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Reduction of Nitric Oxide-Mediated γ-Amino Butyric Acid Release in Rostral Ventrolateral Medulla Is Involved in Superoxide-Induced Sympathoexcitation of Hypertensive Rats

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Background: The rostral ventrolateral medulla (RVLM) in the brainstem is responsible for regulation of the sympathetic nervous system. In the RVLM, nitric oxide (NO)-mediated γ-amino butyric acid (GABA) is a major sympathoinhibitory amino acid neurotransmitter and superoxide is a major sympathoexcitatory factor. In this study, we investigated whether or not NO-mediated GABA release is involved in superoxide-induced sympathoexcitation in the RVLM of hypertensive rats.

Methods and Results: For our model hypertensive rats with sympathoexcitation, we used stroke-prone spontaneously hypertensive rats (SHRSP). GABA levels in the RVLM were measured by in vivo microdialysis. Microinjection of tempol, a superoxide scavenger, into the RVLM decreased arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) with an increase in GABA release in the RVLM. Microinjection of N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, into the RVLM increased AP, HR, and RSNA with a decrease in GABA release in the RVLM. Prior microinjection of L-NMMA into the RVLM attenuated the tempol-induced changes in AP, HR, RSNA, and GABA release in the RVLM. Microinjection of bicuculline, a GABA receptor blocker, into the RVLM attenuated the tempol- and L-NMMA-induced changes in AP, HR, and RSNA.

Conclusions: The findings suggest that reduction of NO-mediated GABA release in the RVLM is partly involved in superoxide-induced sympathoexcitation of SHRSP. (*Circ J* 2012; **76:** 2814–2821)

Key Words: Amino acids; Brain; Nitric oxide; Oxidative stress; Sympathetic nervous system

Activation of the sympathetic nervous system (SNS) has an important role in the pathogenesis of hypertension[1] and central mechanisms are crucially involved in sympathetic hyperactivity.[1,2] The rostral ventrolateral medulla (RVLM) in the brainstem contains the pre-sympathetic neurons that maintain baseline sympathetic tone,[2,3] and previous studies have demonstrated that nitric oxide (NO) in the RVLM inhibits the activation of the SNS.[4–7] The sympathoinhibitory effect of NO in the RVLM is considered to be reduced in spontaneously hypertensive rats (SHR).[8,9] Furthermore, γ-amino butyric acid (GABA) is a major inhibitory neurotransmitter[10] and GABA receptors in the RVLM have been demonstrated.[11,12] Activating the $GABA_A$ receptor inhibits the activity of the RVLM neurons,[10,13] and it has been reported that, in SHR, there is a GABAergic disinhibition of neuronal activity in the RVLM.[9,10,14–16] NO is an important mediator in the autonomic nuclei, such as RVLM, paraventricular nucleus (PVN), and nucleus tractus solitarii,[4,7,17–22] for cardiovascular regulation, and acting on presynaptic terminals to increase vesicular GABA release.[17,20,21] NO has been shown to increase the release of GABA in the PVN and decrease arterial pressure (AP), although the release of excitatory amino acids in the PVN was also increased by NO.[22] We also have demonstrated that increased NO production by overexpression of endothelial NO synthase (eNOS) caused GABAergic inhibition in the RVLM.[7,9]

Many previous studies in experimental animal models of hypertension have indicated that superoxide in the brain con-

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tributes to the activation of the SNS, thereby increasing AP.[23–31] It has already been determined that superoxide causes sympathoexcitation in the RVLM.[23,26–28] Although several mechanisms of superoxide-induced sympathoexcitation have already been determined,[23] the aim of the present study was to determine whether or not NO-mediated GABA release is involved in superoxide-induced sympathoexcitation in the RVLM of hypertensive rats with sympathoexcitation, which has not been fully clarified. For this purpose, we used stroke-prone SHR (SHRSP), and infused either 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol), a superoxide dismutase (SOD) mimetic, or N^G-monomethyl-L-arginine (L-NMMA), an NO synthase (NOS) inhibitor, into the RVLM. GABA levels in the RVLM of SHRSP were measured before and during infusion of each drug. Furthermore, bicuculline was also injected into the RVLM to confirm the effects of GABA on superoxide-induced sympathoexcitation and NO-induced sympathoinhibition in the RVLM.

Methods

Animals and General Procedures

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at the Kyushu University Graduate School of Medical Sciences and conducted according to the Guidelines for Animal Experiments of Kyushu University. Experiments were performed on male SHRSP and Wistar-Kyoto rats (WKY) (280–340 g, 14–18 weeks old; SLC Japan, Hamamatsu, Japan). Rats were initially anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal followed by 20 $mg \cdot kg^{-1} \cdot h^{-1}$ intravenous infusion). A catheter was inserted into the femoral artery to record mean AP (MAP) and heart rate (HR), and another catheter was inserted into the femoral vein to allow for intravenous drug injections. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. The left renal nerve was exposed with a left retroperitoneal flank incision. Stainless steel bipolar electrodes were placed beneath the renal nerve to record multifiber renal sympathetic nerve activity (RSNA).[32,33] The rats were placed in a stereotaxic frame with incisor bar and the dorsal surface of the medulla was surgically exposed to allow for positioning of the microinjection pipettes into the RVLM (with the pipette angled rostrally 18°, 1.8 mm lateral, 3.5 mm below the calamus scriptorius), as described previously.[7,33] After identification of the RVLM by monitoring the response to an injection of a small dose of L-glutamate,[6–9,27,33] microinjection or microinfusion studies with in vivo microdialysis were performed. At the end of each experiment, microinjection of the vehicle solution containing Evans blue dye (100 nl) was made into the RVLM injection or infusion site, using the same coordinates as for the drug injections. The animal was then killed by an overdose of sodium pentobarbital, and the brain removed and placed in 10% formalin for at least 48 h. Subsequently, 100-μm thick coronal sections of the brainstem were cut on a microtome. The labeled sites of microinjection were identified by examining the sections using a microscope.

Measurement of GABA Levels by In Vivo Microdialysis

A microdialysis probe with juxtapositional infusion cannula (MI-C-I-12–01FEP; Eicom, Kyoto, Japan) was inserted in one (unilateral) of the RVLM. The RVLM was perfused with Ringer solution (140 mmol/L NaCl, 4 mmol/L KCl, 1.26 mmol/L $CaCl_2$, and 1.15 mmol/L $MgCl_2$, pH 7.4) at a constant flow rate of 3 μl/min through a microdialysis probe. To measure GABA levels, the perfused dialysates were collected every 5 min, and GABA levels were measured by high-performance liquid chromatography with an electrochemical detector (HTEC-500, Eicom, Kyoto, Japan).[7,34] GABA levels were quantitated by averaging 2 consecutive dialysate samples, which were obtained at approximately ≥1 h after starting the brain perfusion with Ringer's solution.

Experimental Protocols

(1) To confirm the role of superoxide in the RVLM in the regulation of AP, HR, and sympathetic nerve activity, tempol (1 nmol in 100 nl) was acutely microinjected into both (bilateral) RVLM of SHRSP and WKY. The dose of tempol was chosen because there is a dose-response relationship between different doses of tempol (0.01, 0.1, and 1 nmol) and effects on MAP, as determined in a previous study,[27] and was used for subsequent microinjection experiments.

(2) To explore the role of superoxide in the RVLM in the regulation of the GABA release, tempol (0.01, 0.1, or 1 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the juxtapositional infusion cannula attached to the microdialysis probe, while recording AP, HR, and RSNA, and collecting the perfused dialysates. We chose the dose of tempol as 1 nmol/min for subsequent infusion experiments, because of the apparent sympathoinhibitory response to it.

(3) To explore the role of endogenous NO in the RVLM in the regulation of AP, HR, RSNA, and the GABA release, L-NMMA (10 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the juxtapositional infusion cannula. This dose of L-NMMA was chosen because the increases in MAP induced by infusion of 100 nmol (10 nmol/min for 10 min) and 1 μmol (100 nmol/min for 10 min) in the unilateral RVLM did not different (data not shown), and we considered that a total infusion of 100 nmol (10 nmol/min for 10 min) L-NMMA into the unilateral RVLM would be sufficient to inhibit the effects of NO in the RVLM.

(4) To explore the role of endogenous NO in the RVLM in superoxide-induced changes, tempol (1 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the cannula following the infusion of L-NMMA into the ipsilateral RVLM (10 nmol/50 nl/min for 10 min).

(5) To confirm that changes in AP, HR, and RSNA caused by infusion of tempol or L-NMMA for 10 min were the result of an increase or decrease in GABA release, bicuculline (200 pmol in 100 nl) was acute microinjected into the bilateral RVLM of SHRSP followed by acute microinjection of tempol (1 nmol in 100 nl) or L-NMMA (10 nmol in 100 nl). The dose of bicuculline was chosen because of the results in previous studies.[7,9] The dose of L-NMMA was chosen because we confirmed the pressor and sympathoexcitatory responses by infusion of 10 nmol/min L-NMMA in the unilateral RVLM at 1 min after the initiation.

(6) To investigate whether glutamatergic excitatory inputs into the RVLM are involved in superoxide-induced sympathoexcitation, a glutamate receptor antagonist, kynurenic acid (2.7 nmol in 100 nl) was acutely microinjected into the bilateral RVLM of SHRSP, followed by acute microinjection of tempol (1 nmol in 100 nl). This dose of kynurenic acid was chosen because of the results in previous studies.[7,35,36] Tempol was acutely microinjected at 20–30 min after the injection of kynurenic acid, because we confirmed that the decreases in AP and RSNA occurred rapidly and reached a peak value within 15–20 min after the acute microinjection of kynurenic acid into the bilateral RVLM, lasting for 40 min in the preliminary experiments.

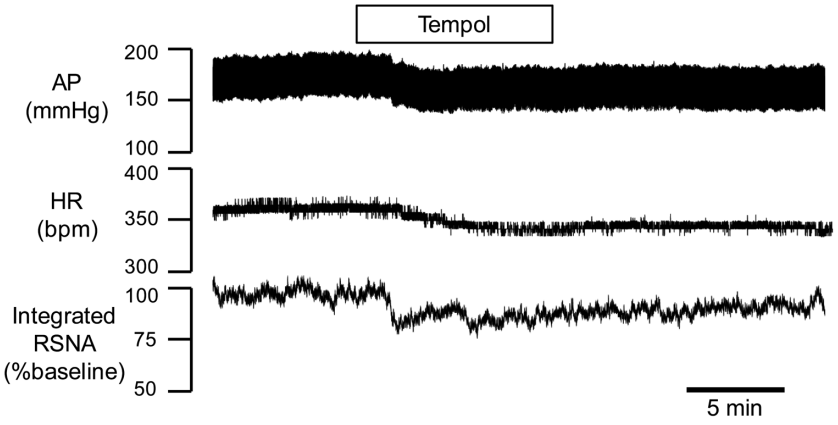

Figure 1. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. bpm indicates beats/min.

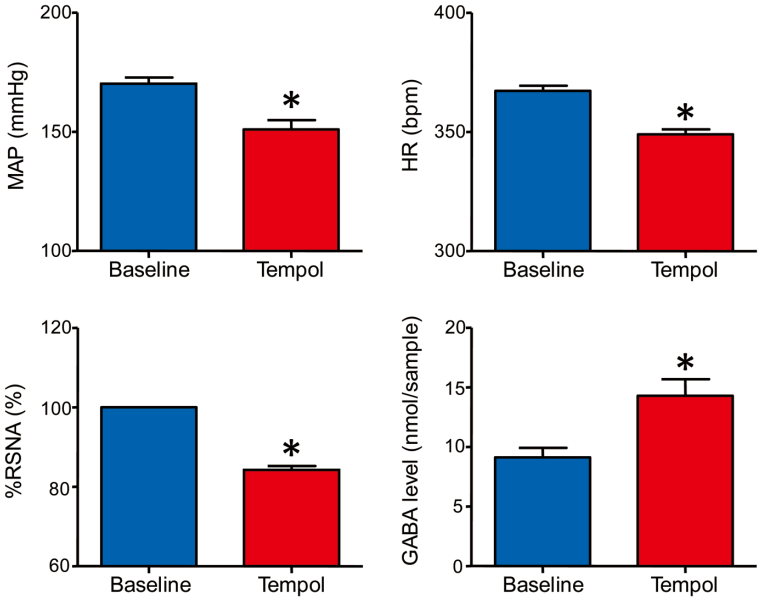

Figure 2. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the unilateral rostral ventrolateral medulla (RVLM) to infusion of tempol (1 nmol/50 nl/min for 10 min) of stroke-prone spontaneously hypertensive rats. Group data of tempol-induced changes in mean AP (MAP), HR, RSNA, and GABA levels in the RVLM. *P<0.01 (n=5 for each). bpm indicates beats/min.

Statistical Analysis

All values are expressed as the mean±SEM. A paired t-test was used to compare the changes in MAP, HR, RSNA, and GABA values with a few exceptions. An unpaired t-test was used to compare the baselines and changes in MAP, HR, and RSNA between SHRSP and WKY, and to compare the changes in MAP, HR, RSNA, and GABA values between the infusion of tempol and L-NMMA plus tempol during the experiments. Values of P<0.05 were considered significant.

Results

Effects of Tempol in the RVLM of SHRSP and WKY

Basal MAP and HR were significantly higher in SHRSP than in WKY (182±2 vs. 101±3 mmHg, 365±3 vs. 305±3 beats/min, P<0.01, n=5 for each). Acute microinjection of tempol into the bilateral RVLM decreased MAP, HR, and RSNA (ΔMAP, −33±8 mmHg; ΔHR, −29±5 beats/min; ΔRSNA %baseline, −19±2 %; n=5) in SHRSP, but not in WKY (ΔMAP, −4±1 mmHg; ΔHR, −3±1 beats/min; ΔRSNA %baseline, −3±1 %; n=5). The magnitude of the decreases in these variables was significantly greater in SHRSP than in WKY (P<0.01).

Effects of Tempol on GABA Levels in the RVLM of SHRSP

Infusion of tempol for 10 min into the unilateral RVLM decreased MAP (−19±2 mmHg from baseline 170±3 mmHg, n=5), HR (−18±2 beats/min from baseline 367±2 beats/min, n=5), and RSNA (−16±1%, n=5), and increased the level of GABA in the dialysates (5.2±1.0 nmol/sample from baseline 9.1±0.8 nmol/sample, n=5) in SHRSP (Figures 1,2).

Effects of L-NMMA in the RVLM of SHRSP

Infusion of L-NMMA for 10 min into the unilateral RVLM increased MAP (9±1 mmHg from baseline 169±2 mmHg, n=5), HR (15±1 beats/min from baseline 367±4 beats/min, n=5), and RSNA (5±1 %, n=5), and decreased the level of GABA in the dialysates (−1.1±0.2 nmol/sample from baseline 10.6±1.4 nmol/sample, n=5) in SHRSP (Figure 3).

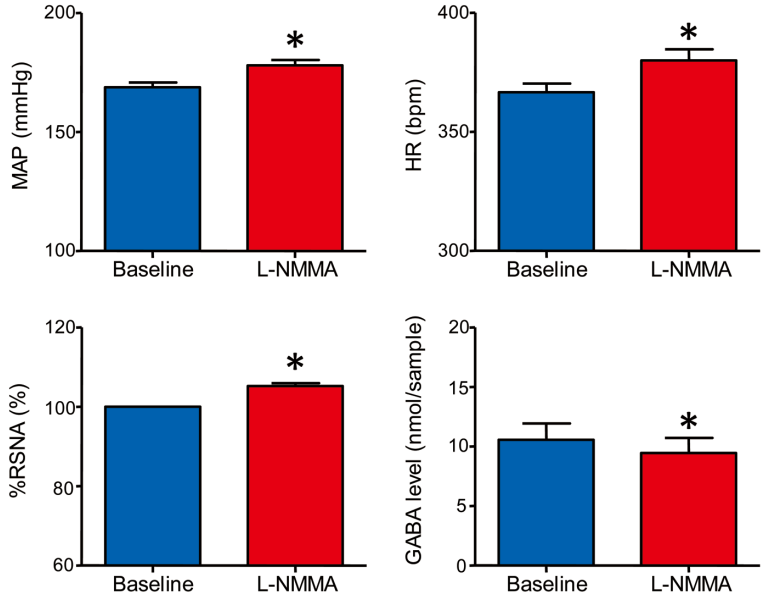

Figure 3. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the rostral ventrolateral medulla (RVLM) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats. Group data of L-NMMA-induced changes in mean AP (MAP), HR, RSNA, and GABA levels in the RVLM. *P<0.01 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

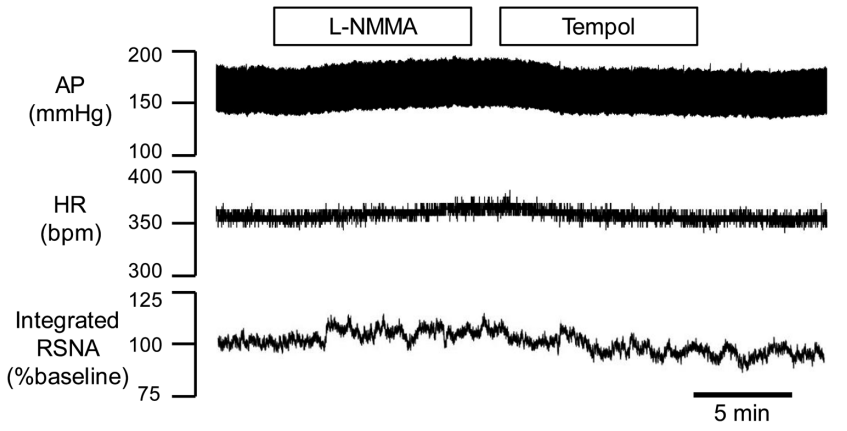

Figure 4. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) and subsequent tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

Effects of L-NMMA on the Responses to Tempol in RVLM of SHRSP

Following the infusion of L-NMMA for 10 min into the unilateral RVLM, infusion of tempol for 10 min into the ipsilateral RVLM decreased MAP (−11±1 mmHg from baseline 176±2 mmHg, n=5), HR (−11±2 beats/min from baseline 377±5 beats/min, n=5), and RSNA (−8±1 %, n=5), and increased the level of GABA in the dialysates (2.3±0.5 nmol/sample from baseline 9.5±1.3 nmol/sample, n=5) in SHRSP (Figures 4,5). The tempol-induced changes in these variables were significantly attenuated by prior infusion of L-NMMA (Figure 5). Although prior infusion of L-NMMA changed the basal values before the infusion of tempol, the percentage changes from baseline induced by tempol were also significantly attenuated by L-NMMA.

Effects of Bicuculline on the Responses to Tempol or L-NMMA in the RVLM of SHRSP

Prior acute microinjection of bicuculline into the bilateral RVLM of SHRSP attenuated the tempol-induced depressor and sympathoinhibitory responses and L-NMMA-induced pressor and sympathoexcitatory responses (Figure 6). Although prior acute microinjection of bicuculline changed the basal values before acute microinjection of tempol or L-NMMA, the percentage changes from baseline induced by tempol or L-NMMA were also significantly attenuated by bicuculline.

Effects of Kynurenic Acid on the Responses to Tempol in the RVLM of SHRSP

Acute microinjection of kynurenic acid into the bilateral RVLM significantly decreased MAP (−58±5 mmHg from baseline 182±4 mmHg, n=5), HR (−37±6 beats/min from baseline 361±3 beats/min, n=5), and RSNA (−25±3 %, n=5) in SHRSP. The depressor and sympathoinhibitory responses caused by the acute microinjection of tempol into the bilateral RVLM were unchanged between before and after acute microinjection of kynurenic acid (Figure 7). Although kynurenic acid changed the basal values before acute microinjection of tempol, the percentage changes from baseline induced by tempol were also unchanged.

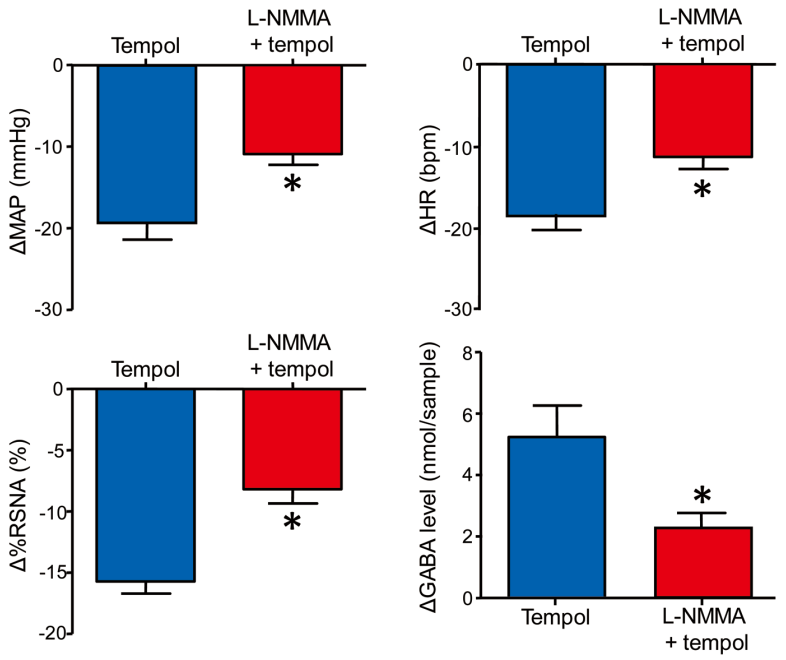

Figure 5. Effect of inhibition of nitric oxide (NO) production by L-NMMA (10 nmol/50 nl/min for 10 min) in the rostral ventrolateral medulla (RVLM) on the responses to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats (SHRSP). Prior infusion of L-NMMA attenuated the tempol-induced changes in mean arterial pressure (MAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the RVLM of SHRSP. *P<0.05 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

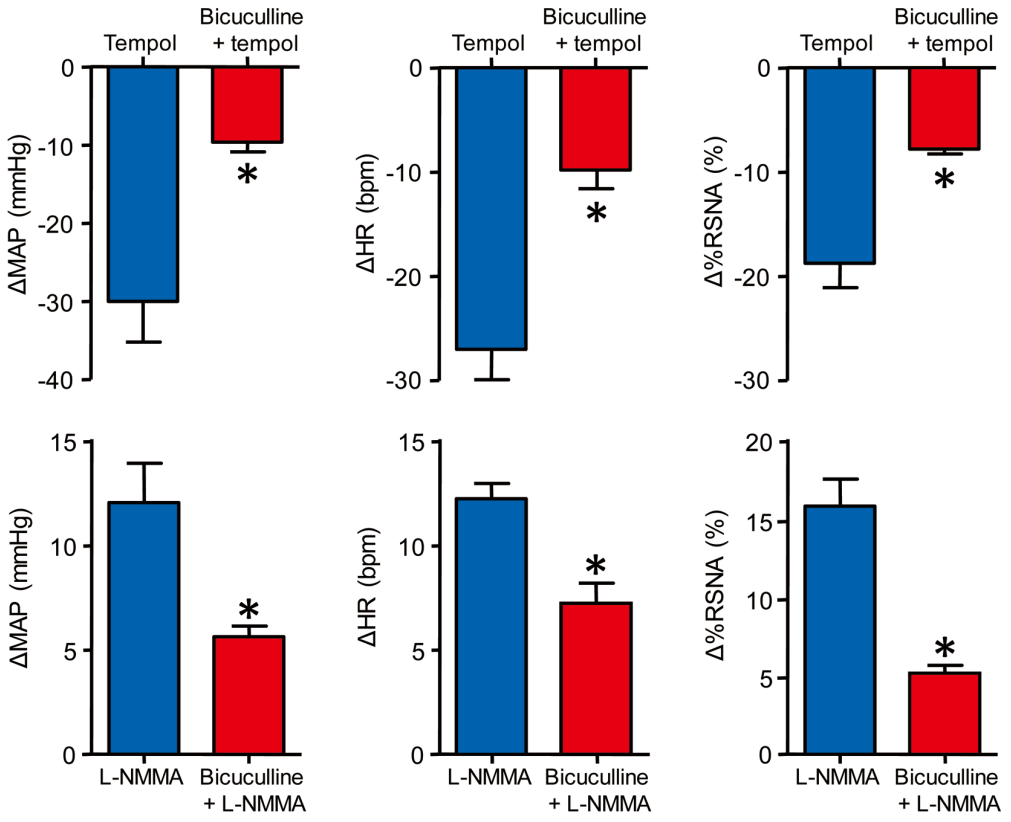

Figure 6. Effect of bicuculline (200 pmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) or L-NMMA (10 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior microinjection of bicuculline attenuated the tempol-induced and L-NMMA-induced changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA). *P<0.05 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

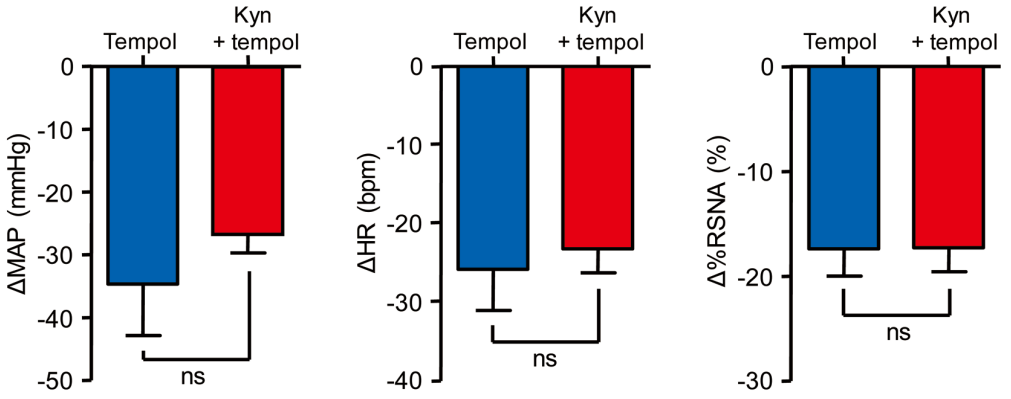

Figure 7. Effect of kynurenic acid (2.7 nmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior acute microinjection of kynurenic acid did not affect the tempol-induced changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) (n=5 for each). kyn, kynurenic acid; ns, not significant. bpm indicates beats/min.

Discussion

We have demonstrated 3 major findings. First, infusion of tempol into the RVLM increased GABA release in the RVLM with sympathoinhibition in SHRSP, and these responses were attenuated by prior infusion of bicuculline into the RVLM. Second, infusion of L-NMMA into the RVLM decreased GABA release in the RVLM with sympathoexcitation in SHRSP. Third, prior infusion of L-NMMA into the RVLM attenuated the tempol-induced increase in GABA release with sympathoinhibition in SHRSP. In particular, we were able to detect changes in GABA release using a microdialysis technique. Thus, in the present study, we provide the first evidence that superoxide inhibits NO-mediated GABA release in the RVLM of SHRSP, thereby increasing the activity of the SNS.

Many previous studies have indicated the microinjection of tempol, widely used as an antioxidant agent,[37] into the RVLM causes sympathoinhibition in animal models of hypertension, probably via reduction of superoxide.[26,27,38–40] We also confirmed that microinjection of tempol into the RVLM resulted in sympathoinhibition in SHRSP, but not in WKY, in the present study. In addition, we also showed that prior injection of bicuculline into the RVLM attenuated the tempol-induced decrease in MAP and HR with sympathoinhibition. These results suggest that the increase in GABA release in the RVLM caused by a reduction of superoxide is functionally relevant to sympathoinhibition. Moreover, in the present study, we confirmed that NO-mediated GABAergic inputs into the RVLM are involved in tonic inhibition of the SNS in SHRSP, consistent with the results from previous studies in normotensive rats.[7,41,42] NO acts on presynaptic terminals to increase vesicular GABA release,[17,20,21] although postsynaptic inhibitory effects of NO on neuronal firing have also been reported.[17,43]

In the present study, we also demonstrated that prior infusion of L-NMMA into the RVLM attenuated the tempol-induced increase in GABA release with sympathoinhibition in the RVLM of SHRSP. Although we did not measure superoxide production in the RVLM of SHRSP, these results suggest that a reduction of NO-mediated GABA release in the RVLM is involved in sympathoexcitation, probably induced by superoxide in SHRSP, because tempol is as effective as native SOD in preventing superoxide production.[37] It has been demonstrated that superoxide reacts with and inactivates NO and thereby modulates its bioavailability,[17,44] and that superoxide in the RVLM of hypertensive rats is increased compared with normotensive rats.[23,26–28] In addition, previous reports indicate that, in the RVLM, the modulatory effect of NO on GABA release and the interaction between superoxide and NO are respectively involved in the pathogenesis of hypertension in hypertensive rats.[9,26] Moreover, our findings suggest that superoxide suppresses NO-mediated GABAergic inhibition in the RVLM of SHRSP.

The RVLM is known to receive both excitatory and inhibitory inputs,[2,45] and glutamate is the major excitatory neurotransmitter. In the present study, tempol-induced sympathoinhibition was attenuated by prior injection of bicuculline, but not kynurenic acid, into the RVLM of SHRSP. Although we did not measure glutamate levels in the RVLM of SHRSP, these results suggest that GABAergic disinhibition might contribute to superoxide-induced sympathoexcitaion in SHRSP. Furthermore, the majority of GABAergic neuronal terminals in the RVLM come from the caudal ventrolateral medulla (CVLM) and inhibit the neuronal excitability of RVLM neurons.[13,15,45,46] It has also been reported that inhibition of the CVLM or blockade of its GABAergic inhibitory inputs to the RVLM by injection of bicuculline into the RVLM caused a smaller pressor response in SHR than in WKY.[10,14–16] In the present study, we focused only on superoxide and NO-mediated GABA release in the RVLM, because the effects of GABA in the RVLM are mainly caused by GABAergic inputs from the CVLM into the RVLM. However, further examination is necessary to clarify the relationship between the RVLM and CVLM in the pathway of superoxide-NO-GABA-sympathetic nerve activity. It would be important to investigate this issue further, because abnormal activation of the SNS is the target of treatments for various cardiovascular diseases.[47–49]

Study Limitations

First, we used L-NMMA, a non-selective inhibitor of all NOS isoforms (neuronal, endothelial, and inducible) and we could not explore the role of each isoform. These NOS isoforms generate superoxide depending on the availability of L-argi-

nine and tetrahydrobiopterine, a co-factor of NOS.[17,50] Second, the NO concentration and superoxide levels in the RVLM remain to be determined. It is still difficult to measure NO concentration in vivo,[51] causing it to be difficult to investigate the role of NO, and we could not conclude whether NO production itself or NO-mediated GABA release is reduced in the RVLM of SHRSP. Further studies are needed to develop a quantitative understanding of NO and superoxide. Third, we did the examinations in SHRSP only, not WKY. However, the aim of the present study was to investigate the further mechanisms by which superoxide in the RVLM causes sympathoexcitation in SHRSP. We speculate that infusion of tempol into the RVLM will not change GABA levels in the RVLM of WKY, because our recent previous study demonstrated that chronic inhibition of superoxide did not change the MAP and RSNA responses to microinjection of bicuculline into the RVLM of WKY.[36]

Conclusions

Our results suggest that a reduction of NO-mediated GABA release is partly involved in superoxide-induced sympathoexcitation in the RVLM of SHRSP. Superoxide-induced reduction of GABA release in the RVLM may play an important role in the mechanism of sympathoexcitation in SHRSP, and NO-mediated GABA release in the RVLM should be more of a focus in studies of the central mechanisms of sympathoexcitation, in addition to the direct action of superoxide in the brain.

Acknowledgments

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Disclosures

Conflict of Interest: None.

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