Reactive oxygen species in the paraventricular nucleus mediate the cardiac sympathetic afferent reflex in chronic heart failure rats

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Abstract

The aim of this study was to determine whether reactive oxygen species (ROS) in the paraventricular nucleus (PVN) mediate both the cardiac sympathetic afferent reflex (CSAR) and angiotensin II-induced CSAR enhancement in chronic heart failure (CHF) rats. CSAR was evaluated from the responses of renal sympathetic nerve activity (RSNA) to epicardial application of bradykinin. In both CHF and sham-operated rats, PVN microinjection of the superoxide anion scavengers tempol or tiron almost abolished the CSAR, but the superoxide dismutase inhibitor DETC potentiated the CSAR. PVN pretreatment with tempol or tiron abolished, whereas DETC augmented, the angiotensin II-induced CSAR enhancement. In CHF rats, superoxide anion and malondialdehyde (MDA) levels in the PVN were increased, but were normalized by the AT₁ receptor antagonist losartan. PVN microinjection of tempol decreased superoxide anion and MDA levels, but epicardial application of bradykinin or PVN microinjection of angiotensin II increased superoxide anion and MDA to higher levels in CHF rats than in sham-operated rats. These results indicate that ROS in the PVN mediates the CSAR and the effect of angiotensin II in the PVN on the CSAR in both CHF and sham-operated rats. Increased ROS in the PVN are involved in the enhanced CSAR in CHF.

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1. Introduction

It is well known that sympathetic activity is enhanced in chronic heart failure (CHF). This enhanced sympathetic activity contributes to further haemodynamic deterioration and the degree of sympathoexcitation is prognostic for survival in this disease [1,2]. In CHF, the cardiac sympathetic afferent reflex (CSAR) is over-activated and contributes to the increases in sympathetic outflow [3–5]. Inhibition of this enhanced CSAR to reduce the over-excitation of the sympathetic nervous system may be beneficial in CHF.

The paraventricular nucleus (PVN) is an important integrative centre for the control of cardiovascular activity and sympathetic outflow. In a previous study we reported that Ang II and AT₁ receptors in the PVN play an important role in the central modulation of CSAR, and contribute to the pathogenesis of enhanced CSAR in CHF [5–10]. Furthermore, microinjection of the GABA A receptor agonist muscimol into the PVN has been shown to attenuate the CSAR induced by epicardial application of bradykinin (BK) [11]. Another study reported an increase in c-Fos immunoreactive cells in the PVN after epicardial application of BK in cats [12].

It has been reported that reactive oxygen species (ROS) are novel molecules involved in the intracellular signalling mechanisms of Ang II in the brain [13,14]. We found that NAD(P) oxidase-derived ROS in the PVN modulated the CSAR and contributed to the effect of Ang II in the PVN on the CSAR in normal rats [6,7]. The present study was designed to investigate whether ROS in the PVN is involved in the pathogenesis of enhanced CSAR in CHF rats, and...
whether ROS in the PVN mediates the Ang II-induced CSAR enhancement in CHF rats.

2. Methods

Experiments were carried out on 228 male Sprague-Dawley rats weighing between 300 and 400 g. The procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

2.1. CHF model

CHF was induced by coronary artery ligation as previously described [15]. Briefly, all rats were anesthetized with phenobarbital (50 mg/kg, i.p.) and instrumented using sterile techniques. The left coronary artery was ligated near its branch point from the aorta. The sham-operated rats underwent the same operative procedure, but their coronary arteries were not ligated. Laboratory chow and tap water were available ad libitum. Mortality was about 30% in the rats with coronary ligation, and death occurred primarily during the first day after ligation.

The final experiment was carried out 6–8 weeks after coronary ligation or sham surgery. The criteria for CHF were an elevated left ventricular end-diastolic pressure (LVEDP, \( \geq 13 \) mm Hg), and a 40% decrease in maximum of the first differentiation of left ventricular pressure (+dp/dt\text{max}).

2.2. Acute experimental procedure

Each rat was anesthetized with urethane (800 mg/kg, ip) and \( \alpha \)-chloralose (40 mg/kg, ip). Supplemental anesthesia was administered at 1/10 of the initial dose per hour. The trachea and right carotid artery were cannulated for mechanical ventilation and measurement of arterial pressure respectively. Baroreceptor denervation and vagotomy were carried out and confirmed as previously reported [7,10]. Baroreceptor denervation was assumed to be complete if HR changed by less than 5 beats/min in response to an intravenous injection of phenylephrine (20 \( \mu \)g/kg).

Rats were placed in a stereotaxic instrument (Stoelting, Chicago). The location of the PVN was determined according to the Paxinos and Watson rat atlas (1.8 mm caudal from bregma, 0.4 mm lateral to the midline, and 7.9 mm ventral to the dorsal surface). The PVN microinjection volume was 100 nl and administration was completed within 1 min. A representative photograph of microinjection sites in the PVN is shown in Fig. 1. Renal sympathetic nerve activity (RSNA) was recorded as previously described [7]. The signal was amplified with an AC/DC differential amplifier (Model 3000, A-M System Inc.) with a low-frequency cut-off at 60 Hz and a high-frequency cut-off at 3 kHz. The amplified and filtered signals were integrated at time constant of 10 ms. The background noise was determined after section of the central end of the renal nerve at the end of the experiment and subtracted from all the integrated values of the RSNA. The RSNA and arterial pressure were recorded with PowerLab data acquisition system (8SP, ADInstruments). The RSNA was expressed as the percent change from control.

The CSAR was elicited by application of a piece of filter paper (3×3 mm) containing bradykinin (BK, 0.4 \( \mu \)g in 2.0 \( \mu \)l) to the non-infarcted area of the epicardial surface of the left ventricle. Each piece of paper was applied for 1 min, and then removed. The epicardium was rinsed three times with 10 ml of warm normal saline (38 °C). The CSAR was evaluated by the response of the RSNA to epicardial application of BK.

At the end of the experiment, 100 nl of Evans blue (2%) was injected into the microinjection site for histological identification. Only the data from rats whose microinjection sites were within the boundaries of the PVN were used for analysis.

2.3. Measurement of superoxide anion and malondialdehyde (MDA) levels

The rat brain was removed quickly, flash-frozen in liquid nitrogen and stored at \(-70\) °C. Coronal sections of the brain were made using a cryostat microtome (CM1900, Leica LTD), and the PVN area was punched out with a 15-gauge needle. The punched PVN tissue was homogenized and then centrifuged. Protein concentrations in the supernatant were measured with the Bradford assay [16]. Malondialdehyde (MDA) was used as an indirect marker of ROS level and was determined by the thiobarbituric acid spectrometric method of Ohkawa et al. [17]. The pink-coloured chromogen formed by the reaction of thiobarbituric acid with the MDA in an acidic medium was read at 532 nm. 1,1,3,3-tetraethoxypropane was used as the external standard. The MDA level was expressed as nanomoles per milligram of tissue protein.
Lucigenin-derived chemiluminescence is a valid probe for detecting superoxide anion [18–22]. The lucigenin reacts with the superoxide anion in the sample to yield an unstable dioxetane intermediate. The lucigenin dioxetane breaks down into two molecules of N-methylacridone, one of which is in an electronically excited state, which becomes the ground state by emitting a photon. The superoxide anion level is determined by measuring the photon emission. The reaction was started by addition of dark-adapted lucigenin (5 μM). Light emission was measured with a luminometer (20/20n, Turner, CA), and values were expressed as mean light unit (MLU) per minute per milligram of protein, which represents the superoxide anion level. Background chemiluminescence in the buffer containing lucigenin (5 μM) was measured. Specificity for superoxide anion was determined by adding superoxide dismutase (SOD, 350 U/ml) to the incubation medium.

2.4. Drugs

BK, Ang II, tempol, tiron, diethyldithio-carbamic acid (DETC) and lucigenin were obtained from Sigma Chemical Co. Losartan was a gift from Merck.

2.5. Protocols

First, the effects of PVN microinjection of saline, tempol (0.2, 2 and 20 nmol), tiron (10 nmol) and diethyldithio-carbamic acid (DETC, 10 nmol) on the CSAR, baseline RSNA and MAP were determined in 6 groups of CHF rats and 6 groups of sham-operated rats (n=6 for each). CSAR was determined 10 min after the PVN microinjection.

Secondly, the effect of pretreatment with PVN microinjection of saline, tempol (0.2, 2 and 20 nmol), tiron (10 nmol) and DETC (10 nmol) on the CSAR, RSNA and MAP responses to PVN microinjection of Ang II (0.3 nmol) were investigated in 6 groups of CHF rats and 6 groups of sham-operated rats (n=6 for each). The PVN microinjection of Ang II was carried out 8 minutes after pretreatment, and the CSAR was determined 2 min after Ang II.

Thirdly, the effects of epicardial application of saline and BK (0.4 μg) on MDA and superoxide anion levels in the PVN were determined in 2 groups of CHF rats and 2 groups of sham-operated rats (n=6 for each).

Fourthly, the effects of microinjection of saline, Ang II (0.3 nmol), losartan (10 nmol) and tempol (20 nmol) on MDA and superoxide anion levels in the PVN were determined in 4 groups of CHF rats and 4 groups of sham-operated rats (n=6 for each).

Finally, to exclude the possibility that the effects of tempol were caused by diffusion into other areas of the brain, the effects of microinjection of tempol (20 nmol) into the anterior hypothalamic area, which is adjacent to the PVN, on the CSAR and Ang II-induced responses were determined in CHF and sham-operated rats (n=3 for each).

2.6. Statistics

Comparisons between two observations (before and after administration) in the same animal were assessed by Student’s paired t test. One-way ANOVA, followed by the Student’s t test, was used when multiple comparisons were made. All data were expressed as mean±SE. P<0.05 was considered statistically significant.

3. Results

3.1. Anatomic and haemodynamic data in CHF and sham-operated rats

The mean infarct area was about 34% of the left ventricle in CHF rats, no obvious infarcts were found in sham-operated rats. The heart weight and the heart-to-body weight ratio were increased in CHF rats, suggesting myocardial hypertrophy in the non-infarcted region of the myocardium. The systolic arterial pressure, pulse pressure, left ventricle...
peak systolic pressure, developed pressure and +dP/dt max decreased, while LVEDP increased in CHF rats. Table 1 shows anatomic and haemodynamic data for a representative group of CHF rats and sham-operated rats. There were no significant differences between each group of sham-operated rats or each group of CHF rats. These haemodynamic and anatomical data indicate the presence of myocardial damage and suggest decreased contractile function in CHF rats.

3.2. Effects of tempol, tiron and DETC on baseline RSNA and MAP

Microinjection of the superoxide anion scavengers, tempol or tiron, into the PVN decreased baseline RSNA and MAP in both CHF and sham-operated rats, but the superoxide dismutase inhibitor DETC showed opposite effects. In addition, the RSNA change caused by DETC in CHF rats was greater than that in sham-operated rats (Fig. 2).

3.3. Effects of tempol, tiron and DETC on CSAR

Microinjection of tempol into the PVN dose-dependently inhibited the CSAR (data not shown). Representative recordings in Fig. 3 show that CSAR was enhanced in the CHF rat, and that microinjection of high dose of tempol into the PVN abolished the CSAR in both CHF and sham-operated rats. Microinjection of a high dose of tempol or tiron into the PVN inhibited the CSAR, but DETC enhanced the CSAR in both
CHF and sham-operated rats (Fig. 4). Microinjection of a high dose of tempol into the anterior hypothalamic area (adjacent to the PVN) failed to abolish the CSAR.

3.4. Effects of pretreatment with tempol, tiron and DETC on RSNA and MAP responses to Ang II

Microinjection of Ang II into the PVN increased RSNA and MAP in both CHF rats and sham-operated rats. Pretreatment with a high dose of tempol or tiron abolished the effects of Ang II on the RSNA and MAP in both CHF rats and sham-operated rats. However, DETC did not further potentiate the effects of Ang II on RSNA and MAP (Fig. 5).

3.5. Effects of pretreatment with tempol, tiron and DETC on enhanced CSAR responses to Ang II

Microinjection of Ang II into the PVN significantly potentiated the CSAR in both sham-operated and CHF rats, which is similar to our previous report [9]. Microinjection of tempol into the PVN dose-dependently inhibited the enhanced CSAR response induced by Ang II (data not shown). Pretreatment with microinjection of tiron or a high dose of tempol into the PVN almost abolished the enhanced CSAR response induced by Ang II in both CHF and sham-operated rats. DETC augmented the enhanced CSAR response induced by Ang II only in CHF rats (Fig. 6). Microinjection of a high dose of tempol into the anterior hypothalamic area (adjacent to the PVN) failed to abolish the enhanced CSAR response induced by Ang II.

3.6. Superoxide anion and MDA levels in CHF rats and sham-operated rats

The superoxide anion and MDA levels in the PVN were higher in CHF rats than in sham-operated rats (Superoxide anion, 9.07±0.89 vs. 3.37±1.01 MLU/min/mg protein, P<0.05; MDA, 2.12±0.11 vs. 1.59±0.16 nmol/mg protein, P<0.05).

3.7. Effects of epicardial application of BK on superoxide anion and MDA levels

Epicardial application of BK increased MDA and superoxide anions levels in the PVN in both sham-operated and CHF rats. The MDA and superoxide anion levels in CHF rats were much higher than in sham-operated rats after administration of BK (Fig. 7).

3.8. Effects of microinjection of Ang II, losartan and tempol into the PVN on superoxide anion and MDA levels

Microinjection of Ang II into the PVN increased superoxide anion and MDA levels in the PVN in both CHF and sham-operated rats. The superoxide anion and MDA levels in CHF rats were higher than those in sham-
operated rats after administration of Ang II. Tempol had opposite effects to Ang II. However, the AT$_1$ receptor antagonist losartan only normalized the elevated superoxide anion and MDA levels in CHF rats without significant effects in sham-operated rats (Fig. 7).

4. Discussion

Our previous studies have indicated that CSAR is enhanced in rats and dogs with CHF [15,23]. AT$_1$ receptors in the PVN have been shown to be involved in the enhanced CSAR in CHF rats [5,8–10]. ROS in the PVN mediates the CSAR in normal rats [6,7]. We have now shown that scavenging superoxide anions in the PVN abolish the CSAR, and block the Ang II-induced CSAR enhancement in CHF rats. These results suggest that the enhanced CSAR in CHF rats is mediated by the ROS in PVN, and that the augmented effect of Ang II in the PVN on the CSAR is also mediated by the ROS in the PVN.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) and tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid) are stable membrane-permeable superoxide anion scavengers which mimic superoxide dismutase to scavenge superoxide anions [24–27]. It has been reported that intracerebroventricular infusion of tempol decrease arterial pressure and inhibit RSNA in rats [28]. Kishi et al. found that bilateral microinjection of tempol into the rostral ventrolateral medulla decreased blood pressure; and that superoxide dismutase over-expression in the rostral ventrolateral medulla decreased blood pressure and inhibited sympathetic nerve activity in stroke-prone spontaneously hypertensive rats [29]. These results suggest that central ROS is involved in regulating arterial pressure and sympathetic activity. The present study found that microinjection of tempol or tiron into the PVN abolished CSAR, inhibited RSNA, and decreased MAP, but the superoxide dismutase inhibitor DETC showed opposite effects in both CHF and sham-operated rats. Furthermore, epicardial chemical stimulation of the cardiac afferents with BK increased, but microinjection of tempol into the PVN decreased the superoxide anion and MDA levels in the PVN in both CHF and sham-operated rats. These results indicate that ROS in the PVN mediates the CSAR in both CHF and sham-operated rats. It is interesting that superoxide anion scavengers abolished not only normal CSAR, but also the enhanced CSAR in CHF rats. On the other hand, DETC further potentiated the enhanced CSAR in CHF rats although the CSAR was already enhanced. Furthermore, superoxide anion and MDA levels in the PVN increased in CHF rats. Epicardial application of BK in CHF rats resulted in much higher levels of the superoxide anion and MDA in the PVN with enhanced CSAR compared with sham-operated rats. These results suggest that the generation of ROS in the PVN could be involved in the pathogenesis of enhanced CSAR in CHF.

Our previous study showed that the enhanced CSAR in CHF rats was normalized by microinjection of the AT$_1$ receptor antagonist losartan, AT$_1$ receptor mRNA antisense or the angiotensin-converting enzyme inhibitor captopril, into the PVN. Furthermore, AT$_1$ receptor protein in the PVN was up-regulated in CHF rats [5,8,9]. It was reported that the changes in blood pressure, heart rate and drinking elicited by intracerebroventricular injection of Ang II were abolished by prior treatment with AdSOD in the brain [30]. Gao et al. found that intracerebroventricular administration of tempol or apocynin (a NAD(P)H oxidase inhibitor) inhibited the increased RSNA responses induced by intracerebroventricular administration of Ang II, and decreased the baseline RSNA in rabbits with heart failure, suggesting a close relationship between Ang II and ROS in the brain in contributing to sympathoexcitation in heart failure [18]. However, the location of the central sites in which the ROS is involved in sympathoexcitation in CHF, and whether central ROS contributes to the enhanced CSAR in CHF rats, is not known. Recently, we found that ROS in the PVN mediated the Ang II-induced CSAR enhancement in normal rats [6,7]. In the present study, microinjection of Ang II into the PVN potentiated the CSAR, and increased RSNA as well as MAP. The effects of Ang II in CHF rats were much greater than those in sham-operated rats. Pretreatment with microinjection of tempol or tiron into the PVN almost abolished the effect of Ang II on the CSAR, RSNA and MAP in both CHF rats and sham-operated rats. Microinjection of Ang II into the PVN significantly increased the MDA and superoxide anion levels in the PVN. However, DETC augmented the effect of Ang II only in CHF rats. Microinjection of Ang II into the PVN in CHF rats resulted in much higher levels of superoxide anion and MDA in the PVN and larger CSAR than sham-operated rats. The AT$_1$ receptor antagonist losartan normalized the increased superoxide anion and MDA levels in CHF rats without significant effects in sham-operated rats. This is consistent with our previous findings that losartan normalized the enhanced CSAR in CHF rats without significant effects on the CSAR in normal rats [9]. Combining the present study with our previous findings, that increased activity of AT$_1$ receptors in the PVN contributed to the enhanced CSAR [5,8,9], we concluded that the augmented effect of Ang II on the CSAR in CHF rats was mediated by ROS in the PVN in CHF rats.

It is interesting to compare the present study with our previous studies. Both blockade of AT$_1$ receptors and decreased Ang II in the PVN, normalized the enhanced CSAR, but did not completely abolish the CSAR in CHF rats. Also, in addition, there were no significant effects on the CSAR in normal rats [5,8,9]. However, scavenging superoxide anions in the PVN almost completely abolished CSAR in both CHF rats and sham rats, although the CSAR in CHF rats was much greater than that in sham rats. We therefore conclude that ROS in the PVN mediate the CSAR in either normal or CHF rats, but the AT$_1$ receptor activity in the PVN only partially mediates or modulates the CSAR. The ROS in the PVN mediate the effects of Ang II in the PVN on CSAR in both sham-operated rats and CHF rats. It is
possible that the enhanced AT1 receptor activity in the PVN in CHF increases the generation of ROS and then contributes to the enhanced CSAR and sympathetic activity in CHF rats.

One limitation to the present study is that the neurotransmitter which mediates the CSAR upstream of the ROS in the PVN is unknown. We plan to investigate the possible neurotransmitters which mediate the CSAR in the near future.

In summary, ROS in the PVN mediate the CSAR and the effect of Ang II in the PVN on the CSAR in both CHF and sham-operated rats. Increased ROS in the PVN is involved in the enhanced CSAR in CHF.

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