rest, +11 ± 3%; Exercise, +4 ± 1%, $P = 0.015$, **Figure 3**). The FC-induced changes in Q were larger during exercise compared with the resting condition (Rest, −1.0 ± 1.5%; Exercise, +4.1 ± 2.6%, $P = 0.08$). The percent changes in MCA V_{mean} induced by FC were not significantly correlated with the percent changes in $SkBF_{face}$ ($r = 0.095$, $P = 0.709$).

DISCUSSION

The findings of the present study provide new information regarding CBF regulation to FC with water mist (FC) during dynamic exercise. As expected, FC was associated with an increase in MCA V_{mean} at rest and during exercise. FC elicited a greater restriction of $SkBF_{face}$ during exercise compared with the resting condition. However, this larger decrease in $SkBF_{face}$ did not result in a larger increase in MCA V_{mean} during exercise. In addition, changes in MCA V_{mean} with the FC did not correlate with the concomitant changes in $SkBF_{face}$. These findings suggest that other physiological mechanisms apart from decreased $SkBF_{face}$, such as cardiovascular components of the diving reflex, may affect CBF during FC at least during mild dynamic exercise.

Anatomically, the ECA supplies blood to the face, anterior neck, and cranial surface. The ECA blood flow gradually increases with exercise intensity (Sato et al., 2011). In addition, this increase in ECA blood flow significantly correlates with the concomitant change in forehead cutaneous vascular conductance (Sato et al., 2011), suggesting that ECA blood flow is likely increased selectively for thermoregulatory purposes. Similarly, in the present study, $SkBF_{face}$ was increased even during mild exercise as skin temperature rose. Moreover, selective FC decreased $SkBF_{face}$ both at rest and during exercise (**Table 2**, **Figures 1** and **2**). This $SkBF_{face}$ response may be due to a similar mechanism seen in the peripheral circulation, e.g., the local cooling-induced vasoconstriction (Johnson and Kellogg, 2010). As expected, the decrease in $SkBF_{face}$ was significantly greater during exercise than that at rest ($P = 0.012$). These different $SkBF_{face}$ responses may reflect an exercise-induced increase in facial capillary volume (+132%, $P = 0.009$) for thermoregulation rather than different autonomic

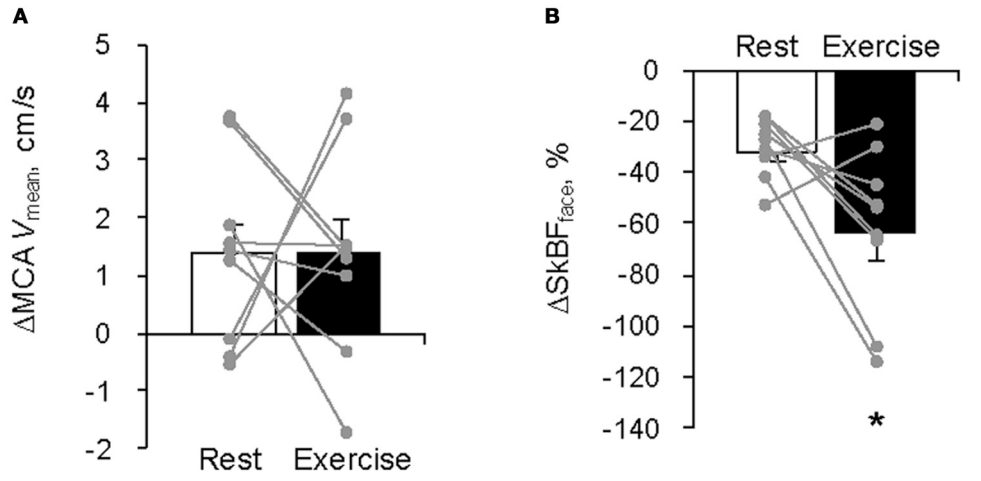

FIGURE 2 | Facial cooling-induced hemodynamic changes (Δ) at rest and during exercise: (A) middle cerebral artery mean blood velocity (MCA V_{mean}) and (B) forehead skin blood flow ($SkBF_{face}$). Each gray plot represents an individual response. Values are means ± S.E.M. $^{*}P < 0.05$ different from rest.

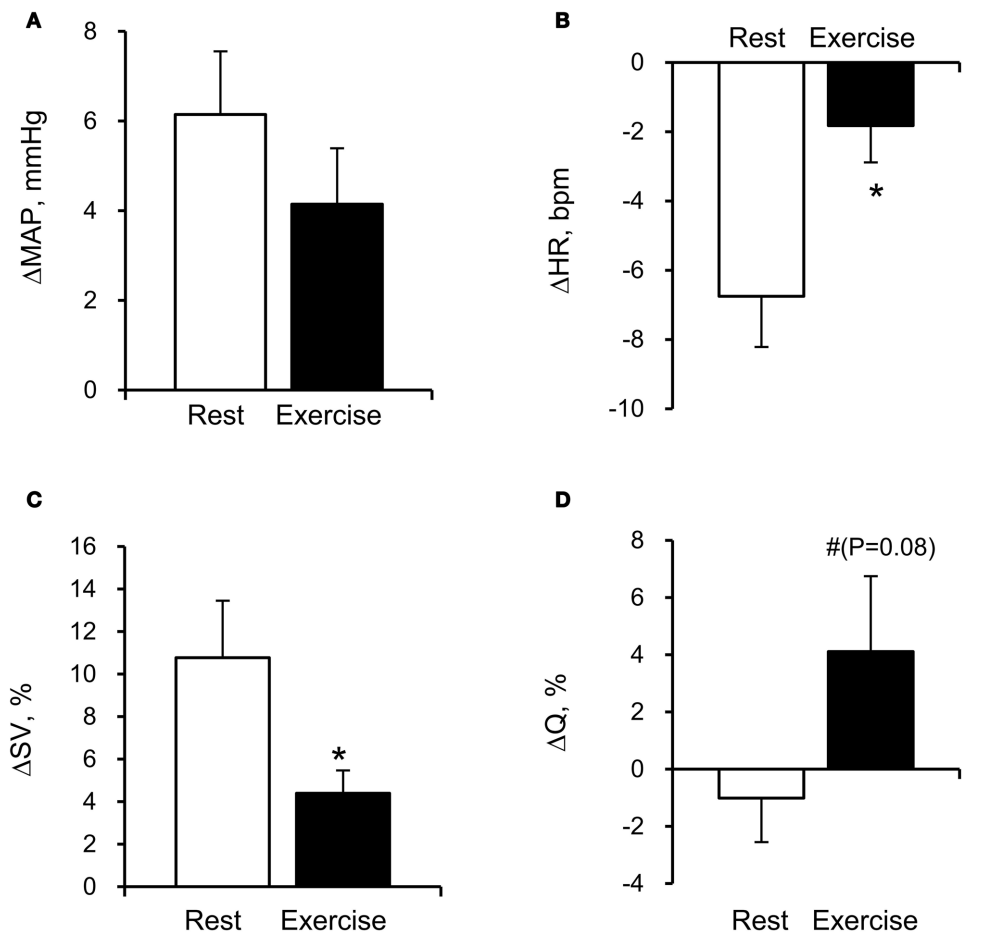

FIGURE 3 | Facial cooling-induced hemodynamic changes (Δ) at rest and during exercise: (A) mean arterial pressure (MAP), (B) heart rate (HR), (C) stroke volume (SV), and (D) cardiac output (Q). Values are means ± S.E.M. $^{*}P < 0.05$, $^{\#}P < 0.1$ different from rest.

regulation. In any case, these findings provide evidence that exercise-induced heat stress modifies the blood circulation in the head.

The common carotid artery bifurcates into the ECA and ICA; therefore, it is possible that changes in ECA blood flow could affect ICA flow and thus CBF. Indeed, during exercise changes in ECA blood flow were related to changes in ICA blood flow (Sato et al., 2011). Since fatigue and perception of effort during exercise may be related to CBF (Ide and Secher, 2000; Secher et al., 2008; Ogoh and Ainslie, 2009), we hypothesized that FC-induced effects on exercise time to fatigue (Ansley et al., 2008) and lowering of RPE (Mundel et al., 2007; Ansley et al., 2008) in the heat may have been associated with changes in blood flow to the face. In the present study, however, the FC-induced larger decreases in $SkBF_{face}$ but did not lead to proportional changes in MCA V_{mean} during exercise (**Figure 2**). Furthermore, there were no significant correlations between FC-induced changes in MCA V_{mean} and $SkBF_{face}$ at rest and during exercise ($P = 0.709$). These results suggest that the FC-induced increase in CBF could not be explained solely by changes in $SkBF_{face}$ at least during mild exercise.

The interaction between facial blood flow and CBF may be mediated by factors associated with exercise intensity or the diving reflex. Sato et al. (2011) demonstrated that the increase in ECA blood flow from moderate to heavy intensity exercise was negatively correlated with the decrease in ICA blood flow. They suggested that a large increase in ECA blood flow contributes to the decrease in ICA blood flow observed during heavy exercise. In the present study, however, the relatively low exercise intensity and thermal stress may have been insufficient to cause significant diversion of blood from the brain (ICA) to the face (ECA). Indeed, FC did not change RPE during exercise in the present study despite an increase in MCA V_{mean} (**Table 2**). During prolonged exercise at higher intensity or ambient temperature, the decrease in CBF, perhaps due to diversion of blood to the face, may still influence the development of central fatigue (Nybo and Nielsen, 2001; Nybo et al., 2002; Dalsgaard et al., 2004). In these conditions, $SkBF_{face}$ largely increases for thermoregulatory purposes and FC may attenuate facial vasodilatation and help preserve CBF.

Another possibility is that FC elicits the diving reflex in which facial cold receptors stimulate cardiovascular changes, including bradycardia and peripheral vasoconstriction (Fagius and Sundlof, 1986; Foster and Sheel, 2005; Kinoshita et al., 2006) that may divert blood toward the brain. For this to occur there would need to be a larger increase in peripheral relative to cerebral vascular resistance. Indeed, the increase in TPR was larger than CVRi at with FC at rest (+7.4% vs. +4.7%). Interestingly, this difference in the FC-induced change between TPR and CVRi was not evident during exercise (+1.3% vs. +1.7%). While CBF is generally believed to be maintained within a narrow range despite changes in MAP between 60 and 150 mmHg—commonly known as cerebral autoregulation (Lassen, 1959). Lucas et al. (2010) indicated that CBF closely follows pharmacological-induced changes in blood pressure in humans. Therefore, it is possible that the increase in MAP induced by FC might have affected the MCA V_{mean} response during exercise. Further studies are needed for confirming the possibility. Also, Q affects CBF at rest and during exercise (Ogoh et al., 2005). In the present study, the changes in Q evoked by FC were negligible and not significant at rest and during exercise (**Table 2**). Therefore, it would appear that the changes in Q had a small effect on the FC-induced increase in CBF. However, higher Q response to FC ($P = 0.08$) may contribute to increase in MCA V_{mean} during exercise. In addition, the diving reflex to FC enhances cardiac parasympathetic nerve activity via activation of facial receptors and the trigeminal nerve pathways (Heistad et al., 1968; Khurana et al., 1980). In the present study, FC decreased HR at rest and during exercise. The parasympathetic system has a potential vasodilator effect on brain vessels by mediators such as vasoactive intestinal peptide, acetylcholine, and nitric oxide (Hamel, 2006). More recently, it has been demonstrated that parasympathetic involvement might have a vasodilatory role in the regulation of the cerebral resistance vessels during mild dynamic exercise in humans (Seifert et al., 2010). Thus, it is likely that the increase in CBF evoked by FC might be associated with parasympathetic activation. However, HR responses to FC were different between rest and exercise. Taken together, the relative distribution of blood flow between the brain and the face appears to be regulated by different mechanisms depending on the level of exercise intensity and thermal stressors.

The present study involves some technical considerations. First, the transcranial Doppler ultrasonography-derived MCA V_{mean} is an index of regional CBF, and it is acknowledged that this estimate is justified as a measure of CBF only if the vessel diameter is maintained (Valdueza et al., 1997; Serrador et al., 2000). However, the MCA diameter appears to change little during several conditions such as acute hemodynamic perturbations (Giller et al., 1993) and increases in sympathetic activity (Serrador et al., 2000). Additionally, the changes in MCA V_{mean} during submaximal dynamic exercise appears to reflect transient changes in CBF determined by other exercise validated techniques [e.g., internal carotid artery blood flow (Hellstrom et al., 1996) and ^{133}Xe clearance technique (Jorgensen et al., 1992a,b)]. Second, we used laser Doppler flowmetry to estimate the change in skin blood flow at the forehead. Although this method was used and validated in previous studies for estimation of flow changes in cutaneous regions, this technique has some acknowledged limitations, most notably the inability to provide quantitative measurements of $SkBF_{face}$ in absolute units (Johnson et al., 1984). Therefore, we conducted the rest and exercise trials in the same day and did not move the laser Doppler probes throughout whole experiment to provide quantitative metrics for our comparisons. Finally, the protocol was not designed to identify the mechanism of FC induced-increases in CBF. It is possible that FC-induced hemodynamic changes (i.e., blood pressure) might modify the effects on $SkBF_{face}$ and CBF regulation. Also, it is possible that the contribution of $SkBF_{face}$ to FC induced-changes in MCA V_{mean} was altered by exercise itself. Therefore, to address the actual mechanism of FC induced-increases in CBF, further studies, which manipulate $SkBF_{face}$ without altering systemic circulation, will be needed.

SUMMARY

FC evokes reflexes that reduce $SkBF_{face}$ at rest and to a greater extent during exercise. However, this larger decrease in $SkBF_{face}$ did not result in a larger increase in MCA V_{mean} during exercise. Because changes in $SkBF_{face}$ did not correlate with changes in MCA V_{mean}, FC induced increases in MCA V_{mean} during exercise could not be explained solely by changes in $SkBF_{face}$. These results suggest that regulation of skin blood flow during low-intensity exercise has little impact on overall CBF and those other mechanisms.

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Sympathetic limitation of exercise hyperemia: even hypoperfused muscle is not exempted

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Exercise requires major adjustments in cardiovascular performance to accommodate the large increases in blood flow to active skeletal muscle groups. These cardiovascular adjustments involve alterations in autonomic control, most notably an increase in sympathetic activity, that act not only to increase cardiac output but also redistribute blood flow away from visceral organs and inactive skeletal muscle to enable a sufficient increase in flow to the active muscle while maintaining aortic perfusion pressure (Laughlin et al., 2012). Sympatholysis in active skeletal muscle facilitates the increase in flow that occurs during exercise, but there is evidence that even during severe exercise sympathetic vasoconstriction limits blood flow in the active skeletal muscle (Laughlin et al., 2012). Under normal inflow conditions this sympathetic restraint of flow has minimal effects on muscle oxygenation, as the limitation of flow can be compensated for by an increase in muscle oxygen extraction (Laughlin et al., 2012). In contrast, in the presence of a flow-limiting artery stenosis the sympathetic vasoconstrictor influence may interfere with autoregulation of muscle blood flow, thereby aggravating tissue hypoperfusion. There is evidence that intense sympathetic activity can limit ischemic vasodilation of skeletal muscle resistance vessels, as sympathetic activation was shown to limit reactive hyperemia in the human fore-arm (Ardill et al., 1967). However, the effect of a flow-limiting stenosis on sympathetic control of skeletal muscle flow during exercise remains to be established.

In a series of studies published over the past 5 years, Casey and Joyner and colleagues have explored the cardiovascular adjustments in response to skeletal muscle hypoperfusion during exercise (Casey and Joyner, 2011). For this purpose they developed an elegant model of forearm blood flow limitation in humans by use of an inflatable balloon positioned in the brachial artery. Using this model they have shown that inflation of the balloon during exercise results in an immediate decrease in blood flow followed by a partial (∼80% of normal) restoration of blood flow. The latter is, at least in part, dependent on nitric oxide and adenosine (Casey and Joyner, 2011). In this special issue of *Frontiers in Physiology*, Casey and Joyner addressed the important question whether the incomplete restoration of blood flow distal to an acute stenosis during exercise could be the result of sympathetic activity (Casey and Joyner, 2012). The results demonstrate that the incomplete restoration of blood flow was indeed the result of a sympathetic vasoconstrictor influence in the forearm microcirculation, as the non-selective α-adrenoceptor antagonist phentolamine facilitated blood flow recovery to pre-inflation levels. These findings imply that, even during exercise in conjunction with a flow-limiting stenosis, sympathetic activity continues to limit muscle perfusion. Another interesting observation was that the level of flow recovery in an individual was inversely correlated with the vasoconstriction produced by tyramine-induced release of endogenous norepinephrine, suggesting that significant inter-individual variability in sensitivity to sympathetic activation exists. These are important observations because it suggests, for the first time, that the limited exercise capacity observed in patients with obstructive peripheral artery disease (Stewart et al., 2002) is not only due to the presence of a proximal artery stenosis but also due to incomplete vasodilation of the skeletal muscle microcirculation, as a consequence of competition between α-adrenergic vasoconstriction and autoregulation. The observations are in good agreement with observations in the canine coronary circulation where α-adrenergic constriction limits resistance vessel dilation distal to a flow-limiting stenosis in dogs during treadmill exercise, thereby aggravating cardiac muscle hypoperfusion (Laxson et al., 1989).

As is usually the case with interesting data, several questions arise from the present study, which should be the subject of future studies. First, it would be important to determine whether similar results are obtained with (1) exercise involving a larger number of muscle groups, e.g., leg exercise or even whole body exercise, (2) higher intensities of exercise, and (3) more severe degrees of stenosis. Second, it would be important to repeat studies in the presence of β-adrenergic receptor blockade. Thus, it is possible that the phentolamine-induced improvement in flow restoration was, at least in part, due to presynaptic α_2-adrenergic blockade resulting in enhanced norepinephrine release and subsequent (unopposed) vascular β_1- and β_2-adrenergic receptor stimulation (Laughlin et al., 2012). Third, the α-adrenergic receptor subtype(s) mediating the sympathetic vasoconstriction was not determined. It is well known that α_1- and α_2-receptors are differentially expressed in small arteries (α_1) vs. arterioles (α_2) (Faber, 1988), partly as a result of which α_2-receptors are more sensitive

to metabolic inhibition (Anderson and Faber, 1991; Wray et al., 2004). It is thus possible that α_1-adrenergic receptors mediated the sympathetic restraint of flow. Fourth, studies should also be performed with other techniques (e.g., PET or MRI) allowing investigation of the regional blood flow responses separately in various tissues of the forearm or leg, in order to establish whether the α-adrenergic constriction actually occurred in the active skeletal muscle or that its effects on whole forearm blood flow were principally due to constriction in the inactive muscle fibers and non-muscular tissues such as bone, fat, and skin (Heinonen et al., 2012). Finally, it would be of significant interest to determine whether these findings apply to patients with peripheral artery disease, who oftentimes suffer from vascular endothelial dysfunction due to reduced nitric oxide bioavailability (Stewart et al., 2002). The authors have actually already shown in healthy human subjects that restoration of flow during hypoperfusion is critically dependent on nitric oxide (Casey and Joyner, 2009). It is likely that sympathetic limitation of flow during exercise would be even more pronounced in patients with peripheral artery disease, as lack of nitric oxide may leave the microcirculation more vulnerable to sympathetic vasoconstriction (Dinenno and Joyner, 2006; Joyner and Green, 2009). Notwithstanding these considerations, the study Casey and Joyner in this issue of *Frontiers in Physiology* provides further evidence for the flow restraining influence of sympathetic activity that is apparently strong enough to limit metabolic vasodilation in the microcirculation of exercising human skeletal muscle even in the presence of hypoperfusion.

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