Superoxide anions in the paraventricular nucleus mediate the enhanced cardiac sympathetic afferent reflex and sympathetic activity in renovascular hypertensive rats

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Superoxide anions in the paraventricular nucleus mediate the enhanced cardiac sympathetic afferent reflex and sympathetic activity in renovascular hypertensive rats. J Appl Physiol 110: 646–652, 2011. First published December 16, 2010; doi:10.1152/japplphysiol.00908.2010.—An enhanced cardiac sympathetic afferent reflex (CSAR) is involved in the sympathetic activation in renovascular hypertension. The present study was designed to determine the role of superoxide anions in the paraventricular nucleus (PVN) in mediating the enhanced CSAR and sympathetic activity in renovascular hypertension in the two-kidney, one-clip (2K1C) model. Sinoaortic denervation and vagotomy were carried out, and renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) were recorded under anesthesia. The CSAR was evaluated by the response of RSNA to the epicardial application of capsaicin. Superoxide anion levels and NAD(P)H oxidase activity in the PVN increased in 2K1C rats and were much higher in 2K1C rats than in sham-operated (sham) rats after the epicardial application of capsaicin or PVN microinjection of ANG II. In both 2K1C and sham rats, PVN microinjection of the superoxide anion scavenger tempol or the NAD(P)H oxidase inhibitor apocynin abolished the CSAR, whereas the SOD inhibitor diethyldithiocarbamic acid (DETC) potentiated the CSAR. Tempol and apocynin decreased but DETC increased baseline RSNA and MAP. ANG II in the PVN caused larger responses of the CSAR, baseline RSNA, and baseline MAP in 2K1C rats than in sham rats. The effects of ANG II were abolished by pretreatment with tempol or apocynin in both 2K1C and sham rats and augmented by DETC in the PVN in 2K1C rats. These results indicate that superoxide anions in the PVN mediate the CSAR and the effects of ANG II in the PVN. Increased superoxide anions in the PVN contribute to the enhanced CSAR and sympathetic activity in renovascular hypertension.

angiotensin II; hypothalamus; reactive oxygen species; autonomic nervous system; blood pressure

SYMPATHETIC OUTFLOW is enhanced in patients with essential hypertension (9, 14, 23a), secondary hypertension in chronic kidney disease (24, 28), diabetes (8, 32), or obesity-related hypertension (3, 17) and also in a variety of experimental hypertensive models, such as spontaneously hypertensive rats (18), salt-sensitive hypertensive rats (10), two-kidney, one-clip (2K1C) rats (16), and obesity-induced hypertensive rats (31). This elevated sympathetic activity participates in the pathogenesis of hypertension and the progression of organ damage (11, 21, 23, 27). Inhibition of the enhanced sympathetic tone may be beneficial for hypertension (3, 15, 30).

The cardiac sympathetic afferent reflex (CSAR) is known as a positive-feedback, sympathoexcitatory reflex, which has been demonstrated in rats, cats, and dogs (1, 12, 13, 19). Epicardial application of bradykinin, capsaicin, adenosine, or hydrogen peroxide stimulates cardiac sympathetic afferents in the ventricular surface and reflexly increases sympathetic outflow and arterial blood pressure (ABP). Similar responses are caused by endogenous chemicals released from the myocardium during myocardial ischemia (7, 20). The paraventricular nucleus (PVN) is an important component of the central neurocircuity of the CSAR (36) and projects to autonomic nuclei in the brain stem and spinal cord and are responsible for the integration of the sympathetic outflow and regulation of cardiovascular activity (2, 6). The CSAR may play a regulatory role in increasing the ABP and cardiac output via sympathetic activation in the normal state with a complex interaction of central integration and peripheral inhibitory and excitatory reflexes (20). However, a persistently enhanced CSAR in hypertension (5, 39) or chronic heart failure (CHF) (38, 40) partially contributes to excessive sympathetic activity and thereby deteriorates these diseases.

The superoxide anion scavenger tempol in the PVN inhibits the CSAR, whereas ANG II augments the CSAR in rats with CHF. The effects of ANG II in the PVN are prevented by pretreatment with the ANG II type 1 (AT1) receptor antagonist losartan or tempol in CHF rats (13, 38). Recently, we (5) have found that the enhanced CSAR in 2K1C rats is normalized by the PVN administration of losartan. However, whether superoxide anions in the PVN in 2K1C hypertensive rats are involved in mediating the enhanced CSAR and sympathetic activity is unknown. The present study was designed to investigate the role of superoxide anions in the PVN in the enhanced CSAR and sympathetic activation in renovascular hypertensive rats induced by 2K1C. First, the effects of the SOD inhibitor diethyldithiocarbamic acid (DETC), the superoxide anion scavenger tempol, and the NAD(P)H oxidase inhibitor apocynin in the PVN on the CSAR and renal sympathetic nerve activity (RSNA) were determined. Second, the effects of PVN pretreatment with DETC, tempol, or apocynin on ANG II-induced CSAR and RSNA responses were investigated. Finally, the changes in superoxide anion levels and NAD(P)H oxidase activity in the PVN caused by the chemical stimulation of cardiac sympathetic afferents or PVN microinjection of ANG II were determined.

METHODS

Experiments were carried out in male Sprague-Dawley rats. The procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and complied with the
National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Rats were kept in a temperature-controlled room on a 12:12-h light-dark cycle with standard chow and tap water ad libitum.

Renovascular Hypertensive Model

Renovascular hypertension was induced by the 2K1C method as we have previously reported (39). Briefly, rats (weight: 160–180 g) were anaesthetized with an intraperitoneal administration of chloral hydrate (300 mg/kg). The right renal artery was exposed and partly occluded with a U-shaped silver clip (0.2 mm inner diameter) to induce renovascular hypertension with sterile techniques. Normotensive sham-operated (sham) rats received a similar surgical intervention except that the clip was not used. Acute experiments were carried out at the end of the fourth week after the surgery. The criterion of hypertension in the present study was set as systolic blood pressure (SBP) > 160 mmHg (39). Only rats with SBP > 160 mmHg underwent acute experiments in 2K1C rats. In 97 2K1C rats and 87 sham rats, 8 2K1C rats were excluded because SBP in these rats was not high enough to meet the criterion mentioned above and 5 2K1C rats and 3 sham rats were excluded because of unsuccessful animal surgical preparation.

SBP Measurements

SBP of the tail artery was measured in the conscious state using a noninvasive computerized tail-cuff system (NIBP, AD Instruments). Rats were warmed for 10–20 min at 28°C before measurements to allow the detection of tail artery pulsations and to achieve the pulse level ready. SBP was obtained by averaging 10 measurements. To minimize stress-induced fluctuations in SBP, all rats were trained by measuring SBP daily for 10 days. After 2K1C or sham operation, SBP was measured at weekly intervals.

General Procedures of Acute Experiments

At the end of the fourth week after surgery, each rat was intraperitoneally anesthetized with urethane (800 mg/kg) and α-chloralose (40 mg/kg). Adequate depth of anesthesia was assessed by the absence of corneal reflexes and paw withdrawal response to a noxious pinch. Supplemental doses of urethane and α-chloralose were administered to maintain an adequate depth of anesthesia. A midline incision in the neck was made, and the trachea was cannulated and connected to a rodent ventilator (model 683, Harved Apparatus) for mechanical ventilation. ABP was measured with a pressure transducer (MLT0380, AD Instruments) through a catheter placed into the right carotid artery.

Baroreceptor Denervation and Vagotomy

To minimize the confounding effect of the baroreflex on sympathetic drive, baroreceptor denervation and vagotomy were carried out and identified as previously reported (37). Bilateral carotid sinus areas were exposed. The carotid sinus nerves and other nerve fibers visible in the carotid sinus area were cut. Adventitial tissues were stripped from the common carotid arteries and carotid bifurcation from 4 mm above the bifurcation to 4 mm below. To destroy any remaining nerve fibers in this area, vessels were painted with 10% phenol solution. Bilateral cervical vagi were then identified, tied, and sectioned.

RSNA Recordings

RSNA was taken as representative of sympathetic outflow. On an opposite side of the clipped renal artery, a retroperitoneal incision was made, and the left renal nerve was identified. The renal nerve was cut distally to eliminate renal afferent activity. The nerve was placed on a pair of silver recording electrodes and immersed in mineral oil. RSNA was amplified with an alternating current/direct current differential amplifier (3000, A-M System) with a low-frequency cutoff at 60 Hz and a high-frequency cutoff at 3,000 Hz. RSNA was integrated at a time constant of 10 ms. Background noise was determined after section of the central end of the nerve. Raw RSNA, integrated RSNA, ABP, mean arterial pressure (MAP), and heart rate (HR) were simultaneously recorded with a PowerLab data-acquisition system (8SP, AD Instruments). The RSNA change after intervention was expressed as the percent change from baseline.

Evaluation of the CSAR

A limited left lateral thoracotomy was performed to expose the heart, and the pericardium was removed. The CSAR was elicited by the epicardial application of a piece of filter paper (3 × 3 mm) containing capsaicin (1.0 nmol in 2.0 μl) on the anterior wall of the left ventricle. A minute later, the filter paper was removed, and the epicardium was rinsed three times with 10 ml normal saline (38°C). The CSAR was evaluated by the RSNA response to the epicardial application of capsaicin (37).

PVN Microinjections

Rats were placed in a stereotaxic frame (Stoelting). The stereotaxic coordinates for the PVN were 1.8 mm caudal from the bregma, 0.4 mm lateral to the midline, and 7.9 mm ventral to the dorsal surface according to Paxinos and Watson’s rat atlas. Bilateral PVN microinjections were completed within 1 min, and the microinjection volume was 50 nl for each side of the PVN. At the end of the experiment, 50 nl Evans blue dye (2%) was injected into each microinjection site. The microinjection sites were histologically verified with a microscope (Fig. 1). Rats with microinjection sites outside the PVN were excluded from data analysis.

PVN Samples

The brain of the rat was removed from the skull quickly, flash frozen in liquid nitrogen, and stored at −70°C. Coronal sections of the brain were made with a cryostat microtome (CM1900, Leica LTD), and the PVN area was punched out with a 15-gauge needle. The punched tissue of the PVN was homogenized and then centrifuged. Protein concentrations in the supernatants were measured with the Bradford assay (4).

Measurement of Superoxide Anion Levels

Lucigenin-derived chemiluminescence was used to determine superoxide anion levels in the PVN as we have previously reported (13). Light emission was measured with a luminometer (20/20n, Turner), and values were expressed as mean light units per minute per milligram of protein.

Measurement of NAD(P)H Oxidase Activity

NAD(P)H oxidase activity in the PVN was measured by lucigenin-enhanced chemiluminescence as we have previously reported (35). Light emission was measured with a luminometer (20/20n, Turner),

Fig. 1. Representative brain slice showing the sites of paraventricular nucleus (PVN) microinjections. Arrows point to the microinjection sites.
and values were expressed as mean light units per minute per milligram of protein.

Protocols

Acute experiments were carried out at the end of the fourth week after the 2K1C or sham surgery. At first, rats were kept in the supine position. The trachea and right carotid artery were cannulated, and sinoaortic denervation and vagotomy were carried out. Rats were kept in a prone position with the head fixed in the stereotaxic frame for PVN positioning. The surgery mentioned above was completed in 20–25 min. Finally, the rat body was kept in the right lateral position with the head fixed in the stereotaxic frame. A left lateral thoracotomy was performed to prepare for the epicardial application of chemicals. A left retroperitoneal incision was made to place electrodes for RSNA recordings. The surgery and related operation were also completed in 20–25 min. All the surgery finished in 50 min. Rats were allowed to stabilize for 30 min before interventions.

Protocol 1. Either sham or 2K1C rats were randomly divided into two groups (n = 6 rats/group), which were, respectively, subjected to a bilateral PVN microinjection of saline or ANG II (0.3 nmol). Three minutes after the microinjection, rats were euthanized, and superoxide anion levels and NAD(P)H oxidase activity in the PVN were determined.

Protocol 2. Either sham or 2K1C rats were randomly divided into two groups (n = 6 rats/group), which were, respectively, subjected to an epicardial application of saline or capsaicin (1 nmol). Three minutes later, rats were euthanized, and superoxide anion levels and NAD(P)H oxidase activity in the PVN were determined.

Protocol 3. Either sham or 2K1C rats were randomly divided into five groups (n = 6 rats/group), which were, respectively, subjected to bilateral PVN microinjection of DETC (10 nmol), tempol (20 nmol), apocynin (1 nmol), normal saline (vehicle for DETC and tempol), and DMSO (1% DMSO in normal saline, vehicle for apocynin). Baseline changes in RSNA and MAP were determined. Eight minutes after PVN microinjection, the CSAR induced by an epicardial application of capsaicin (1 nmol) was evaluated.

Protocol 4. Pretreatment with PVN microinjection of DETC (10 nmol), tempol (20 nmol), apocynin (1 nmol), saline, and DMSO were, respectively, carried out in five groups of sham rats and five groups of 2K1C rats (n = 6 rats/group). Eight minutes after the pretreatment, the effects of PVN microinjection of ANG II (0.3 nmol) on RSNA and MAP were determined. Two minutes after the administration of ANG II, the CSAR induced by an epicardial application of capsaicin (1 nmol) was evaluated.
ANG II, capsaicin, tempol, DETC, apocynin, and lucigenin were obtained from Sigma Chemical (St. Louis, MO). ANG II, capsaicin, tempol, and DETC were dissolved in normal saline. Apocynin was dissolved in normal saline containing 1% DMSO.

Statistical Analysis

Comparisons between two observations in the same animal were assessed by Student’s paired t-test. One-way ANOVA followed by the Newman-Keuls test for post hoc analysis was used when multiple comparisons were made. All data are expressed as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

General Data

At the end of the fourth week, either SBP of the tail artery in the conscious state or MAP of the carotid artery under anesthesia in 2K1C rats was significantly higher than that in sham rats (Table 1). There were no significant differences in SBP and MAP among any subgroups within sham or 2K1C rats.

Superoxide Anion Levels and NAD(P)H Oxidase Activity in the PVN

Both superoxide anion levels and NAD(P)H oxidase activity in the PVN were higher in 2K1C rats than those in sham rats. Either microinjection of ANG II into the PVN or epicardial application of capsaicin increased superoxide anion levels and NAD(P)H oxidase activity in the PVN in both 2K1C and sham rats. Superoxide anion levels and NAD(P)H oxidase activity in the PVN were higher in 2K1C rats than those in sham rats after ANG II or capsaicin treatment (Fig. 2).
Effects of DETC, Tempol, and Apocynin on Baseline RSNA and MAP

Baseline MAP before PVN microinjection in each subgroup of 2K1C rats was much higher than that of sham rats (Table 2). PVN microinjection of the SOD inhibitor DETC increased baseline RSNA and MAP in both sham and 2K1C rats, but the RSNA response of 2K1C rats was greater than that of sham rats (Fig. 3). PVN microinjection of the superoxide anion scavenger tempol or the NAD(P)H oxidase inhibitor apocynin decreased baseline RSNA and MAP in both sham and 2K1C rats.

Effects of DETC, Tempol, and Apocynin on the CSAR

The CSAR was enhanced in 2K1C rats. DETC potentiated the CSAR in both sham and 2K1C rats, but the CSAR was enhanced in 2K1C rats compared with sham rats after DETC treatment. Tempol and apocynin abolished the CSAR in both sham and 2K1C rats (Fig. 4).

Effects of Pretreatment With DETC, Tempol, and Apocynin on ANG II-Induced RSNA and MAP Responses

Microinjection of ANG II into the PVN significantly enhanced the CSAR in both 2K1C and sham rats. ANG II-induced CSAR responses were greater in 2K1C rats than in sham rats. Pretreatment with DETC further potentiated the effects of ANG II on RSNA and MAP in 2K1C rats. Pretreatment with either tempol or apocynin abolished the effects of ANG II on RSNA and MAP in both 2K1C and sham rats (Fig. 5).

**Effects of Pretreatment With DETC, Tempol, and Apocynin on ANG II-Induced CSAR Responses**

Microinjection of ANG II into the PVN significantly enhanced the CSAR in both 2K1C and sham rats. ANG II-induced CSAR responses were greater in 2K1C rats than in sham rats. Pretreatment with DETC further potentiated the ANG II-induced CSAR enhancement in 2K1C rats. Pretreat-
ment with either tempol or apocynin abolished the effects of ANG II on the CSAR in both 2K1C and sham rats (Fig. 6).

DISCUSSION

Sympathetic activity and plasma norepinephrine levels are increased in 2K1C-induced hypertensive rats, and the enhanced CSAR in 2K1C rats partially contributes to the sympathetic activation (39). There is an increase in oxidative stress in the PVN in renovascular hypertensive rats, and this is an important mechanism to maintain RSNA in 2K1C rats (25). Tempol is known to be a stable membrane-permeable superoxide anion scavenger that mimics SOD to scavenge superoxide anions (33). In the present study, microinjection of tempol into the PVN decreased RSNA and MAP and abolished the CSAR, whereas the SOD inhibitor DETC in the PVN increased RSNA and CSAR in both 2K1C and sham rats. RSNA and the CSAR were more enhanced in 2K1C rats than in sham rats after the administration of DETC. Superoxide anion levels in the PVN were increased in 2K1C rats. Stimulation of cardiac afferents with the epicardial application of capsaicin further increased superoxide anion levels in the PVN in both sham and 2K1C rats, but superoxide anion levels in the PVN were much higher in 2K1C rats than in sham rats after capsaicin. These results indicate that superoxide anions in the PVN mediate the CSAR, and the increased superoxide anions in the PVN contribute to the enhanced CSAR and sympathetic activation in 2K1C rats.

The PVN contains AT1 receptors and the necessary components to synthesis its own ANG peptides (26). Recently, we have found that microinjection of losartan into the PVN normalizes the enhanced CSAR and RSNA and decreases MAP in 2K1C rats but not in sham rats. Pretreatment with losartan in the PVN abolishes the effects of ANG II in the PVN on the CSAR, RSNA, and MAP in both sham and 2K1C rats. The results show endogenous ANG II is involved in the enhanced CSAR and RSNA via activation of AT1 receptors (5). In the present study, microinjection of ANG II into the PVN increased RSNA and MAP and enhanced the CSAR in both 2K1C and sham rats. However, exogenous ANG II was more reactive in 2K1C rats than in sham rats. A potential mechanism responsible for the intensified responses to ANG II may be the increased AT1 receptor expression in the PVN in 2K1C rats (5). Pretreatment with tempol in the PVN abolished the effects of ANG II in both 2K1C and sham rats. Conversely, the SOD inhibitor DETC augmented the effects of ANG II in 2K1C rats. After microinjection of ANG II into the PVN, superoxide anion levels in the PVN were higher in 2K1C rats than in sham rats. These results indicate that superoxide anions in the PVN mediate the role of ANG II in the PVN in enhancing the CSAR and RSNA. The increased superoxide anions in the PVN contribute to the enhanced CSAR and sympathetic activation partially by mediating the effects of ANG II in the PVN in 2K1C rats, which is supported by the findings that NAD(P)H oxidase is activated by ANG II in the brain and that NAD(P)H oxidase-derived ROS are involved in ANG II signaling (22, 29).

Our previous study (35) has shown that NAD(P)H oxidase in the PVN is a major source of ROS in modulating the CSAR and that ROS originating from NAD(P)H oxidase in the PVN contributes to the effects of ANG II on the CSAR in normal rats. In the present study, PVN microinjection of the NAD(P)H oxidase inhibitor apocynin decreased baseline RSNA and MAP and abolished the CSAR and the effects of ANG II in the PVN in both 2K1C and sham rats. Furthermore, NAD(P)H oxidase activity in the PVN of 2K1C rats was higher than that of sham rats. After the epicardial application of capsaicin or PVN microinjection of ANG II, NAD(P)H oxidase activity in the PVN increased to a higher level in 2K1C rats than in sham rats. These results indicate that NAD(P)H oxidase in the PVN is an important source of superoxide anions, which is involved in the enhanced CSAR and the effects of ANG II in the PVN in 2K1C rats.

PVN microinjection of tempol and apocynin significantly decreased RSNA and MAP and abolished the CSAR in both sham and 2K1C rats, indicating the important fact that endogenous superoxide anions play a tonic role in sympathetic activation and mediate the CSAR. Reducing superoxide anions in the PVN may be helpful in inhibiting sympathetic activation, which is supported by our recent study (34) showing that persistently scavenging of superoxide anions in the PVN with Cu/ZnSOD overexpression not only normalized sympathetic activity and the CSAR but also decreased ABP in spontaneously hypertensive rats. However, it is worthwhile to note that there were no significantly greater decreases in RSNA in 2K1C rats than in sham rats after PVN microinjection of tempol and apocynin, although endogenous superoxide anions and NAD(P)H oxidase activity significantly increased. A possible reason is that increased superoxide anion levels in the PVN is only one mechanism related to sympathetic activation in 2K1C rats and that other mechanisms or some compensatory mechanisms are involved in sympathetic activation.

In conclusion, superoxide anions in the PVN not only mediate the CSAR but also mediate the effects of ANG II in the PVN on the CSAR, RSNA, and MAP. Increased superoxide anions in the PVN contribute to the enhanced CSAR and sympathetic activity in 2K1C rats. NAD(P)H oxidase in the PVN is the major source of superoxide anions in mediating the CSAR and the effects of ANG II in the PVN in 2K1C rats.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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