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# Inhibition of reactive oxygen species in hypothalamic paraventricular nucleus attenuates the renin–angiotensin system and proinflammatory cytokines in hypertension

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ABSTRACT

*Aims:* To explore whether reactive oxygen species (ROS) scavenger (tempol) in the hypothalamic paraventricular nucleus (PVN) attenuates renin–angiotensin system (RAS) and proinflammatory cytokines (PICs), and decreases the blood pressure and sympathetic activity in angiotensin II (ANG II)-induced hypertension.
*Methods and results:* Male Sprague–Dawley rats were infused intravenously with ANG II (10 ng/kg per min) or normal saline (NS) for 4 weeks. These rats were treated with bilateral PVN infusion of oxygen free radical scavenger tempol (TEMP, 20 μg/h) or vehicle (artificial cerebrospinal fluid, aCSF) for 4 weeks. ANG II infusion resulted in increased mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA). These ANG II-infused rats also had higher levels of $gp91^{phox}$ (a subunit of NAD(P)H oxidase), angiotensin-converting enzyme (ACE), and interleukin-1beta (IL-1β) in the PVN than the control animals. Treatment with PVN infusion of TEMP attenuated the overexpression of $gp91^{phox}$, ACE and IL-1β within the PVN, and decreased sympathetic activity and MAP in ANG II-infused rats.
*Conclusion:* These findings suggest that ANG II infusion induces elevated PICs and oxidative stress in the PVN, which contribute to the sympathoexcitation in hypertension. Inhibition of reactive oxygen species in hypothalamic paraventricular nucleus attenuates the renin–angiotensin system, proinflammatory cytokines and oxidative stress in ANG II-induced hypertension.



## Introduction

Hypertension is considered as a condition of neurohumoral activity in both central and peripheral tissues. Recent studies indicate that renin–angiotensin system (RAS), proinflammatory cytokines (PICs), and reactive oxygen species (ROS) are involved in the pathophysiology of hypertension (Davisson, 2003; Ferrario and Strawn, 2006; Gao et al., 2005; Kang et al., 2006, 2009; Zhu et al., 2004; Zimmerman et al., 2002). The hypothalamic paraventricular nucleus (PVN) is the center of arterial blood pressure control, and a predominant region for coordinating nervous signals of the blood pressure (Cardinale et al., 2012; Davisson et al., 2000). Angiotensin II (ANG II) infusion induces RAS activation in the PVN and sympathoexcitation. Central blockade of RAS decreases mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) (Kang et al., 2008). In addition, the overexpression of PICs (tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 (IL-6), and interleukin (IL)-1beta (IL-1β)) in the PVN also contributes to sympathoexcitation (Cardinale et al., 2012; Kang et al., 2009). These studies suggest that RAS and PICs in the PVN are closely related to the regulation of blood pressure and renal sympathetic nerve activity in the progress of hypertension.

It is well known that the binding of excess ANG II to angiotensin type 1-receptor ($AT_1$-R) in the PVN makes dysfunctional mitochondria and produces excessive amounts of ROS, such as superoxide ($O_2^{\bullet -}$) (Hitomi et al., 2007; Oskarsson and Heistad, 1997; Zhang et al., 2007). The expression of superoxide protein $gp91^{phox}$ (a membrane associated oxidase protein) is aggrandized within the peripheral tissues and in the nerve cells (Gao et al., 2004; Li et al., 2005; Serrano et al., 2003;

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Sundaresan et al., 1996). It implies that the production of ROS from neuron was also augmented in the PVN. Until recently, there is little available information in literatures on the precise mechanisms of hypertension accounting for the increase of oxidative stress in the PVN.

In this study, we explore whether ANG II infusion induces elevated PICs and oxidative stress in the PVN, which contribute to the sympathoexcitation in hypertension. We hypothesized that inhibition of reactive oxygen species in the PVN attenuates ANG II-induced sympathoexcitation and hypertension by attenuating elevated PICs, activated RAS and oxidative stress in the PVN.

## Material and methods

*Animals.* Experiments were conducted with adult male Sprague–Dawley rats (250–275 g). All rats were housed in a climate-controlled room with a 12:12 h light: dark cycle and allowed access to normal rat chow and tap water *ad libitum*. All experimental procedures were based on all applicable principles set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (the US National Institutes of Health Publication No. 85-23, revised 1996). All of the animal procedures were approved by the Animal Care and Use Committees of Xi'an Jiaotong University.

*General experimental protocol.* Rats were anesthetized and implanted with osmotic minipumps (ALZET, model 1004; infusion rate of 0.11 μl/h) into the bilateral PVN. The osmotic minipumps were filled with oxygen free radical scavenger tempol (TEMP, 20 μg/h; Sigma) dissolved in artificial cerebrospinal fluid (aCSF) (Cardinale et al., 2012; Yu et al., 2013), implanted subcutaneously in the back of the neck and connected to the cannulae for chronic infusion in the bilateral PVN for 4 weeks. Simultaneously, ANG II (dissolved in sterile saline) was infused into the femoral vein at a rate of 10 ng/kg per min for 4 weeks *via* an osmotic minipump (ALZET, model 2004; infusion rate of 0.25 μl/h). The doses used in this study are based on our previous research (Kang et al., 2008) and other effective studies (Edgley et al., 2003; Zhang et al., 2008). The success rate of bilateral microinjection and vein infusion is respectively 65% and 78%.

*Implantation of bilateral PVN cannulae for chronic infusion studies.* Rats were anaesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture *via* intraperitoneal injection (ip), and the rats were placed into a stereotaxic apparatus. According to rats atlas (Paxinos et al., 1987) rats atlas, coordinates for the PVN were determined at 1.8 mm posterior, 0.4 mm lateral to the bregma, and 7.9 mm ventral to the zero level (Yu et al., 2013). The minipumps connected with TEMP for the continuous infusion, were implanted subcutaneously in the back of the neck. Then catheters were advanced into the femoral vein for minipump infusion of ANG II or NS as previously described (Edgley et al., 2003; Reckelhoff et al., 2000).

*Blood pressure measurements.* Arterial pressure was measured noninvasively *via* tail-cuff instrument and their recording system. The recordings of method and data analysis have been described previously (Elks et al., 2011). Mean arterial pressure and heart rate data were collected between 8 and 11 a.m. and analyzed until the end of this study.

After 4 weeks of blood pressure recordings, animals were anaesthetized for terminal electrophysiological studies, and then euthanized to collect brain tissue for molecular and immunohistochemical analysis.

*Sympathetic neural recordings.* Rats were anaesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (ip). After retroperitoneal laparotomy, the left renal nerves were isolated *via* glass microelectrode technique under an inversion microscope. The renal nerve was hung by a platinum electrode which is connected with the recording system. In order to moisturize the nerves and isolate electrical disturbance, the nerve should be covered by paraffin oil tampons. The recordings of rectified and integrated RSNA were analyzed using methods described as previously (Kang et al., 2008, 2009; Zhang et al., 2003).

*Immunohistochemistry and immunofluorescence.* The methods for immunohistochemistry and immunofluorescence were performed as described previously (Sriramula et al., 2011). The primary antibodies for $gp91^{phox}$ (sc-5827, an affinity purified goat polyclonal antibody), IL-1β (sc-1251, an affinity purified goat polyclonal antibody) and ACE (sc-20781, a rabbit polyclonal antibody) were from Santa Cruz Biotechnology. Superoxide generation in the PVN was determined by fluorescent-labeled dihydroethidium (DHE; Molecular Probes) staining as previously described (Miller et al., 1998).

*Western blot.* After measurement of RSNA, the rats were euthanized to collect PVN tissue. Western blot was used for measurement of $gp91^{phox}$, IL-1β and ACE expressions in PVN. The primary antibodies for $gp91^{phox}$ (sc-5827, an affinity purified goat polyclonal antibody raised for detection of $gp91^{phox}$ of rat origin), IL-1β (sc-1251, an affinity purified goat polyclonal antibody raised for detection of IL-1β of rat origin) and ACE (sc-20781, a rabbit polyclonal antibody raised for detection of ACE of rat origin) were from Santa Cruz Biotechnology. Protein loading was controlled by probing all western blots with β-actin antibody which was from Santa Cruz Biotechnology and normalizing $gp91^{phox}$, IL-1β and ACE protein intensities to that of β-actin. Band densities were analyzed using NIH Image software (Mariappan et al., 2007, 2010).

*Statistical analysis.* All data are expressed as mean ± SEM. The significance of differences between mean values was analyzed by ANOVA followed by Tukey's test. Blood pressure data were analyzed by repeated measures ANOVA. A probability value of $P < 0.05$ was considered to be statistically significant.

## Result

*Effect of PVN infusion of TEMP on mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA)*

ANG II infusion induced a significant increase in MAP compared with control rats. MAP remained elevated throughout the 28 days of the study. Treatment with PVN reduced MAP in hypertensive rats (Fig. 1).

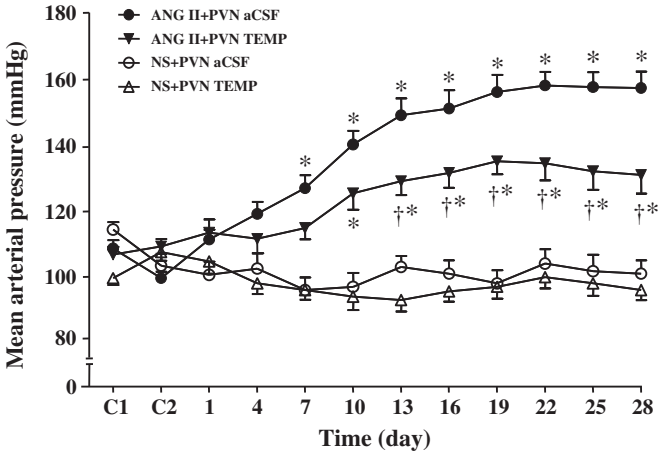


**Fig. 1.** Effects of PVN infusion of oxygen free radical scavenger (TEMP) or aCSF on mean arterial pressure (MAP) of ANG II-infused rats and control rats. ANG II infusion induced an increase in MAP compared with controls. PVN infusions of TEMP attenuated ANG II-induced presser response. Values are expressed as means ± SEM. $n = 7$ per group; $^{*}P < 0.05$ versus control (NS + PVN infusion of TEMP or NS + PVN infusion of aCSF); †$P < 0.05$ versus ANG II + PVN infusion of aCSF.