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Central command dysfunction in rats with heart failure is mediated by brain oxidative stress and normalized by exercise training

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Key points

- In heart failure, sympathoexcitation elicited by central command, a parallel activation of the motor and autonomic neural circuits in the brain, is exaggerated.
- Mechanisms underlying central command dysfunction in heart failure were unexplored, and effects of exercise training on central command dysfunction in heart failure were not determined.
- Data presented here suggest that oxidative stress in the medulla in heart failure mediates central command dysfunction, and that exercise training in heart failure is capable of normalizing central command dysfunction through its antioxidant effects in the medulla.
- The present study contributes to our understanding of brain mechanisms underlying abnormal autonomic adjustments to exercise in heart failure.

Abstract Sympathoexcitation elicited by central command, a parallel activation of the motor and autonomic neural circuits in the brain, has been shown to become exaggerated in chronic heart failure (CHF). The present study tested the hypotheses that oxidative stress in the medulla in CHF plays a role in exaggerating central command-elicited sympathoexcitation, and that exercise training in CHF suppresses central command-elicited sympathoexcitation through its antioxidant effects in the medulla. In decerebrate rats, central command was activated by electrically stimulating the mesencephalic locomotor region (MLR) after neuromuscular blockade. The MLR stimulation at a current intensity greater than locomotion threshold in rats with CHF after myocardial infarction (MI) evoked larger ($P < 0.05$) increases in renal sympathetic nerve activity and arterial pressure than in sham-operated healthy rats (Sham) and rats with CHF that had completed longterm (8–12 weeks) exercise training (MI + TR). In the Sham and MI + TR rats, bilateral microinjection of a superoxide dismutase (SOD) mimetic Tempol into the rostral ventrolateral medulla (RVLM) had no effects on MLR stimulation-elicited responses. By contrast, in MI rats, Tempol treatment significantly reduced MLR stimulation-elicited responses. In a subset of MI rats, treatment with Tiron, another SOD mimetic, within the RVLM also reduced responses. Superoxide generation in the RVLM, as evaluated by dihydroethidium staining, was enhanced in MI rats compared with that in Sham and MI + TR rats. Collectively, these results support the study hypotheses. We suggest that oxidative stress in the medulla in CHF mediates central command dysfunction, and that exercise training in CHF is capable of normalizing central command dysfunction through its antioxidant effects in the medulla.

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Abbreviations Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; CHF, chronic heart failure; FS, fractional shortening; HR, heart rate; MAP, mean arterial pressure; MI, myocardial infarction; MLR, mesencephalic locomotor region; NADPH, nicotinamide adenine dinucleotide phosphate; RAS, renin angiotensin system; RSNA, renal sympathetic nerve activity; RVLM, rostral ventrolateral medulla; SNA, sympathetic nerve activity; SOD, superoxide dismutase.

Introduction

Supervised exercise training interventions in patients with chronic heart failure (CHF) have now been accepted as therapeutic treatment. Such treatment not only improves quality of life and functional class, but also decreases resting sympathetic overactivity, alleviates peripheral inflammation, and reverses morphometric and histochemical features of skeletal myopathy (Middlekauff, 2010; Downing & Balady, 2011; Piepoli *et al.* 2011). However, sympathoexcitation seen during a bout of exercise is augmented in patients with CHF in comparison with healthy individuals (Sterns *et al.* 1991; Negrão *et al.* 2001). The augmented sympathoexcitation during exercise is a possible cause of exercise intolerance, a hallmark of CHF (Notarius *et al.* 1999). Thus, understanding regulatory mechanisms of sympathetic nerve activity (SNA) during exercise in CHF is clinically important.

Central command is a feedforward neural mechanism that evokes parallel modifications of motor and autonomic functions during exercise (Goodwin *et al.* 1972). This neural activation elicits sympathoexcitation by exciting the medullary cardiovascular pathway containing the rostral ventrolateral medulla (RVLM), in which sympathetic premotor neurones are located (Nolan & Waldrop, 1997; Padley *et al.* 2007). Central command function in patients with CHF has been suggested to become exaggerated, thereby contributing to augmented sympathoexcitation during exercise (Sterns *et al.* 1991; Negrão *et al.* 2001). This suggestion was supported by the findings of a subsequent rat study which showed that sympathoexcitatory responses to central command activation, caused by electrical stimulation of the mesencephalic locomotor region (MLR) after neuromuscular blockade (Eldridge *et al.* 1985; Bedford *et al.* 1992), were larger in rats with CHF than in healthy controls (Koba *et al.* 2006*a*). However, the mechanisms underlying central command dysfunction in CHF have so far remained unknown.

Oxidative stress developing in CHF may contribute to central command dysfunction. Superoxide overproduction in the central nervous system has been suggested to lead to neurocardiovascular dysregulation, such as resting sympathetic overactivity (Gao *et al.* 2004, 2005, 2007; Kishi *et al.* 2004, 2011; Lindley *et al.* 2004; Zimmerman *et al.* 2004; Nishi *et al.* 2013). It was reported that in conscious CHF rabbits in which the RVLM was exposed to oxidative stress, intracerebroventricular administration of a superoxide dismutase (SOD) mimetic Tempol decreased resting SNA (Gao *et al.* 2004). Moreover, *in vitro* studies have suggested that superoxide increases the sensitivity of neuronal cells, which respond to excitatory input by regulating membrane ion channels and controlling action potential generation. For example, voltage-gated potassium current in neuronal cells was shown to be inhibited by superoxide (Sun *et al.* 2005; Yin *et al.* 2010). These findings led us to hypothesize that oxidative stress in the medulla of CHF may play a role in sensitizing the RVLM neurones which respond to central command activation, thereby exaggerating central command-elicited sympathoexcitation (Hypothesis 1).

As stated, longterm exercise training has beneficial effects on various clinical parameters in CHF. However, there has been no information about the effect of exercise training on central command dysfunction. Longterm exercise training in rabbits with CHF reportedly had an antioxidant effect in the RVLM, thereby decreasing resting sympathetic overactivity (Gao *et al.* 2007). Given these findings, and if the present Hypothesis 1 is correct, it is reasonable to hypothesize that exercise training in CHF suppresses central command-elicited sympathoexcitation through its antioxidant effects in the medulla (Hypothesis 2).

The purposes of this study were to test Hypotheses 1 and 2 in rats. In the experiments, we employed three rat groups: (i) rats with CHF after myocardial infarction (MI); (ii) rats with CHF that had completed longterm exercise training, and (iii) sham-operated control rats. In these rats, renal SNA (RSNA) and cardiovascular responses to electrical stimulation of the MLR were examined. The effects of an antioxidant treatment within the rat RVLM on MLR stimulation-elicited responses were also investigated. Moreover, *in situ* superoxide production in the rat medulla was studied.

Methods

All procedures outlined in the present study complied with the Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences of the Physiological Society of Japan, and were approved by the

Animal Care Committee of Tottori University. The present experiments were performed in male Sprague–Dawley rats ($n = 70$). Rats were housed in standard rodent cages in a temperature-controlled room (24°C) and regulated on a 12 : 12 h light–dark schedule. Food and water were made available *ad libitum*.

Ligation surgery, exercise training and echocardiography

Coronary artery ligation surgery to induce MI was performed as described previously (Koba *et al.* 2006*a*, 2009). Rats (aged 5–6 weeks, 170–220 g, $n = 47$) were anaesthetized with a mixture of isoflurane (<4%) and oxygen, intubated and artificially ventilated with a respirator (model SN-480-7; Shinano Co., Tokyo, Japan). An incision between the fourth and fifth ribs was made, and the left ventricular wall was exposed through left thoracotomy. The left coronary artery was then ligated (MI rats). In another set of rats ($n = 18$), sham operations were performed without ligation of the coronary artery (Sham rats). The thorax was closed, the tracheal tube was removed, and the rat was allowed to recover from anaesthesia.

At 6–9 weeks after the ligation surgery, a subset of MI rats was randomly assigned to a training group (MI + TR rats). MI + TR rats ($n = 17$) were treadmill-trained for 8–12 weeks according to a progressive exercise protocol adapted from a previous study (Musch *et al.* 1986). In the present study, the treadmill exercise was conducted five times per week between 09.00 hours and 11.00 hours. On the first day of the protocol, the rats were acclimatized to a custom-built treadmill (MK-680C; Muromachi Kikai Co. Ltd, Tokyo, Japan) by running at 20 m min^{-1} on a 5% incline for 5 min. The duration of running was then increased by 0–10 min per day over a 3 week period until rats ran for 60 min per day. Rats that exhibited refusal to run during the training programme were excluded from further training. The protocol employed in the present study is considered sufficient to have exercise training effects; exercise training protocols previously employed by others in which the training period was equivalent to or shorter than that used in the present study had significant effects in terms of increasing maximal oxygen consumption, skeletal muscle succinate dehydrogenase activities, and/or skeletal muscle citrate synthase activities in rats with CHF (Musch *et al.* 1986; Kleiber *et al.* 2008). Sedentary MI or Sham rats had limited activity in the cages in which they were housed during the exercise training for MI + TR rats.

Transthoracic echocardiography (model 5189002; GE Healthcare Ltd, Little Chalfont, UK) was performed in rats anaesthetized with 1.5% isoflurane in oxygen to assess left ventricular function, prior to the experiments described below. Rats that underwent ligation were excluded from the study if they did not meet the present criteria for CHF [i.e. left ventricular fractional shortening (FS) of <35%].

Experiment preparation

The surgery and experiments to observe MLR stimulation-elicited responses were conducted as reported previously (Koba *et al.* 2006*a*,*b*). In rats anaesthetized with a mixture of isoflurane (<4%) and oxygen, the trachea was cannulated and the lungs were mechanically ventilated with a respirator with 6 ml kg^{-1} tidal volume at a frequency of 70 per min. The left jugular vein and common carotid artery were cannulated to administer drugs and to measure arterial pressure (AP), respectively. The arterial catheter was attached to a pressure transducer (P23XL; Becton, Dickinson & Co., Newark, DE, USA). Two needle electrodes were placed on the chest for the purposes of electrocardiography (ECG), the signals of which were amplified with an AC amplifier (P511; Grass Instruments, Natus Neurology, Inc., Warwick, RI, USA). Heart rate (HR) was calculated beat to beat with detection of the time between successive R waves in the ECG. Arterial pH was monitored with a pH meter (B-212; Horiba Corp., Kyoto, Japan) and maintained within normal limits (pH 7.4) by an I.V. infusion of a sodium bicarbonate solution (8.4%). Rectal body temperature was monitored with a digital thermometer, and adequately maintained at 37.5–38.5°C with an external heating lamp. To measure RSNA, a bipolar electrode made of a Teflon-insulated stainless steel wire (790600; A-M Systems, Inc., Sequim, MA, USA) was connected to the renal nerve directed to the left kidney. The RSNA signal was amplified with an amplifier (MEG-5200; Nihon Kohden Corp., Tokyo, Japan) with a bandpass low-frequency filter of 150 Hz and a high-frequency filter of 3 kHz, and made audible. The rats were held in a stereotaxic head unit (900LS; David Kopf Instruments, Inc., Tujunga, CA, USA). The dorsal surface of the rat brainstem was exposed by a middle incision made at the back of the head, a dissection of muscles overlaying the base of the skull, and an incision made through the atlanto-occipital membrane. For the decerebration procedures, a parietal craniotomy was performed and cortical tissue was removed by aspiration. Then, the brain was sectioned coronally with a blade at the precollicular level, and all neural tissue rostral to the section and the cortical tissues covering the cerebellum were aspirated. Anaesthesia was withdrawn. A recovery period of at least 60 min after decerebration was allowed before the identification of brain sites of the MLR and RVLM.

The site of the rat MLR was functionally identified as described previously (Bedford *et al.* 1992; Koba

et al. 2006*a*,*b*). A needle-type bipolar microelectrode (CBBPE75; FHC, Inc., Bowdoin, ME, USA) connected to an electronic stimulator (SEN-7103; Nihon Kohden Corp.) through an isolator (SS-202J; Nihon Kohden Corp.) was inserted into the midbrain at an angle vertical to the surface of the superior and inferior colliculus with the aid of an operating microscope. Initial stereotaxic coordinates for the MLR were 0.7 mm rostral and 1.9–2.1 mm lateral to the border of the inferior and superior colliculi, and 3.5–4.5 mm ventral to the dorsal surface of the colliculi. Determination of the site of the MLR was confirmed by the physiological criteria observed when the site was electrically stimulated (60 Hz frequency, 1 ms duration) as follows: (i) threshold of locomotion with reciprocal limb movement at <35 μA; (ii) stimulus-bound locomotion, and (iii) graded activity of locomotion and gait changes with increased stimulation current. It should be noted that electrical stimulation of the rat MLR at 35 μA, which initially evoked rhythmic alternating locomotor activity, gradually resulted in a wide variation in the expression of motor activity including tonic contraction, short bursts of locomotor activity which started, stopped and started again, and odd forms of locomotion such as 'goose stepping' as the stimulating current was applied. Having identified the site of the MLR, we subjected the rats to neuromuscular blockade with an I.V. infusion of pancuronium bromide (0.5 mg kg^{-1} body weight).

The bilateral sites of the rat RVLM were then functionally identified as described previously (Kishi *et al.* 2004; Mueller 2007). A glass micropipette was inserted into the brainstem at an angle vertical to the dorsal surface of the brainstem with the aid of the microscope. Initial stereotaxic coordinates for the RVLM were 1.0 mm rostral and 1.9–2.1 mm lateral to the calamus scriptorius, and 3.5–3.8 mm ventral to the dorsal surface of the medulla. In all studies, the RVLM was identified functionally by observing a pressor response (>10 mmHg) when glutamate (10 mM, 50.6 nl) was pressure ejected using a calibrated microinjection system (Nanoject II; Drummond Scientific, Co., Broomall, PA, USA). A period of at least 20 min was allowed to elapse after the identification of the MLR and RVLM prior to the initiation of the experimental protocols.

Experimental protocols to observe MLR stimulation-elicited responses

RSNA and cardiovascular responses to electrical stimulation of the MLR before and after bilateral microinjection into the RVLM of Tempol, a compound that mimics the enzymatic activity of SOD, were examined in 11 Sham, 12 MI and 10 MI + TR rats. The MLR of decerebrated rats under neuromuscular blockade was electrically stimulated at 35 μA for 30 s. The MLR was also electrically stimulated at 20 μA for 30 s. The order of current intensity was random and intervals of at least 5 min were allowed between manoeuvres. In the present study, the MLR was not stimulated at 50 μA, a level we had previously employed (Koba *et al.* 2006*b*), in order not to evoke huge pressor responses that may accidentally cause brain bleeding. Subsequently, Tempol diluted in saline [10 mM, 92 nl (23.0 nl $\times$ 4)] was microinjected into the RVLM bilaterally using the Nanoject II. At 30–40 min after the Tempol microinjection, the MLR was again electrically stimulated at 20 μA or 35 μA for 30 s. The amount of Tempol microinjected into the rat RVLM was based on findings in a previous rat study (Kishi *et al.* 2004).

In a subset of MI rats ($n = 7$), the effect on MLR stimulation-elicited responses of microinjection into the RVLM of Tiron, which is not chemically related to Tempol but has a similar superoxide scavenging activity, was examined. The MLR was electrically stimulated at 20 μA or 35 μA for 30 s before and 30–40 min after bilateral microinjection of Tiron [10 mM, 92 nl (23.0 nl $\times$ 4)] into the RVLM. The amount of Tiron was equivalent to that of Tempol because the same amounts of Tempol and Tiron bilaterally injected into the RVLM in conscious rabbits reportedly caused equivalent reductions in the pressor response to air-jet stress (Mayorov *et al.* 2004).

In cases in which stimulation of the MLR induced actual locomotion during the experiments, supplemental doses of pancuronium bromide (0.25 mg kg^{-1} body weight) were given I.V. At the end of data collection, the MLR was stimulated at 150 μA for 10 s in order to obtain maximal values of RSNA (Koba *et al.* 2006*a*). After all of the observations had been conducted, the renal nerve was cut between the electrode and the neural axis in order to measure the background noise of RSNA. The microinjection sites for the RVLM were marked using India ink for histological verification. At the conclusion of the experiment, the rats were humanely killed with an I.V. infusion of sodium pentobarbital (75 mg kg^{-1}) followed by an I.V. infusion of saturated potassium chloride solution (1 ml).

In another set of experiments using normal control rats ($n = 5$, 361 $\pm$ 30 g body weight), we tested if electrical stimulation of the MLR at 35 μA would increase α-motoneurone discharge. In rats anaesthetized with a mixture of isoflurane ($<4\%$) and oxygen, a laminectomy exposing the lower lumbar portion of the spinal cord (L1–L6) was performed. The meningial layers surrounding the cord were cut and reflected laterally. Two nerve bundles obtained from L4 and L5, or L5 and L6 ventral roots on the left side were carefully isolated and sectioned. The exposed neural tissue was immersed in mineral oil. The central end of the roots was placed on an insulated bipolar recording electrode. The neural activity signal was amplified with the amplifier with a bandpass

low-frequency filter of 150 Hz and a high-frequency filter of 3 kHz, and made audible. In decerebrate rats after the withdrawal of anaesthesia, the site of the MLR was identified with the procedure described above. In decerebrated rats under neuromuscular blockade, electrical stimulation of the MLR at 35 μA, greater than the locomotion threshold, continuously increased the ventral root discharge as long as the stimulating current was applied; Fig. 1 indicates the typical response. These observations are consistent with the findings of previous studies (Eldridge *et al.* 1985; Degtyarenko & Kaufman, 2000; Koba *et al.* 2006*b*).

In situ superoxide production in the rat medulla

In other subsets of Sham ($n = 7$), MI ($n = 7$) and MI + TR ($n = 7$) rats, in which neither Tempol nor Tiron were microinjected into the RVLM, intracellular superoxide generation in the medulla was evaluated with dihydroethidium (DHE) staining (Zimmerman *et al.* 2004; Nishi *et al.* 2013). The brains including the medulla of rats anaesthetized with isoflurane (<4% in oxygen) were quickly removed, embedded in optimal cutting temperature compounds, and cryostat-sectioned (30 μm, coronal). To exclude the possible effects of a bout of exercise acutely increasing superoxide generation in the brain, the brains of MI + TR rats were harvested 2 days after the cessation of the exercise training programme. However, acute exercise (treadmill running to exhaustion) in rats reportedly had little effect on brain oxidative stress biomarkers (Liu *et al.* 2000*a*). Brain sections containing the bilateral RVLM obtained from a set of one Sham, one MI and one MI + TR rat were prepared on the same day, and two sections from each rat brain were used in the following process. Sections were incubated with DHE (1 μM) in the dark for 30 min at 37°C. Images of red-fluorescent ethidium, which result from the oxidation of DHE, were then obtained using an epifluorescence microscope system with a camera (DMRB; Leica Microsystems AG, Wetzlar, Germany). The RGB confocal images were loaded into ImageJ Version 1.47 (National Institutes of Health, Bethesda, MD, USA), and converted to 8-bit grey scale. Fluorescence intensity was then measured. For each sample, an average fluorescence intensity was obtained from bilateral measurements within the RVLM of the two sections. Another *in situ* experiment was also performed to determine if Tempol/Tiron has antioxidant effects on brain tissues. Sections obtained in another subset of MI rats ($n = 4$) were pretreated in saline, Tempol (10 mM diluted in saline) or Tiron (10 mM diluted in saline) for 30 min, and incubated with DHE (1 μM) in the dark for 1 h at 37°C. Then, fluorescence intensity was measured using the procedure described above.

Table 1. Morphometric and echocardiographic characteristics of rats used for this study

	Sham	MI	MI + TR
Rats, *n*	18	30	17
Body weight, g	490 ± 10	503 ± 14	477 ± 16
Heart weight/body weight, mg g^{-1}	2.87 ± 0.08	3.40 ± 0.06*	3.41 ± 0.14*
Left ventricular end-diastolic diameter, mm	7.8 ± 0.2	10.5 ± 0.2*	10.1 ± 0.4*
Left ventricular end-systolic diameter, mm	4.2 ± 0.2	8.5 ± 0.2*	8.0 ± 0.4*
Fractional shortening, %	46.9 ± 1.1	20.2 ± 1.2*	20.7 ± 1.5*

Values are means ± SEM. Abbreviations: Sham, sham-operated rats; MI, rats with myocardial infarction; MI + TR, MI and exercise-trained rats. *$P < 0.05$ *versus* Sham detected by Tukey's *post hoc* test following one-way ANOVA.

Statistics

In the experiments to observe MLR stimulation-elicited responses, all measured variables were displayed continuously on a computer monitor and stored on a hard disk at a sampling rate of 1 kHz through an analog–digital interface (PowerLab/8 s; AD Instruments, Dunedin, New Zealand). Baseline data were obtained from averaged values for 30 s immediately prior to electrical stimulation of the MLR. RSNA was expressed as a percentage of maximum RSNA induced by electrical stimulation of the MLR at 150 μA.

Data are expressed as means ± S.E.M. To assess significant differences, data were analysed with a paired *t* test or one-way or two-way repeated-measures ANOVA followed by the appropriate *post hoc* test (Dunnet's or Tukey's method). The level of significance was set at $P < 0.05$.

Results

Characterization of the model of CHF

All the Sham rats had FS of >40%, whereas all the MI and MI + TR rats had FS of <35%. Averaged FS values in the MI and MI + TR groups were significantly less than that in the Sham group (Table 1). No significant differences were found in FS between the MI and MI + TR groups (Table 1). Moreover, left ventricular end-diastolic and end-systolic dimensions were significantly increased in ligated rats irrespective of exercise training (Table 1). These results indicate that ligation developed a left ventricular dilatation, and that, as previously reported (Musch *et al.* 1986), longterm exercise training had no

Table 2. Baseline data prior to electrical stimulation of the mesencephalic locomotor region (MLR) before and 30–40 min after Tempol microinjection into the rostral ventrolateral medulla (RVLM) in sham-operated rats (Sham; $n = 11$), rats with myocardial infarction (MI; $n = 12$), and MI and exercise-trained rats (MI + TR; $n = 10$). The locomotion threshold, the minimum current at which electrical stimulation of the MLR will evoke locomotion, is also presented. Baseline data were obtained from 30 s averaged values immediately prior to MLR stimulation

	Sham		MI		MI + TR	
	Before Tempol	After Tempol	Before Tempol	After Tempol	Before Tempol	After Tempol
Baseline data prior to the MLR stimulation at 20 μA						
RSNA, % of max	30 ± 4	31 ± 4	26 ± 2	33 ± 5	28 ± 4	34 ± 3
Signal-to-noise ratio for RSNA	4.0 ± 0.8	4.1 ± 0.9	3.8 ± 0.8	4.4 ± 1.1	3.3 ± 0.4	4.1 ± 0.6
MAP, mmHg	104 ± 9	105 ± 7	107 ± 6	102 ± 6	116 ± 6	121 ± 5
HR, beats per min	382 ± 11	372 ± 13	380 ± 14	348 ± 13*	392 ± 9	381 ± 10
Baseline data prior to the MLR stimulation at 35 μA						
RSNA, % of max	30 ± 4	31 ± 3	24 ± 2	32 ± 5	27 ± 3	35 ± 3
Signal-to-noise ratio for RSNA	4.0 ± 0.8	4.2 ± 0.8	3.5 ± 0.6	4.4 ± 1.2	3.4 ± 0.4	4.1 ± 0.6
MAP, mmHg	107 ± 8	106 ± 7	108 ± 5	107 ± 7	120 ± 5	120 ± 5
HR, beats per min	381 ± 12	371 ± 14	381 ± 13	345 ± 12*	391 ± 8	382 ± 10
Locomotion threshold, μA	23.7 ± 1.8		26.1 ± 1.5		22.2 ± 3.2	

Values are means ± S.E.M. Abbreviations: RSNA, renal sympathetic nerve activity; MAP, mean arterial pressure; HR, heart rate. *$P < 0.05$ *versus* before Tempol in each group, detected by Tukey's *post hoc* test following two-way repeated-measures ANOVA.

therapeutic effects on deterioration of heart function in CHF.

MLR stimulation-elicited RSNA and cardiovascular responses

We examined the effect of exercise training on central command dysfunction in CHF by comparing RSNA and cardiovascular responses to stimulation of the MLR among Sham ($n = 11$), MI ($n = 12$) and MI + TR ($n = 10$) rats, in which neither Tempol nor Tiron had yet been microinjected into the RVLM. The MLR in decerebrate rats was electrically stimulated after neuromuscular blockade with an I.V. infusion of pancuronium bromide (0.5 mg kg^{-1} body weight). The locomotion threshold, the minimum current intensity at which MLR stimulation evoked locomotion, was <35 μA in all rats and averaged values did not differ significantly among the groups (Table 2). Neither were there any significant differences among the groups in baseline RSNA, MAP and HR (Table 2), as previously reported (Koba *et al.* 2006*a*). Electrical stimulation of the MLR at either 20 μA or 35 μA evoked significant increases in RSNA and MAP in these rats (Figs 2 and 3). This stimulation had no significant effects on HR except in MI rats in which the MLR was stimulated at 35 μA and MI + TR rats in which the MLR was stimulated at 20 μA (Fig. 3). Little effect on HR of MLR stimulation has been reported previously (Koba *et al.* 2006*a*,*b*). In MI rats, stimulation of the MLR at 35 μA evoked significantly larger RSNA and MAP responses, as evaluated by mean changes in RSNA and MAP from baseline during stimulation, than those in Sham and MI + TR rats (Fig. 4). There were no significant differences in responses to MLR stimulation at 35 μA between Sham and MI + TR rats. RSNA and MAP responses to stimulation of the MLR at 20 μA did not differ significantly among the rat groups (Fig. 4).

We also investigated the role played by brain oxidative stress in central command dysfunction in CHF by comparing MLR stimulation-elicited RSNA and

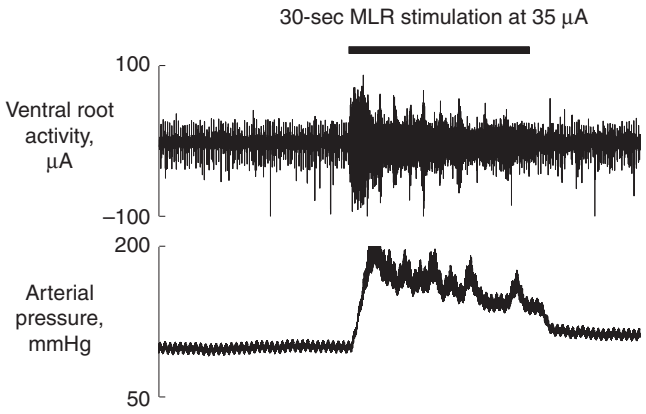

Figure 1. Neural discharge from the cut ends of the left lumbar ventral roots and arterial pressure changes in a healthy rat
Continuous increases in neural discharge recorded from the cut ends of the left lumbar ventral roots (L5 and L6) and arterial pressure changes during 30 s of electrical stimulation of the mesencephalic locomotor region (MLR) at 35 μA in a decerebrate healthy rat under neuromuscular blockade in which the locomotion threshold was 12 μA. In the other four rats tested, continuous increases in motoneurone discharge during MLR stimulation were also observed.

cardiovascular responses before and 30–40 min after bilateral microinjection of Tempol into the RVLM of Sham, MI and MI + TR rats. As previously observed (Kishi *et al.* 2004), Tempol microinjection in these rats acutely decreased RSNA, MAP and HR, and the decreases in RSNA and MAP returned to pre-microinjection levels within 10–15 min. At 30–40 min after Tempol microinjection, this chemical had no effect on RSNA or MAP in any rat group, or on HR in Sham and MI + TR rats, but reduced HR from baseline in MI rats (Table 2). In all rat groups, Tempol administration abolished increases in MAP from baseline during stimulation of the MLR at 20 μA (Fig. 3). In MI rats, Tempol administration significantly reduced MAP responses to stimulation of the MLR at 20 μA and both RSNA and MAP responses to stimulation of the MLR at 35 μA (Fig. 4). In Sham and MI + TR rats, by contrast, Tempol administration had no significant effect on RSNA and MAP responses to MLR stimulation at either 20 μA or 35 μA.

In a subset of MI rats ($n = 7$), we compared MLR stimulation-elicited responses before and 30–40 min after bilateral microinjection of Tiron into the RVLM. The locomotion threshold in this rat group was 24.4 ± 0.7 μA. Tiron administration had no effects on baseline RSNA, MAP or HR at 30–40 min after microinjection (Table 3). Tiron administration significantly reduced MAP responses to stimulation of the MLR at 20 μA and both RSNA and MAP responses to stimulation of the MLR at 35 μA (Fig. 5), as did Tempol administration.

Table 3. Baseline data prior to electrical stimulation of the mesencephalic locomotor region (MLR) before and 30–40 min after Tiron microinjection into the rostral ventrolateral medulla (RVLM) of rats with myocardial infarction (MI; $n = 7$). A paired *t* test was employed to test the effects of Tiron on baseline values and found no significant differences

	MI rats	
	Before Tiron	After Tiron
Baseline data prior to MLR stimulation at 20 μA		
RSNA, % of max	30 ± 5	26 ± 4
Signal-to-noise ratio for RSNA	3.6 ± 0.8	3.4 ± 0.7
MAP, mmHg	119 ± 5	117 ± 8
HR, beats per min	429 ± 15	399 ± 22
Baseline data prior to MLR stimulation at 35 μA		
RSNA, % of max	28 ± 5	27 ± 5
Signal-to-noise ratio for RSNA	3.5 ± 0.7	3.3 ± 0.7
MAP, mmHg	114 ± 8	114 ± 6
HR, beats per min	426 ± 16	395 ± 21

Values are means ± S.E.M. Abbreviations: RSNA, renal sympathetic nerve activity; MAP, mean arterial pressure; HR, heart rate.

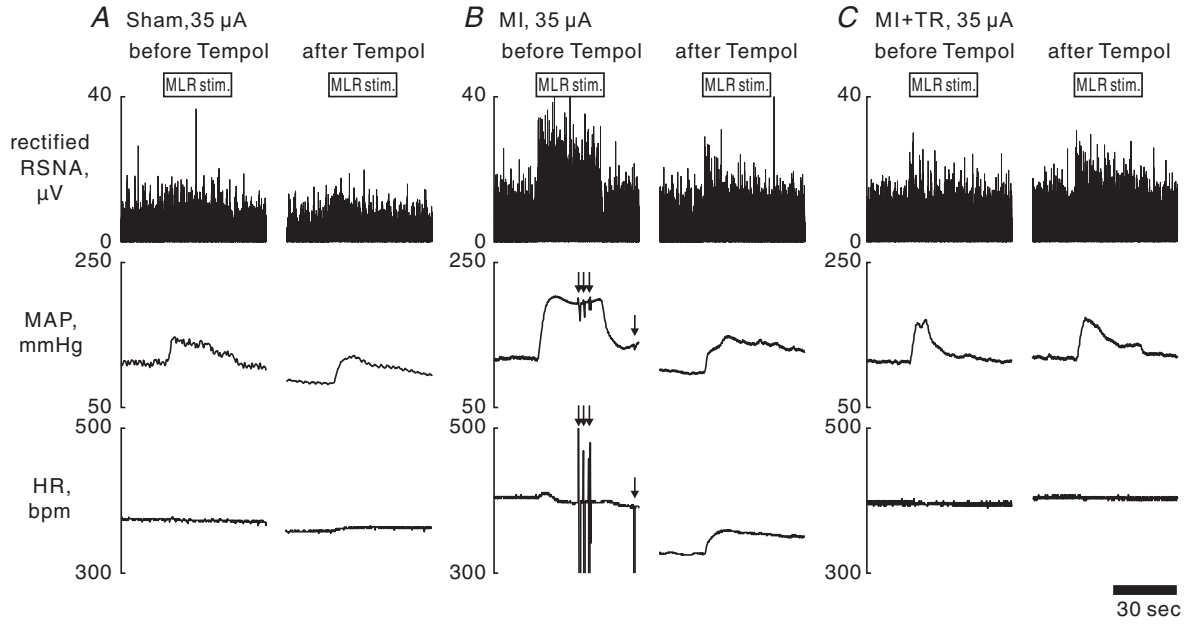

Figure 2. Representative recordings of rectified renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR)
Rectified RSNA, MAP and HR during 30 s of electrical stimulation of the mesencephalic locomotor region (MLR) at 35 μA, before and 30–40 min after bilateral microinjection of Tempol (10 mM, 92 nl) into the rostral ventrolateral medulla of sham-operated [Sham; fractional shortening (FS) = 44%, locomotion threshold (LT) = 32 μA] (*A*), myocardial infarction (MI; FS = 16%, LT = 31 μA) (*B*), and MI and exercise-trained (MI + TR; FS = 16%, LT = 22 μA) (*C*) rats. Arrows in *B* indicate fluctuations of MAP/HR induced by arrhythmia during stimulation of the MLR.

Verification of the RVLM sites

We identified the RVLM functionally by observing pressor responses (increases of >10 mmHg) to microinjections of glutamate in all rats used in the experiments in which Tempol or Tiron would be administered. In addition, we confirmed microinjection sites histologically by examining locations at which India ink was injected. This verified that the pipette tips were located within the regions of the RVLM that have been demonstrated in previous studies (Kishi *et al.* 2004; Mueller *et al.* 2007; Nishi *et al.* 2013) (Fig. 6).

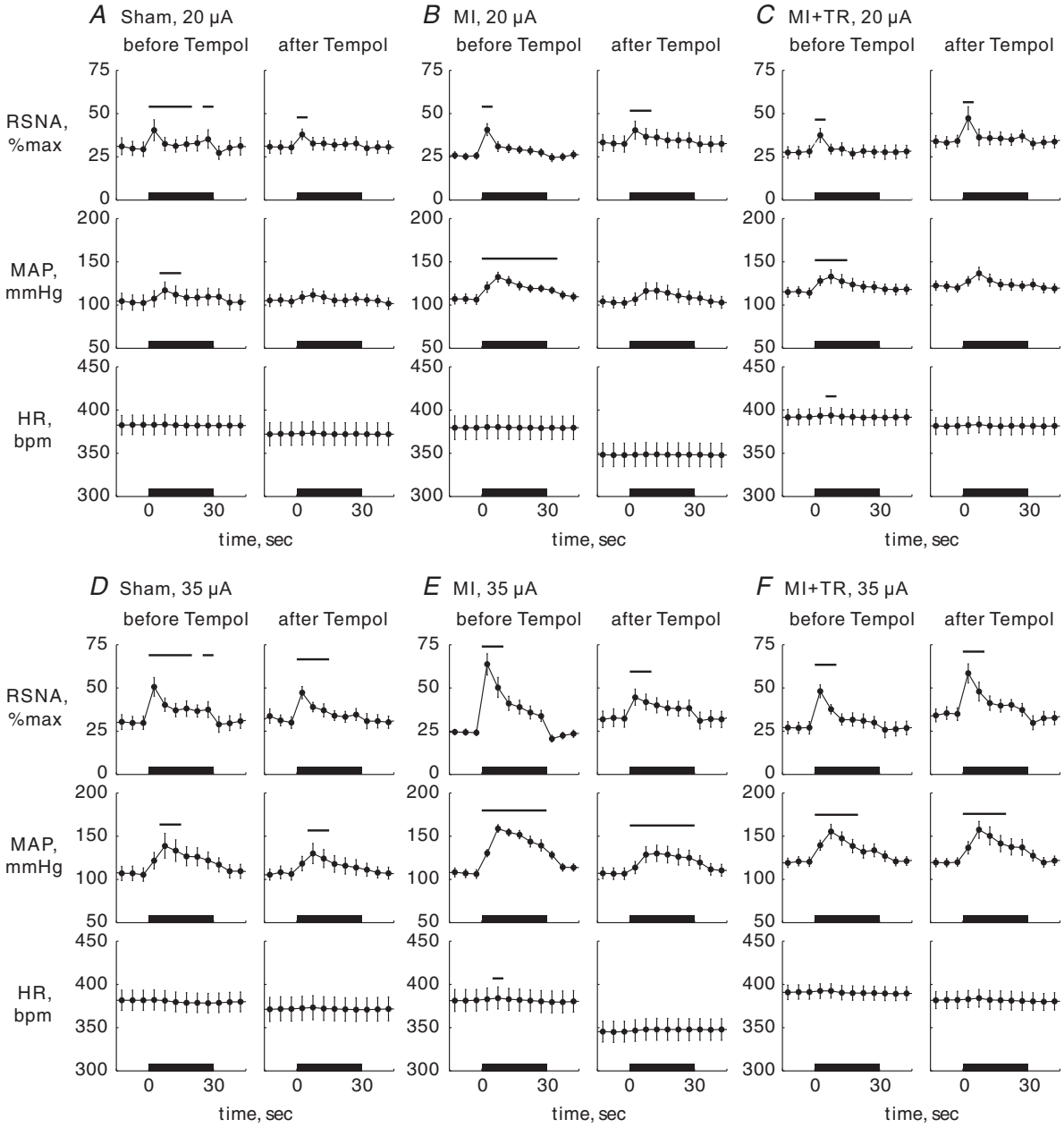

Figure 3. Time course changes in renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR)
Time course changes averaged for 5 s in RSNA per maximal activity ($RSNA_{perMax}$), MAP and HR during 30 s of electrical stimulation of the mesencephalic locomotor region at 20 μA (*A–C*) or 35 μA (*D–F*) before and 30–40 min after bilateral microinjection of Tempol (10 mM, 92 nl) into the rostral ventrolateral medulla in 11 sham-operated (Sham), 12 myocardial infarction (MI) and 10 MI and exercise-trained (MI + TR) rats. Values are means ± S.E.M. Abnormal values of HR caused by arrhythmia were discarded in the analyses. Thick horizontal bars located on the *x*-axis indicate the 30 s stimulation periods. Horizontal bars indicate significant ($P < 0.05$) differences from baseline values, detected by a Dunnett's *post hoc* test following one-way repeated-measures ANOVA.

In situ superoxide production in the medulla

In cryosections of the medulla including the RVLM, ethidium fluorescence was significantly enhanced in MI rats compared with that in Sham or MI + TR rats (Fig. 7). There were no significant differences in ethidium fluorescence between Sham and MI + TR rats (Fig. 7). Another set of DHE staining experiments indicated the antioxidant effects of Tempol/Tiron on superoxide production in rat brain tissue by showing that pre-treatment with either Tempol or Tiron on cryosections of the medulla of MI rats ($n = 4$) significantly reduced the intensity of ethidium fluorescence in the RVLM compared with that of normal saline (Tempol: $-42 \pm 10\%$, Tiron: $-55 \pm 4\%$, *versus* saline, respectively).

Discussion

To test Hypotheses 1 and 2, as stated in the Introduction, RSNA and cardiovascular responses to stimulation of the MLR in healthy rats, rats with CHF, and rats with CHF that had completed longterm exercise training were examined before and after an antioxidant treatment within the RVLM. Before the antioxidant treatment, electrical stimulation of the MLR at 35 μA evoked larger RSNA and pressor responses in MI rats than in Sham or MI + TR rats. Responses in MI + TR rats were similar to those in Sham rats. Moreover, in Sham and MI + TR rats, the SOD mimetic Tempol microinjected bilaterally into the RVLM did not modulate MLR stimulation-elicited responses. In MI rats, by contrast, microinjected Tempol reduced RSNA and pressor responses to stimulation of the MLR. The effect of Tiron, another SOD mimetic, mirrored that of Tempol; when microinjected bilaterally into the RVLM of MI rats, Tiron also reduced MLR stimulation-elicited responses. Furthermore, ethidium fluorescence in the RVLM of MI rats was enhanced in comparison with that in Sham and MI + TR rats, and fluorescence intensity in MI + TR rats was similar to that in Sham rats. These results, indicating superoxide overproduction in the RVLM of CHF and the antioxidant effects of exercise training in the RVLM of CHF, support previous findings (Gao *et al.* 2004, 2005, 2007; Guggilam *et al.* 2011). Taken together, the present observations support Hypotheses 1 and 2 by demonstrating that superoxide overproduction in the RVLM of MI rats played a role in amplifying the sympathoexcitatory response to electrical stimulation of the MLR, and that antioxidant effects in the RVLM

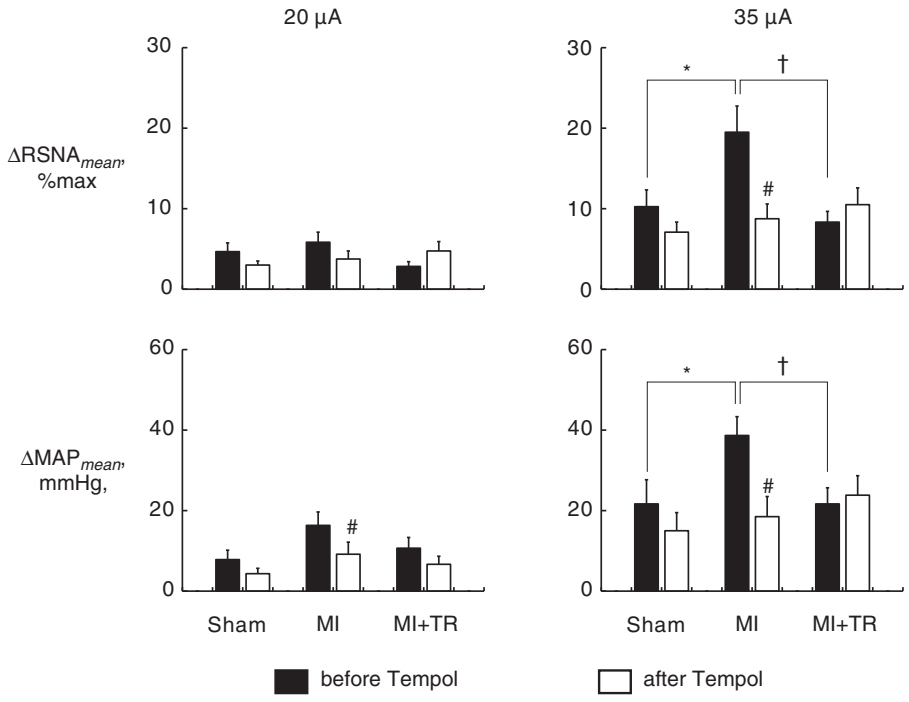

Figure 4. Mean changes from baseline in renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP)
Mean changes in RSNA and MAP from baseline ($\Delta RSNA_{mean}$ and ΔMAP_{mean}) during the 30 s periods of stimulation at 20 μA (left) or 35 μA (right). Filled and open bars represent data before and 30–40 min after, respectively, bilateral microinjection of Tempol into the rostral ventrolateral medulla. Test animals included Sham-operated rats (Sham; $n = 11$), rats with myocardial infarction (MI; $n = 12$), and MI and exercise-trained rats (MI + TR; $n = 10$). Values are means ± S.E.M. *$P < 0.05$, Sham *versus* MI; †$P < 0.05$, MI *versus* MI + TR; #$P < 0.05$ *versus* before Tempol injection. Significant differences were detected by Tukey's *post hoc* test following two-way repeated-measures ANOVA.

of MI + TR rats underlay the normalization of MLR stimulation-elicited responses. We suggest that oxidative stress in the medulla of CHF mediates central command dysfunction. We also suggest that exercise training in CHF is capable of normalizing central command-elicited sympathoexcitation through its antioxidant effects in the medulla.

Autonomic nervous system dysfunction in patients with CHF includes not only resting sympathetic overactivity (Leimbach *et al.* 1986), but also augmented

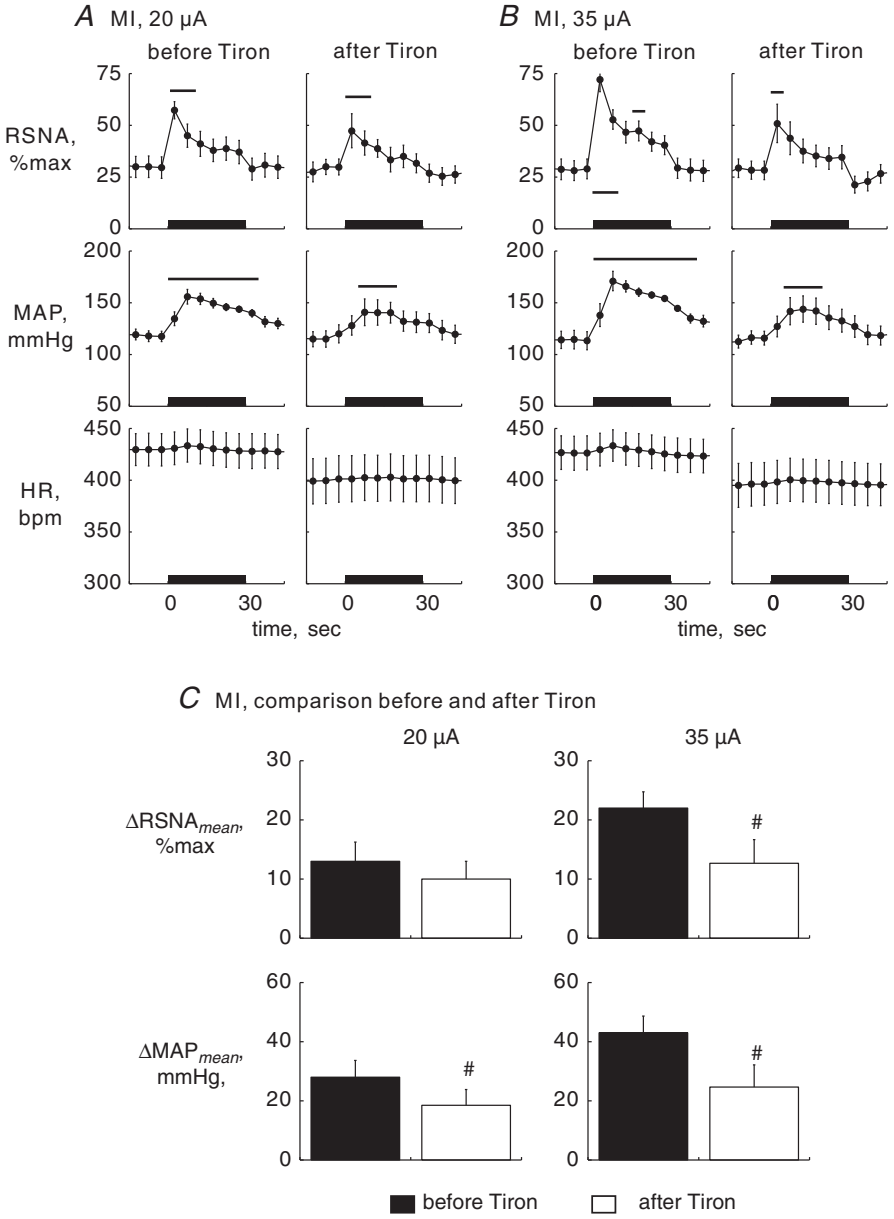

Figure 5. Effects of Tiron on renal sympathetic nerve activity (RSNA) and cardiovascular responses to electrical stimulation

Time course changes averaged for 5 s in $RSNA_{perMax}$, mean arterial pressure (MAP) and heart rate (HR) during 30 s of electrical stimulation of the mesencephalic locomotor region in rats with myocardial infarction (MI; $n = 7$) at 20 μA (*A*) or 35 μA (*B*) before and 30–40 min after bilateral microinjection of Tiron (10 mM, 92 nl) into the rostral ventrolateral medulla. Values are means ± S.E.M. Of note, abnormal values of HR caused by arrhythmia were discarded in this analysis. Thick horizontal bars located on the *x*-axis indicate the 30 s stimulation periods. Horizontal bars indicate significant ($P < 0.05$) differences from baseline values, detected by Dunnett's *post hoc* test following one-way repeated-measures ANOVA. *C*, $\Delta RSNA_{mean}$ and ΔMAP_{mean} at 20 μA (left) and 35 μA (right). Filled and open bars demonstrate the data before and after, respectively, bilateral injection of Tiron. #$P < 0.05$ *versus* before Tiron injection, paired *t* test.

sympathoexcitation during volitional exercise (Sterns *et al.* 1991; Negrão *et al.* 2001). It is suggested that central command dysfunction arising from oxidative stress in the medulla of CHF may contribute to the augmentation of sympathoexcitation during exercise. Moreover, supervised longterm exercise training in patients with CHF has been shown not only to decrease resting sympathetic overactivity (Roveda *et al.* 2003), but also to suppress the sympathoexcitatory response to handgrip exercise towards normal levels (Soares-Miranda *et al.* 2011). The present study suggests that central command function normalized by antioxidant effects in the medulla after exercise training of CHF may have a role in restoring abnormal sympathetic regulation during exercise in this disease.

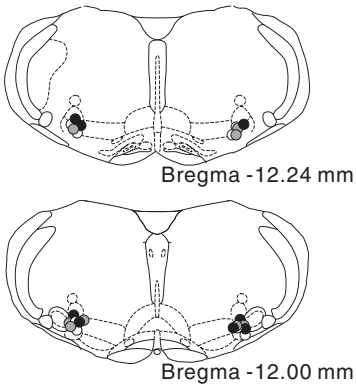

Figure 6. Distribution of injection sites
Injection sites in randomly chosen representative sham-operated rats (black circles), rats with myocardial infarction (MI; white circles), and MI and exercise-trained rats (MI + TR; grey circles) used in the Tempol experiments ($n = 4$ for each), mapped on standard sections from Paxinos & Watson (2007). Injection sites in other rats were located within the area of the rostral ventrolateral medulla shown.

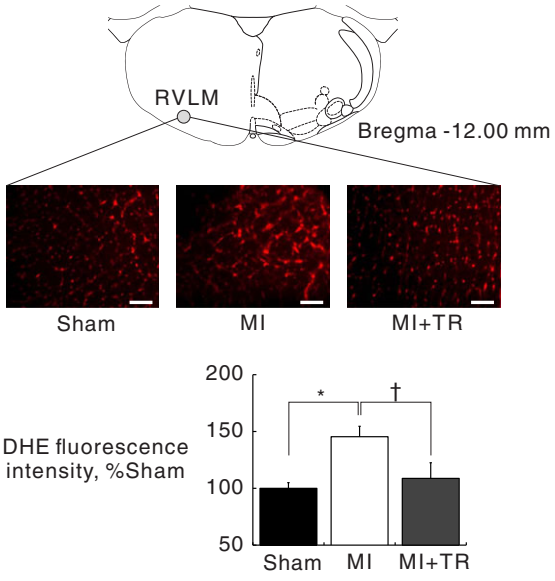

Figure 7. *In situ* superoxide detection in dihydroethidium (DHE)-treated brainstem slices
A, schematic section adapted from Paxinos & Watson (2007) showing sites of DHE intensity measurements in the rostral ventrolateral medulla (RVLM) and representative confocal images of the RVLM in sham-operated rats (Sham), rats with myocardial infarction (MI), and MI and exercise-trained rats (MI + TR). Scale bars: 100 μm. *B*, data are expressed as means ± S.E.M. for each group relative to Sham rats. $^*P < 0.05$, Sham *versus* MI; †$P < 0.05$, MI *versus* MI + TR. Significant differences were detected by Tukey's *post hoc* test following one-way ANOVA.

Sympathoexcitation during exercise is modulated by two principal neural mechanisms: central command, and a reflex originating in exercising skeletal muscle. The muscle-based reflex, termed the 'exercise pressor reflex', is activated by the increase in discharge of mechanically and metabolically sensitive skeletal muscle afferents caused by contraction (Kaufman & Hayes, 2002). Central command dysfunction in CHF has been suggested by human studies in which it was found to be augmented in patients with CHF in whom muscle metaboreceptor responsiveness was impaired in comparison with muscle sympathoexcitation seen during volitional handgrip exercise in healthy subjects (Sterns *et al.* 1991; Negrão *et al.* 2001). However, the human studies could not exclude the possibility that augmented sympathoexcitation during exercise in patients with CHF was not mediated by central command, but instead reflected the activation of the muscle mechanoreceptor reflex, which is accentuated in this disease (Middlekauff *et al.* 2001). The concept of central command dysfunction in CHF was subsequently supported by findings in our rat study, in which we found that rats with CHF displayed larger increases in renal and lumbar SNAs in response to electrical stimulation of the MLR after neuromuscular blockade than healthy rats (Koba *et al.* 2006*a*). Such experimental preparation allowed us to exclude the effects of muscle afferent engagement and enabled us to focus on roles for central command (Eldridge *et al.* 1985; Bedford *et al.* 1992). The current study utilized this preparation to test the present Hypotheses 1 and 2.

The present study proposes a novel role played by oxidative stress in the RVLM of CHF in central command dysfunction, as well as a role played by the antioxidant effects of exercise training in the RVLM of CHF in normalizing central command dysfunction. Previous studies have shown that oxidative stress in the RVLM has a role in resting sympathetic overactivity in pathological conditions. In conscious rabbits with CHF in which the RVLM was exposed to oxidative stress, resting sympathetic overactivity was reportedly decreased by acute intracerebroventricular infusion of Tempol (Gao *et al.* 2004) and suppressed towards normal levels by chronic intracerebroventricular infusion of an antioxidant simvastatin (Gao *et al.* 2005). Roles played by oxidative stress in the RVLM in stroke-prone spontaneously, obesity-induced and renovascular hypertensive rat models in resting sympathetic overactivity have

also been suggested (Kishi *et al.* 2004, 2004; Nishi *et al.* 2013). Further, molecular mechanisms by which oxidative stress is induced in the RVLM of CHF have been previously investigated. A series of studies by Gao *et al.* (2004, 2005, 2007) showed upregulation of NADPH oxidase subunits and downregulation of CuZnSOD and MnSOD in the RVLM of rabbits with CHF. The molecular basis that causes prooxidant effects in the RVLM of CHF may mediate not only resting sympathetic overactivity, but also central command dysfunction. Moreover, longterm exercise training in CHF rabbits has been demonstrated to lead to downregulation of NADPH oxidase subunits and upregulation of CuZnSOD and MnSOD (Gao *et al.* 2007). The molecular basis that causes antioxidant effects after training in the RVLM of CHF may be responsible for not only the decrease in resting SNA but also the normalization of central command dysfunction. However, the mechanisms underlying CHF-induced and training-induced protein expression changes in the brain remain unknown. Further studies are required to address this issue.

Angiotensin II (Ang II), which is increased in CHF, increases NADPH oxidase-derived superoxide production through stimulation of Ang II type 1 receptors (AT1R) in various tissues, including the brain (Bedard & Krause, 2007; Chan & Chan, 2012). Further, previous studies showed that AT1R expression was increased in the RVLM of CHF (Gao *et al.* 2004, 2005, 2007, 2008), and that AT1R stimulation upregulated NADPH oxidase subunits and vice versa (Bedard & Krause, 2007; Liu *et al.* 2008). Thus, increased renin angiotensin system (RAS) activity in the RVLM of CHF may contribute to central command dysfunction by causing superoxide overproduction. Moreover, exercise training in rabbits with CHF has been shown to suppress the increase in RAS activity in the RVLM (Mousa *et al.* 2008; Kar *et al.* 2010). This suppressive effect may be part of the normalization of central command dysfunction.

Mechanisms by which superoxide plays a role in exaggerating MLR stimulation-elicited sympathoexcitation must be elucidated. Experiments employing whole-cell configuration of the patch clamp technique applied to neuronal cells showed that NADPH oxidase-derived (Sun *et al.* 2005) and mitochondria-produced (Yin *et al.* 2010) superoxide inhibited voltage-gated potassium current. Moreover, NADPH oxidase-derived superoxide reportedly increased the intracellular concentration of Ca^{2+} by inducing an influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels in cultured neuronal cells (Wang *et al.* 2004; Zimmerman *et al.* 2005). Thus, superoxide is considered to play a role in sensitizing neuronal cells responding to excitatory input by regulating membrane ion channels. In the present study, superoxide overproduction in the medulla of MI rats may have modulated the functions of those ion channels located on RVLM sympathetic premotor neurones. In turn, the RVLM neurones stimulated by central command activation may have been sensitized, and thus sympathoexcitation in response to stimulation of the MLR may have been amplified in MI rats. Moreover, antioxidant effects in the RVLM of MI + TR rats may have played a role in restoring dysfunctions of these ion channels, thereby normalizing central command dysfunction.

Although resting SNA is overactive in CHF (Leimbach *et al.* 1986; Gao *et al.* 2004, 2005) and resting sympathetic overactivity in CHF is reduced by exercise training (Liu *et al.* 2000*b*; Roveda *et al.* 2003), the present results showed no difference in baseline RSNA among the Sham, MI and MI + TR rats, as reported previously (Koba *et al.* 2006*a*). In the experimental preparation, the hypothalamus, in which the paraventricular nucleus contains sympathetic premotor neurones, was removed for the decerebration. This procedure may buffer resting sympathetic overactivity in MI rats. Other research employing the decerebrate rat preparation showed no difference in resting RSNA between spontaneously hypertensive rats, which would have resting sympathetic overactivity, and normotensive rats (Mizuno *et al.* 2011).

In the present study, microinjection of Tempol into the RVLM decreased HR from baseline in MI rats at 30–40 min after its administration. Tiron mirrored this effect, although the result did not reach statistical significance. We speculate that the prolonged decrease from baseline HR after Tempol or Tiron administration may be attributable to their effects of increasing baroreflex sensitivity because an antioxidant treatment in the RVLM of rabbits with CHF reportedly improved baroreflex function (Gao *et al.* 2005).

The present findings identifying a cause of central command dysfunction in CHF suggest that an antioxidant treatment in central cardiovascular pathways may hold therapeutic potential for autonomic dysfunction during exercise in patients with this disease. In this regard, Deo *et al.* (2012) have reported that, in patients with CHF, 1 month of oral treatment with capsules of simvastatin, which crosses the blood–brain barrier, decreased resting sympathetic overactivity directed to skeletal muscle. Whether this treatment in patients with CHF will also normalize sympathetic regulation during a bout of exercise remains to be determined.

Several limitations should be noted in the interpretation of the findings of this study. Firstly, although we evaluated superoxide generation within the RVLM *in situ* by DHE staining, we did not measure it *in vivo*. Neither do we know how much oxidative stress *in vivo* was indeed reduced by antioxidant treatments with Tempol or Tiron microinjection into the RVLM of the same animal. An experimental approach to determine the amount of *in vivo* superoxide generation in rats is demanded for quantitative

evaluation of prooxidant/antioxidant effects within the medulla to modulate central command function. It is noted that transgenic mice expressing the oxidative stress indicator in which oxidative stress can be monitored *in vivo* are currently available (Oikawa *et al.* 2012).

Secondly, evidence has not been provided to support the speculation described above, that superoxide which mediates central command function might be produced, at least in part, by NADPH oxidases in Ang II signalling in the medulla. In order to examine this hypothetical mechanism, experiments which determine the effects on central command function of administration within the medulla of an AT1R blocker (e.g. losartan) and/or NADPH oxidase inhibitors such as apocynin are considered likely to provide some clues and should be conducted in the future.

Thirdly, although central command functionally interacts with baroreflex (Matsukawa, 2012), the present study did not examine sympathetic baroreflex sensitivity during stimulation of the MLR. Baroreflex sensitivity is attenuated in CHF and enhanced after exercise training in this disease (Liu *et al.* 2000*b*; Mousa *et al.* 2008). It is suggested that attenuated baroreflex sensitivity may contribute to the exaggeration of the MLR stimulation-elicited responses in MI rats and that enhanced baroreflex sensitivity after exercise training may be part of the normalization of the responses in MI + TR rats.

In conclusion, the present results demonstrate that superoxide overproduction in the RVLM of MI rats played a role in amplifying the sympathoexcitatory response to electrical stimulation of the MLR, and that exercise training alleviated superoxide overproduction in the medulla of MI rats, thereby suppressing MLR stimulation-elicited responses. We suggest that oxidative stress in the medulla of CHF mediates central command dysfunction. We also suggest that exercise training in CHF is capable of normalizing central command dysfunction through its antioxidant effects in the medulla.

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Additional information

Competing interests

None declared.

Author contributions

S.K. designed the research, performed the experiments, analysed the data, prepared the figures, interpreted the results, and drafted and edited the manuscript. I.H. and T.W. interpreted the results and edited the manuscript. All authors approved the manuscript. All experiments were performed in the Division of Integrative Physiology, Tottori University Faculty of Medicine.

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