



Augmented fear bradycardia in rats with heart failure

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Received: 14 November 2018 / Accepted: 23 July 2019 / Published online: 30 July 2019
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Abstract

In congestive heart failure (CHF), while resting parasympathetic activity becomes reduced, parasympathetically-mediated responses to stressors have not been described. This study aimed to (1) elucidate the effect of CHF on fear bradycardia, a parasympathetically-mediated response, and (2) examine if brain oxidative stress of CHF mediates fear bradycardia. White noise sound (WNS) exposure to conscious rats induced freezing behavior and elicited bradycardia. WNS exposure-elicited bradycardia was greater in rats with CHF than in controls. Superoxide dismutase mimetics administered in the lateral/ventrolateral midbrain periaqueductal gray (l/vIPAG), a region that contributes to the generation of fear bradycardia, had no effect on the bradycardia response in control and CHF rats. Dihydroethidium staining in situ showed that superoxide generation in the l/vIPAG of CHF rats was increased as compared to controls. These results demonstrate that CHF leads to the augmentation of fear bradycardia. Moreover, oxidative stress in the l/vIPAG of CHF unlikely mediates the augmented fear bradycardia.

Keywords Fear · Heart failure · Heart rate · Parasympathetic nervous system · Midbrain periaqueductal gray

Introduction

Fear is a common emotion that involves the intense anticipation of threat or potential danger and elicits characteristic patterns of defensive behaviors including freezing [1, 2]. Rodent studies have shown that the freezing or immobility is associated with heart rate (HR) deceleration [3, 4], which is mediated via increased parasympathetic outflow to the heart [5].

Congestive heart failure (CHF) is characterized by the steady inability of the myocardium to sufficiently deliver oxygenated blood to meet the metabolic demands in peripheral tissues. In this lethal disease, while sympathetic nerve activity is enhanced at rest [6] as well as in response to stressors in daily lives such as exercise [7, 8], resting parasympathetic control of the heart becomes reduced as assessed by HR variability or effects of atropine administration [9–11].

Such autonomic imbalance is a factor which increases the mortality in patients with CHF [12], and the parasympathetic nervous system has attracted a considerable attention as a possible therapeutic target for the treatment of CHF [11, 13]. Nevertheless, while characteristics and mechanisms of sympathetic hyperactivity in CHF has been extensively studied, relatively less research attention has been paid to abnormal regulation of parasympathetic activity. Specifically, parasympathetically mediated responses to stressors such as fear have not been described. It is, therefore, demanded to elucidate the parasympathetic nervous system pathology that may be present during stressors in CHF as well as mechanisms that lead to the abnormalities.

Oxidative stress is induced in the brain of CHF. Antioxidant treatments within central cardiovascular pathways of CHF alleviate sympathetic hyperactivation at rest [14, 15] and in response to stimulation of the midbrain [16]. In contrast, whether antioxidant treatments in the brain of CHF can ameliorate parasympathetic dysfunction has been undetermined. We recently reported that excitation of neurons in the lateral and ventrolateral part of the midbrain periaqueductal gray (l/vIPAG) drives parasympathetically mediated fear bradycardia, at least in part [5]. Thus, antioxidant treatments in the l/vIPAG of CHF may modify fear bradycardia.

The present study aimed to elucidate the effect of CHF on fear bradycardia and the effect of antioxidant treatments

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in the l/vIPAG on fear bradycardia in CHF. We compared arterial pressure (AP) and HR responses to the exposure of white noise sound (WNS), that induces freezing behavior [5, 17], between rats with CHF after myocardial infarction by left coronary artery ligation and sham-operated control rats. We also examined the effect of microinjection of superoxide dismutase (SOD) mimetics into the l/vIPAG on WNS exposure-elicited cardiovascular changes in control and CHF rats. Moreover, in situ superoxide production in the rat l/vIPAG was examined to assess oxidative stress.

Materials and methods

The experiments were performed on male Sprague–Dawley rats. Rats were housed in standard rodent cages in a temperature-controlled room (24–25 °C) and were regulated on a 12:12 h light–dark schedule. Food and water were made available ad libitum.

Coronary artery ligation surgery and echocardiography

Coronary artery ligation surgery to induce myocardial infarction in rats was conducted as described previously [16]. Briefly, rats ($n = 57$, aged 5–7 weeks, 165–232 g of body weight) were anesthetized with a mixture of isoflurane (1.5–5%) and oxygen, intubated and mechanically ventilated with a respirator (SN-480-7, Shinano, Tokyo, Japan). The left ventricular wall was exposed through left thoracotomy performed between the fourth and fifth ribs. The left coronary artery was then ligated with 6-0 nylon suture to induce chronic myocardial infarction ($n = 39$). Sham operations without ligation of the artery were also performed in another subset of rats (Sham rats, $n = 18$). The thorax was closed, the tracheal tube was removed, and the rat was allowed to recover from anesthesia. Four of the 39 ligated rats died by the next day of the surgery while the other rats survived till the conclusion of the studies.

Transthoracic echocardiography (model 5189002, GE Healthcare Ltd, Little Chalfont, UK) to assess left the ventricular function of anesthetized rats with 1.5% isoflurane and oxygen was performed more than 7 weeks after ligation (12.2 ± 0.7 weeks, mean \pm SEM) or sham (14.5 ± 1.3 weeks) surgery. The echocardiographic data are shown in Table 1. All of the rats that underwent ligation and survived met the present criteria for CHF [i.e., left ventricular fractional shortening (FS) $< 35\%$] while all of Sham rats had $> 40\%$ of FS. Six of the 18 Sham rats and 6 of the 35 CHF rats were used for experiments to evaluate in situ superoxide production in the PAG. The remaining 12 Sham and 29 CHF rats were used for experiments to observe cardiovascular changes in response to WNS exposure.

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

	Sham	CHF
<i>N</i>	18	35
Body weight (g)	496 \pm 21	460 \pm 10
Heart weight/body weight (mg/g)	3.3 \pm 0.1	4.3 \pm 0.2*
Left ventricular end-diastolic diameter (mm)	7.9 \pm 0.2	10.3 \pm 0.2*
Left ventricular end-systolic diameter (mm)	4.2 \pm 0.2	8.4 \pm 0.2*
Fractional shortening (%)	47.6 \pm 1.0	18.3 \pm 0.9*

Values of body and heart weights presented here were collected immediately prior to animal sacrifice. Values are mean \pm SEM

Sham sham-operated control rats, *CHF* rats with congestive heart failure

* $P < 0.05$ vs. Sham, detected by two-sample *t* tests

In situ superoxide production in the rat midbrain

In 6 Sham and 6 CHF rats, intracellular superoxide production in the PAG was evaluated with dihydroethidium (DHE) staining as previously described [16]. On the experimental day, the brains were quickly removed from two sets of one Sham and one CHF rats that were deeply anesthetized with 5% isoflurane in an oxygen air mixture, embedded in optimal cutting temperature compounds, and cryostat-sectioned coronally (35 μ m thickness). Two sections obtained from each rat brain were immediately incubated with DHE (1 μ M, 37291, Sigma–Aldrich, St. Louis, MO, USA) in the dark for 30 min at 37 °C. Digital images of red-fluorescent ethidium, which result from the oxidation of DHE, were then obtained at randomly selected sites in the dorsolateral, lateral, and ventrolateral PAG (dIPAG, lPAG, and vIPAG) with a 40 \times objective lens under a fluorescence microscope (BZ-900, Keyence, Osaka, Japan). The PAG subdivisions were approximated using the atlas of Paxinos and Watson [18]. The RGB confocal images were loaded into ImageJ (NIH, Bethesda, MD, USA), and converted to 8-bit gray scale. Fluorescence intensity in each subdivision was then measured. For each sample, an average fluorescence intensity was obtained from bilateral measurements in each subdivision of the PAG from the two sections. The heart was also harvested and weighed.

Surgeries for cannula, catheters and electrode implantations

In 12 Sham and 27 of the 29 CHF rats, the effect of antioxidant treatments in the midbrain on cardiovascular responses to WNS exposure was examined. The surgery to implant a guide cannula was conducted in accordance with previous studies [5, 19]. Briefly, the rats anesthetized with

a mixture of 1.5–5% isoflurane in oxygen were intubated, mechanically ventilated, and placed in a stereotaxic apparatus (900LS, David KOPF, Tujunga, CA, USA, or SR-6R, Narishige, Tokyo, Japan). The target coordinates for the l/vIPAG were set at 7.5 mm posterior, 0.7 mm lateral, and 5.3–5.5 mm ventral relative to the bregma. A small burr holes were made in the skull. The guide cannulas (Plastic One, Anaheim, CA, USA) were secured by two screws and dental acrylic. Dummy cannulas were inserted to the guides.

Following more than 12 days of recovery (21.0 ± 1.1 days) after the cannula implantation, the rats were processed to the next surgery to chronically implant arterial and venous catheters and an electrode for ECG measurements, as conducted previously [5, 17]. In rats anesthetized with 1.5–5% isoflurane and oxygen, the arterial catheter for AP measurements was implanted via the tail artery on the ventral side, and the catheter tip was placed approximately 10 mm above the aortoiliac bifurcation. The venous catheter for drug infusion was implanted via the right jugular vein, and the catheter tip was placed at the junction of the superior vena cava and right atrium. These catheters were filled with heparinized saline. The bipolar electrode was implanted under the skin at mid-chest level. The catheters and electrode were exteriorized between the ears and protected by a 150 mm coiled spring.

Other two CHF rats were subjected to another experiment to examine the effect of atropine infused intravenously on cardiovascular responses to WNS exposure. The surgeries to implant catheters and the electrode were likewise conducted.

During the recovery periods after the surgeries described above, the rats received daily check if any of them would display signs of inflammation, discomfort, anxiety, or pain. Any rats that would show such sign would have been excluded from the study, and humanely sacrificed. As a result, nevertheless, none of them needed to be excluded.

Observations of arterial pressure (AP) and HR responses to WNS exposure in conscious rats

At least 72 h were allowed for recovery after the catheters and electrode implantations before the first data collection. In the experiment to examine the effect of antioxidant treatments in the midbrain on cardiovascular responses to WNS exposure, each rat was subjected to two different trials, separated by 3 days apart in which bilateral microinjection of either normal saline (first experimental day) or a cell membrane-permeable superoxide scavenger compound [4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) or 4,5-dihydroxyl-1,3-benzenedisulfonic acid (Tiron)] diluted in saline (second experimental day) was followed by WNS exposure as conducted previously [5, 17]. We previously confirmed the antioxidant effects of either Tempol or Tiron in the rat brain tissue [16]. On the day of the

experiment, the rats in their home cages were brought to the experimentation room, in which the temperature was set at 24–25 °C and the ambient noise level was less than 65 dB. The cage was covered with a blackout curtain with a small window to observe rat behavior. The arterial catheter was attached to a pressure transducer (P23XL, Beckton Dickinson, Newark, DE, USA). The electrode was connected to an AC amplifier (P511, Grass, Warwick, RI, USA) to amplify the ECG signal. Noise intensity around the cage was measured by a sound level meter (TM102, Sato Shoji, Kawasaki, Japan). More than one hour was allowed to pass before the conscious rats ($n = 12$ for Sham and $n = 13$ for CHF rats) received bilateral microinjection into the l/vIPAG of saline (100 nl) or Tempol solution (10 mM diluted in 100 nl saline, 176,141, Sigma-Aldrich). Thirty-to-forty min after microinjection, the conscious rats were exposed to WNS at 90 dB for 5 min. In another subset of CHF rats ($n = 14$), saline (100 nl) or Tiron solution (200 mM diluted in 100 nl saline, 172,553, Sigma-Aldrich) was bilaterally microinjected into the l/vIPAG. In this group, 5-min WNS at 90 dB was applied at 5–15 and 30–40 min after saline or Tiron microinjection. The dosages of Tempol or Tiron solution and the time interval of 30–40 min after microinjection were chosen on the basis of a previous study, demonstrating that prior microinjection of either Tempol or Tiron (10 mM diluted in 100 nl saline) into the rostral ventrolateral medulla of rats with CHF after myocardial infarction significantly reduced renal sympathoexcitatory response during fictive locomotion, and that administration of Tempol on brain sections attenuated intracellular superoxide production in the rostral ventrolateral medulla, as evaluated by DHE staining *in situ* [16]. Moreover, Mayorov et al. [20] demonstrated that 20 nmol of either Tempol or Tiron microinjected bilaterally into the rostral ventrolateral medulla of conscious rabbits significantly attenuated pressor and tachycardic responses to air-jet stress. Therefore, in the present study, the dosage of Tempol was equivalent with that used in the previous studies, and the dosage of Tiron was ten-to-twenty times greater than that used in the previous studies [16, 20]. Throughout the protocol, the rat behavior was observed by an investigator through a small window in the curtain covering the cage. After all the observations had been conducted, the rats were deeply anesthetized with 4–5% isoflurane in an oxygen air mixture, and microinjection sites were marked with India ink for histological verification. Then, the rats were humanely euthanized via an intravenous infusion of 1 ml saturated potassium chloride solution, and their brains were removed and sectioned coronally using a cryostat (Leica CM1900, Wetzlar, Germany) to verify the sites in which India ink had been microinjected. Injection sites were approximated using the atlas of Paxinos and Watson [18]. The heart was also harvested and weighed.

In the other two CHF rats, either saline (0.2 ml) or atropine solution (0.1 mg diluted in 0.2 ml saline, A0132, Sigma) [5] was intravenously infused. Five-to-ten min later, cardiovascular responses to WNS exposure were observed as stated above. Saline and atropine trials of each rat were separated by 3 days apart.

Data analysis and statistics

In experiments to observe cardiovascular changes associated with WNS exposure, 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). HR was calculated beat to beat with detection of the time between successive R waves in the ECG. Baseline data were obtained from 5 min averaged values immediately prior to the WNS exposure. HR changes due to sinus arrest and/or escape beats, which frequently occurred during WNS exposure in CHF rats (Fig. 2b), were not included in the present analyses.

Data are expressed as mean \pm SEM. Statistical significances were assessed with a paired *t* test, two-sample *t* test, or one- or two-way repeated-measures analysis of variance (ANOVA) followed by the Dunnett's or Tukey's post hoc test. The analytic test for the figure presentation is described in the relevant legends. The level of significance was set at $P < 0.05$.

Results

In situ superoxide detection in dihydroethidium-treated midbrain slices

Within the subdivisions of the PAG (dIPAG, IPAG, and vIPAG), ethidium fluorescence was significantly enhanced in CHF rats ($n = 6$) compared with that in Sham rats ($n = 6$) (Fig. 1).

Microinjection sites of rats exposed to WNS exposure

Guide cannulas were implanted bilaterally in the brain of 12 Sham and 27 CHF rats. In 7 of the 12 Sham and 16 of the 27 CHF rats, the tips of cannulas for microinjection were located within the l/vIPAG at 7.0–8.0 mm caudal to the bregma, as dyed post hoc by microinjected India ink (Fig. 2a). In the remaining 5 Sham or 11 CHF rats, the cannula tips were located outside of the l/vIPAG (Fig. 2a).

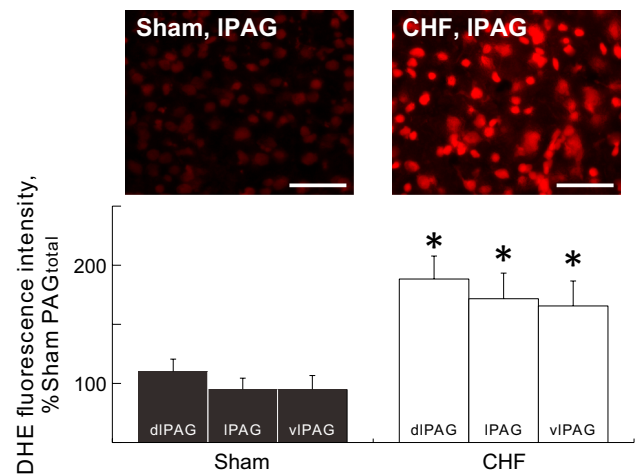


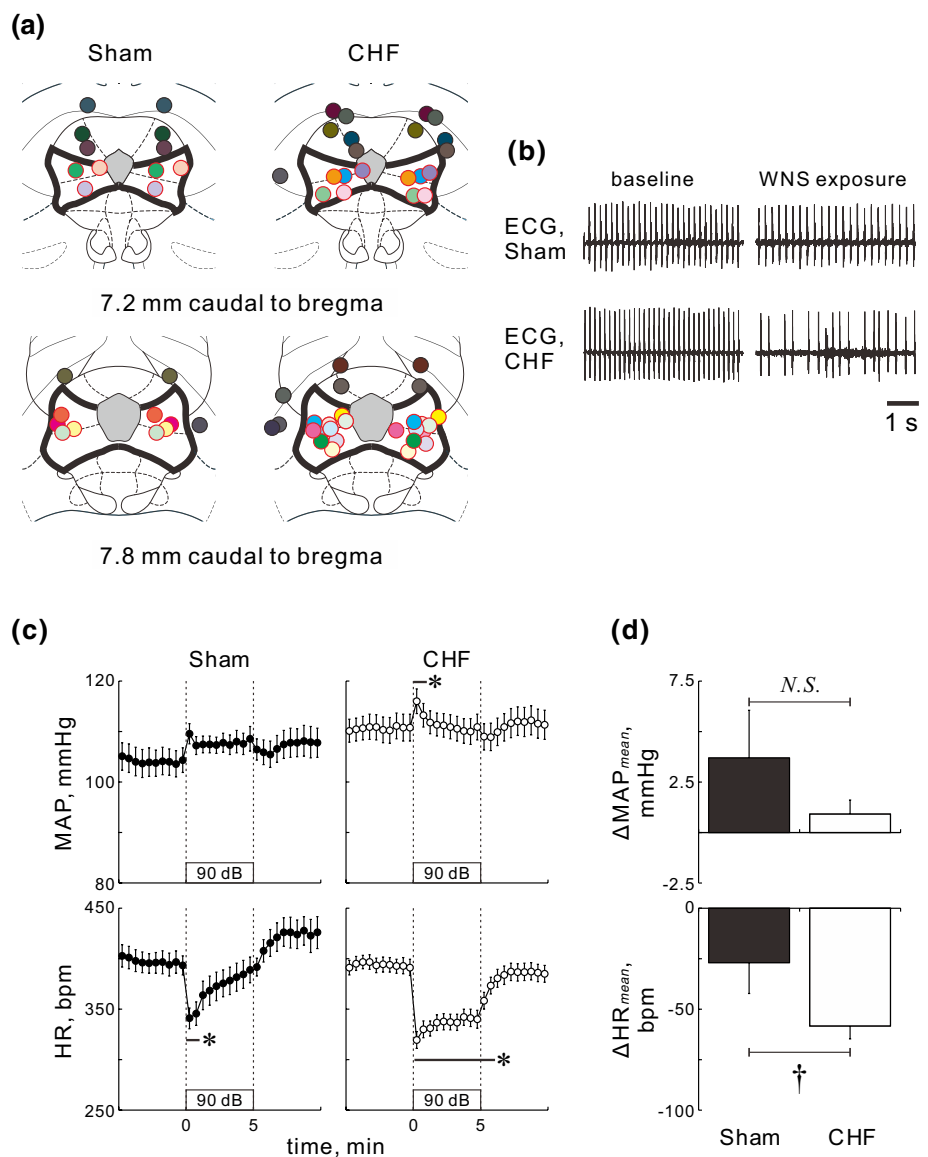
Fig. 1 Top. Examples of confocal images of DHE fluorescence taken in the PAG of sham-operated (Sham) and congestive heart failure (CHF) rats. Scale bars: 50 μ m. Bottom. Comparisons of DHE fluorescence intensities in the dorsolateral, lateral, and ventrolateral parts of the PAG (dIPAG, IPAG, and vIPAG) between 6 Sham and 6 CHF rats. Values represent mean \pm SEM. * $P < 0.05$ vs. Sham rats, detected by two-sample *t* tests

WNS exposure-elicited cardiovascular responses in conscious rats: effect of CHF

To assess the impact of CHF on fear bradycardia, we compared mean AP (MAP) and HR changes in response to 5-min WNS exposure between 12 Sham and 27 CHF conscious rats at 30–40 min after the microinjection of saline into the brain irrespective of the location of the cannula tips. Baseline MAP and HR before WNS exposure were not significantly different between Sham (104 ± 3 mmHg and 396 ± 10 beats/min) and CHF rats (109 ± 2 mmHg and 384 ± 9 beats/min). While WNS exposure was applied to CHF rats, sinus arrest and escape beats occurred frequently (Fig. 2b). In the present analyses of HR, data during sinus arrest and escape beats were excluded. In the following figures, therefore, HR changes due to sinus arrest or escape beats are not expressed. In Sham rats, WNS exposure did not change MAP, but significantly ($P < 0.05$) decreased HR (Fig. 2c). WNS exposure to CHF rats significantly elevated MAP and reduced HR (Fig. 2c). There was no significant difference in MAP changes during WNS exposure between Sham and CHF rats ($P = 0.16$) while the bradycardia response in CHF rats was significantly ($P = 0.03$) greater than that in Sham rats (Fig. 2d).

In other two CHF rats, to examine whether WNS exposure-elicited bradycardia in CHF rats was parasympathetically mediated, cardiovascular parameter changes were studied during WNS exposure 5–10 min after intravenous infusion of saline or atropine solution. In both rats, atropine administration eliminated the response to a great extent by

Fig. 2 a Microinjection sites mapped on standard sections using the atlas of Paxinos and Watson [16] in 12 Sham and 27 CHF rats. These sites were marked with injected Indian ink after data collection. The areas outlined with thick lines represent the lateral/ventrolateral periaqueductal gray (l/vIPAG). In the l/vIPAG at -7.8 mm caudal to the bregma for CHF rats, successful microinjection sites of 2 CHF rats were hidden behind the sites presented. **b** Examples of electrocardiogram tracings obtained from one Sham rat and one CHF rat before and during white noise sound (WNS) exposure. **c** Thirty-second averaged time courses of mean arterial pressure (MAP) and heart rate (HR) while conscious, free-moving Sham (black circle) and CHF (white circle) rats were exposed to 5 min of WNS at 90 dB 30–40 min after microinjection of saline into the brain, irrespective of microinjection site. Values are mean \pm SEM. $*P < 0.05$, vs. baseline, detected by the Dunnett's post hoc test following one-way repeated-measures ANOVA. **d** Comparisons of 5 min averaged changes from baseline in MAP and HR during WNS exposure between Sham and CHF rats. $\dagger P < 0.05$, Sham vs. CHF. *NS* no significant differences between Sham and CHF rats. The statistic differences were examined by two-sample *t* tests



42 and 121%, respectively, as compared to that after saline administration (-73 and -43 beats/min, as assessed by 5-min averaged changes in HR from baseline). This result suggests that a large portion of bradycardia in response to WNS exposure in CHF rats is mediated through parasympathetic outflow.

WNS exposure-elicited cardiovascular responses: effect of Tempol microinjection into the l/vIPAG of Sham and CHF rats

Twelve Sham and 13 CHF rats received microinjection of either saline or Tempol into the brain prior to WNS exposure. In 7 of the 12 Sham and 8 of the 13 CHF rats, the cannula tips were confirmed to be located within the l/vIPAG (Fig. 2a). In these rats, the effect of bilateral microinjection

of Tempol into the l/vIPAG on WNS exposure-elicited AP and HR responses was examined. Baseline MAP and HR at 30–40 min after Tempol microinjection were not significantly different from those after saline microinjection in Sham or CHF rats (Table 2). In both rat groups, WNS exposure did not change MAP but significantly reduced HR after microinjection of saline or Tempol into the l/vIPAG (Fig. 3a, c). The bradycardia responses observed during WNS exposure after Tempol microinjection were not significantly different from that after saline microinjection in either Sham ($P = 0.16$) or CHF ($P = 0.48$) rats (Fig. 3b, d).

Table 2 Baseline mean arterial pressure (MAP) and heart rate (HR) of Sham and CHF rats after bilateral microinjection of saline or Tempol into the lateral/ventrolateral periaqueductal gray (l/vIPAG)

	Sham		CHF	
	30–40 min after saline	30–40 min after Tempol	30–40 min after saline	30–40 min after Tempol
N	7		8	
MAP (mmHg)	105 ± 3	99 ± 1	101 ± 2	100 ± 1
HR (beats per min)	397 ± 13	372 ± 14	387 ± 17	398 ± 23

Basal data were obtained from 5 min averaged values immediately prior to 5 min of white noise sound exposure. Values are mean ± SEM. Paired *t* tests to assess the statistical differences in basal values within each rat group found no significant effect of Tempol in the mediation of baseline MAP or HR

WNS exposure-elicited cardiovascular responses: effect of Tiron microinjection into the l/vIPAG of CHF rats

Fourteen CHF rats received saline or Tiron microinjection into the brain bilaterally and were then exposed to 5-min WNS at 5–15 and 30–40 min after microinjection. In 8 of the 14 CHF rats, the cannula tips were located within the l/vIPAG as post hoc confirmed (Fig. 2a). In the 8 CHF rats, baseline MAP after Tiron microinjection was significantly lower than that after saline microinjection while baseline HR did not differ between saline and Tiron microinjections (Table 3). Although WNS exposure at 5–15 min after saline microinjection, but not 30–40 min after saline microinjection or 5–15/30–40 min after Tiron microinjection, significantly elevated MAP (Fig. 4a), MAP changes in response to WNS exposure did not differ between the protocols (Fig. 4b). WNS exposure-elicited bradycardia responses after Tiron microinjection were not significantly different from those after saline microinjection, irrespective of time intervals after solution administration (Fig. 4a, b).

Discussion

As DHE staining in situ assessed, oxidative stress was induced in the PAG of CHF rats. The present study found that WNS exposure-elicited bradycardia was significantly enhanced in CHF rats compared with that in Sham rats, indicating that CHF leads to an augmentation of fear bradycardia. Moreover, bilateral microinjection of Tempol into the l/vIPAG of either Sham or CHF rats had no effect on WNS exposure-elicited bradycardia. Tiron treatment of the l/vIPAG in CHF rats also did not reduce the bradycardia. These observations suggest that oxidative stress in the l/v

vIPAG of CHF unlikely plays a role in augmenting fear bradycardia.

Bradycardia in response to WNS exposure in conscious rats is principally mediated through parasympathetic outflow as the bradycardia response was abolished by systemic administration of atropine in healthy controls [5]. Atropine administered in CHF rats also eliminated the bradycardia response to a large extent. Therefore, the enhanced bradycardia response to WNS exposure in CHF rats suggests parasympathetic hyperresponsiveness to fear, leading us to hypothesize that parasympathetic responses to stressors that are present in daily lives become abnormal in this pathological condition.

As frequent occurrences of arrhythmia were associated with the augmented bradycardia during WNS exposure in CHF rats (Fig. 2b), it is clinically important to elucidate mechanisms underlying parasympathetic hyperresponsiveness to fear in CHF. While central mechanisms underlying sympathetic hyperactivity in CHF has been extensively studied [21, 22], there is scarce information available regarding the effect of CHF on central mechanisms regulating parasympathetic outflows. A study reported that preganglionic parasympathetic neuronal cells were reduced in the nucleus ambiguus and dorsal vagal motor nucleus in rat medulla with CHF when compared with those in healthy controls, explaining the decreased resting parasympathetic tone [23]. Supraspinal cardiovascular regulatory mechanisms may also be involved in parasympathetic hyperresponsiveness to stressors including fear in CHF. Nevertheless, as the present study did not evidence an involvement of oxidative stress in the l/vIPAG, further studies are necessary to examine whether, and if so how, central nervous system contributed to the augmented fear bradycardia in CHF rats.

Besides central nervous system, the process responsible for parasympathetic control of the heart involves muscarinic cholinergic receptor mechanisms in the sinus node. In the sinus node of CHF, muscarinic receptors are reportedly upregulated [24, 25], muscarinic receptor-mediated regulation of the sinus node is upregulated [25], and acetylcholinesterase activity is diminished [24]. These abnormalities in the sinus node might underlie enhanced parasympathetic control of the heart during fear, thereby causing the augmentation of the bradycardia response as well as frequent occurrences of sinus arrest observed in CHF rats.

It should be noted that cardiac β 1 adrenoreceptors are reportedly desensitized in CHF [26]. Therefore, the lower sympathetic responsiveness to WNS might contribute to the delayed recovery of the HR from the bradycardia, thereby contributing to the augmentation of fear bradycardia in CHF rats. How much the lower sympathetic responsiveness in CHF was involved in the augmented fear bradycardia remains to be investigated.

Fig. 3 **a, c** Thirty-second averaged time courses of MAP and HR while conscious, free-moving Sham (**a**, $n=7$) and CHF (**c**, $n=8$) rats, of which microinjection sites were located within the l/vIPAG, were exposed to 5-min of WNS at 90 dB. At 30–40 min prior to the onset of WNS exposure, saline (100 nl) or Tempol (10 mM diluted in 100 nl saline) was bilaterally microinjected into the l/vIPAG. Values are mean \pm SEM. $*P < 0.05$, vs. baseline, detected by the Dunnett’s post hoc test following one-way repeated-measures ANOVA. **b, d** Comparisons of 5 min averaged changes from baseline in MAP and HR between saline and Tempol microinjections into the l/vIPAG in the Sham (**b**) and CHF (**d**) rats. *NS* no significant differences between saline and Tempol microinjections, examined by paired *t* tests

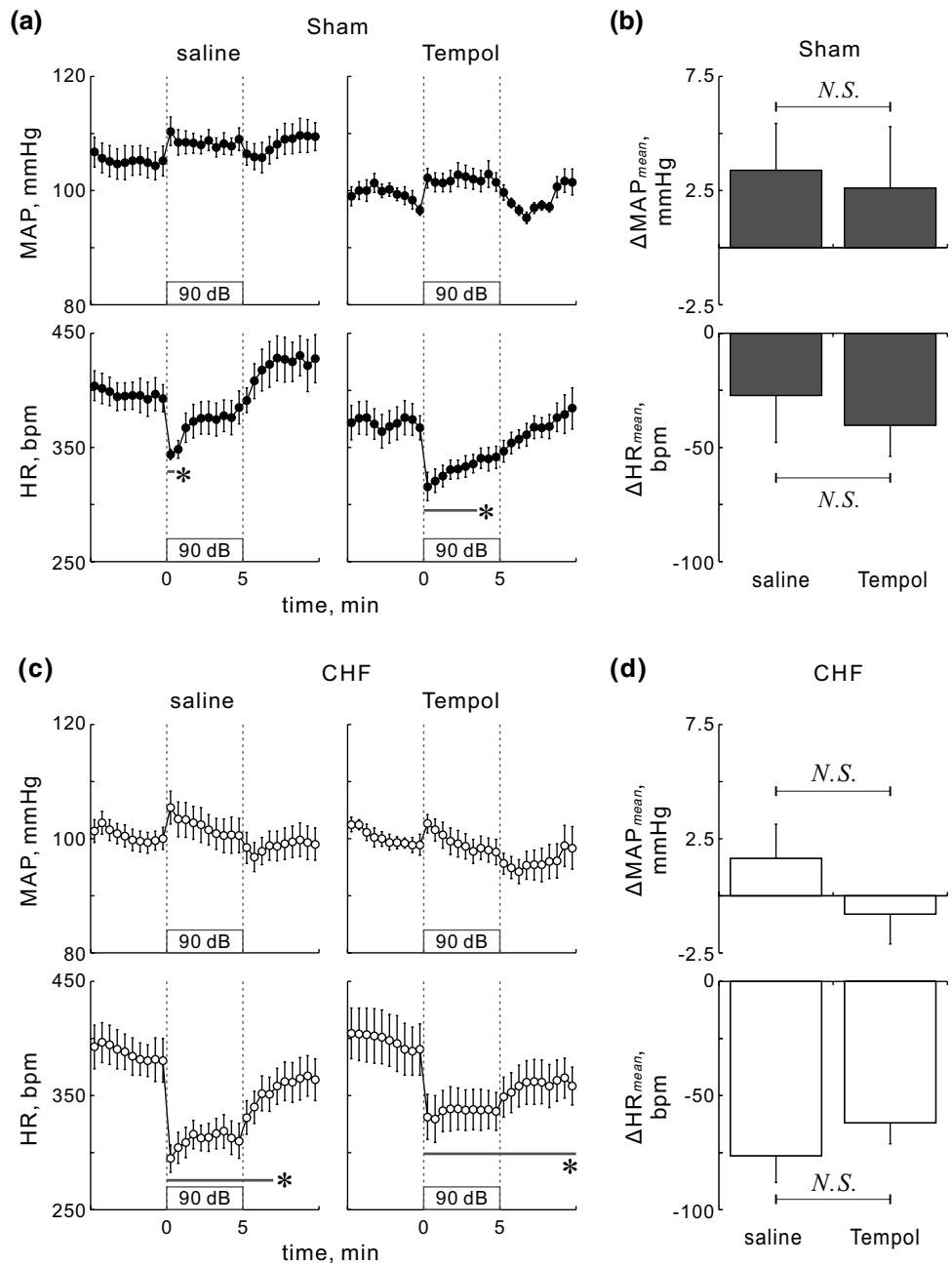


Table 3 Baseline MAP and HR of 8 CHF rats prior to white noise sound exposure starting after bilateral microinjection of saline or Tiron into the l/vIPAG

	5–15 min after saline	5–15 min after Tiron	30–40 min after saline	30–40 min after Tiron
MAP (mmHg)	108 \pm 3	93 \pm 3*	110 \pm 3	98 \pm 5*
HR (beats per min)	415 \pm 26	373 \pm 14	397 \pm 11	398 \pm 18

Values are mean \pm SEM

$*P < 0.05$ vs. saline at the corresponding time interval after administration, detected by two-way repeated-measures ANOVA followed by the Tukey’s post hoc test

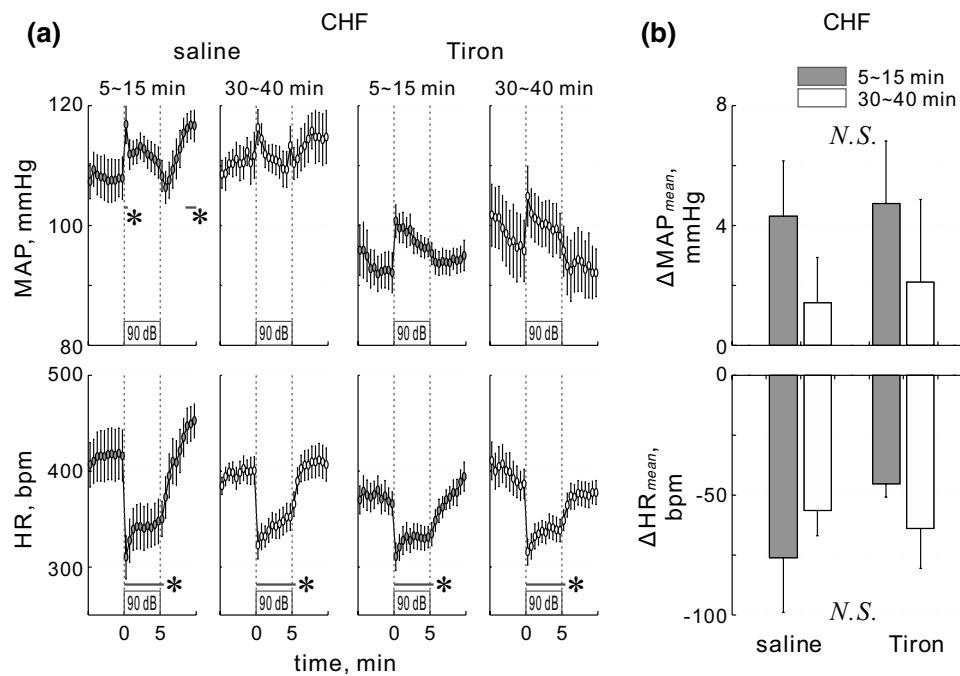


Fig. 4 **a** Thirty-second averaged time courses of MAP and HR while conscious, free-moving CHF ($n=8$) rats, of which microinjection sites were located within the l/vIPAG, were exposed to 5-min of WNS at 90 dB at 5–15 or 30–40 min after bilateral microinjection into the l/vIPAG of saline (100 nl) or Tiron (200 mM diluted in 100 nl saline). Values represent mean \pm SEM * $P < 0.05$, vs. baseline, detected by the Dunnett's post hoc test following one-way repeated-measures

ANOVA. **b** Comparisons of 5 min averaged changes from baseline in MAP and HR during WNS exposure between saline and Tiron microinjections into the l/vIPAG and between 5–15 and 30–40 min after microinjection in the CHF rats. NS no significant differences between saline and Tiron microinjections and between 5–15 and 30–40 min after administration, examined by two-way repeated-measures ANOVA followed by the Tukey's post hoc tests

In conclusion, the present study demonstrated that fear bradycardia becomes augmented in rats with CHF, suggesting parasympathetic hyperresponsiveness to fear. The study also showed that antioxidant treatments with Tempol or Tiron in the l/vIPAG, a brain region that plays a role in generating fear bradycardia, did not modify fear bradycardia in CHF. Further studies are demanded to elucidate mechanisms underlying parasympathetic hyperresponsiveness to fear in CHF.

Acknowledgements We thank Yumiko Inoue for assistance in rat echocardiography, and Ryo Inoue for assistance in rat coronary artery ligation surgery. This study was financially supported by JSPS KAKENHI 26670112 (to SK) and 15H05367 (to SK).

Author contributions SK designed research, performed experiments, analyzed the data, prepared the figures, interpreted the results, and drafted, edited, and revised the manuscript. IH and TW interpreted the results and revised the manuscript critically for important intellectual content.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests and no relationship that may lead to any conflict of interest.

Ethical approval All procedures outlined in this 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 (Reference number: 13-Y-47).

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