Increased Reactive Oxygen Species in Rostral Ventrolateral Medulla Contribute to Neural Mechanisms of Hypertension in Stroke-Prone Spontaneously Hypertensive Rats

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Background—Oxidative stress increases in hypertension. The aim of this study was to determine whether reactive oxygen species (ROS) are increased in the rostral ventrolateral medulla (RVLM) in the brainstem, where the vasomotor center is located, in stroke-prone spontaneously hypertensive rats (SHRSP), and, if so, to determine whether the increased ROS contribute to neural mechanisms of hypertension in SHRSP.

Methods and Results—We measured ROS levels in the RVLM of SHRSP and compared them with those in Wistar-Kyoto rats (WKY). Thiobarbituric acid–reactive substances were increased in SHRSP compared with WKY. ROS were measured by electron spin resonance (ESR) spectroscopy. The ESR signal decay rate in the RVLM of SHRSP was significantly increased compared with that in WKY, and this increase was abolished by dimethylthiourea (a hydroxyl radical scavenger). The increased ESR signal decay rate was reduced to the same extent in the presence of desferrioxamine, catalase, and Tiron, indicating that hydroxyl radicals are derived from superoxide anions and hydrogen peroxide. In addition, total superoxide dismutase (SOD) activity in the RVLM was decreased in SHRSP compared with WKY. Furthermore, bilateral microinjection of tempol into the RVLM decreased blood pressure in SHRSP but not in WKY, and MnSOD overexpression in the RVLM of SHRSP decreased blood pressure and inhibited sympathetic nerve activity.

Conclusions—These results suggest that superoxide anions in the RVLM, which generate hydroxyl radicals, are increased in SHRSP and contribute to the neural mechanisms of hypertension in SHRSP. (Circulation. 2004;109:2357-2362.)

Key Words: blood pressure ■ hypertension ■ brain ■ free radicals ■ nervous system, sympathetic

Reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals are implicated in the pathogenesis of hypertension. 1,2 The production of superoxide anions in aortic vessels is increased in spontaneously hypertensive rats (SHR). 3–6 In addition, the production of superoxide anions is increased in deoxycorticosterone acetate–salt hypertensive rats. 4 Dahl-salt hypertensive rats, 7 and stroke-prone SHR (SHRSP). 1,8 Inhibition of ROS reduces blood pressure in SHR. 9–11 Administration of 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol), a cell membrane–permeable superoxide dismutase (SOD) mimic, decreases blood pressure and renal vascular resistance in SHR 10 and significantly decreases urinary excretion of 8-isoprostaglandin F 2α, which is a marker of oxidative stress. 2 Chronic treatment with tempol prevents the progression of hypertension and vascular remodeling in salt-loaded SHRSP. 11 Furthermore, intravenous infusion of tempol decreases blood pressure, and these effects are mediated by the inhibition of renal sympathetic nerve activity. 12,13

Among the target organs of hypertensive vascular diseases, the brain is most affected by aging and oxidative stress. 14,15 The brain contains a high concentration of polyunsaturated fatty acids in its cell membranes. These fatty acids are targets of oxygen-derived free radicals, which cause chain reactions of lipid peroxidation. 14 Thio-barbituric acid–reactive substances (TBARS), end products of lipid peroxidation and an indirect marker of oxidative stress, are increased in the brains of SHR compared with those of Wistar-Kyoto rats (WKY). 15 There is no direct evidence, however, of an increase in ROS in the brain in hypertension. Furthermore, the effect of ROS in the brain on sympathetic nerve activity has not been determined in hypertension.

The rostral ventrolateral medulla (RVLM) is the vasomotor center that determines basal sympathetic nerve activity. 16 Thus, the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone. 16 Microinjection of SOD into the RVLM of anesthetized pigs produces moderate inhibitory effects on sympathetic nerve activity, and the effects of SOD are greatly enhanced in organic nitrate–treated pigs, in which neuronal oxidative stress was induced. 17 The
role of oxidative stress in the brain in hypertension is unknown.

The aims of this study were to determine whether ROS are increased in the RVLM of SHRSP and if so, to determine which ROS contributes to the increased oxidative stress in SHRSP. SHRSP is a model of hypertension in which sympathetic nerve activity is increased. In addition, we examined the effect of ROS in the RVLM on blood pressure by microinjection of tempol into the RVLM or MnSOD overexpression in the RVLM.

Methods
This study was reviewed and approved by the Committee of Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and was conducted according to the Guidelines for Animal Experiments of Kyushu University.

Animals and General Procedures
Male WKY/Izm and SHRSP/Izm (14 to 18 weeks old, SLC Japan, Hamamatsu, Japan) were used. To obtain RVLM or nucleus tractus solitarii (NTS) tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with PBS (150 mmol/L NaCl, 3 mmol/L KCl, and 5 mmol/L phosphate; pH 7.4, 4°C). The brains were removed quickly, and sections 1 mm thick were obtained with a cryostat at −7 ± 1°C. The RVLM and NTS were defined according to a rat brain atlas, and the NTS and RVLM tissue was obtained by use of a punch-out technique.

Measurement of TBARS
The RVLM or NTS tissues were homogenized in 1.15% KCl (pH 7.4) and 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 minutes, followed by heating to 100°C for 60 minutes. After cooling, the mixture was extracted with distilled water and n-butanolpyridine (15:1) and centrifuged at 1600 g for 10 minutes. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by absorbance with a molecular extinction coefficient of 156 000 and expressed as μmol/g wet wt.

Electron Spin Resonance Spectroscopy
Electron spin resonance (ESR) spectroscopy measurements were performed at room temperature with an X-band (9.45-GHz) ESR spectrometer (JES-RE-1X; Joel) at the following settings: microwave power of 10 mW, external magnetic field range of 20 mT, and scan rate of 10 mT/min. The amount of ROS was quantified by monitoring the time-dependent decay of the amplitude of the ESR spectra elicited by the nitroxide radical 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO) as a spin probe.

Snap-frozen RVLM was homogenized in 50 mmol/L PBS containing protease inhibitors (leupeptin 10 μg/mL, phenylmethylsulfonyl fluoride 100 μg/mL, diethiothreitol 1 mmol/L, and trypsin inhibitor 10 μg/mL). The homogenate was mixed quickly with hydroxy-TEMPO (0.1 mmol/L) in PBS and drawn into glass tubes. ESR spectra were recorded for up to 10 minutes at 10-second intervals. The formation of hydroxyl radicals was confirmed by adding dimethylthiourea (DMTU; 50 mmol/L) to the reaction mixture. The contribution of superoxide and hydrogen peroxide was examined in the presence of 4,5-dihydroxy-1,3-benzenediol (Tiron; 100 μmol/L) and catalase (50 U/mL) as competitive reagents, respectively. The contribution of the interaction of superoxide and hydrogen peroxide through the iron-catalyzed Haber-Weiss reaction and/or the interaction of hydrogen peroxide and iron through the Fenton reaction was examined in the presence of the iron chelator desferrioxamine (DFO; 0.5 mmol/L).

Determination of SOD Activity in the RVLM
Total SOD or MnSOD activity was assayed by monitoring the inhibition of the rate of xanthine/xanthine oxidase-mediated reduction of cytochrome c (pH 7.4). The RVLM tissues were obtained as described above, and snap-frozen RVLM tissues were homogenized in 50 mmol/L PBS containing protease inhibitors.

Microinjection of Tempol into the RVLM
To confirm the role of ROS in the RVLM in blood pressure regulation, tempol, which is a stable, metal-independent, membrane-permeable SOD mimetic, was microinjected bilaterally into the RVLM of SHRSP and WKY that were anesthetized with sodium pentobarbital (50 mg/kg IP followed by 20 mg·kg⁻¹·h⁻¹ IV), as described previously. A catheter was inserted into the femoral artery to record arterial blood pressure. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. The rats were placed in a stereotaxic frame. A glass micropipette was filled with tempol or L-glutamate in PBS and positioned at the injection site. Before the microinjection of tempol, the RVLM was identified by monitoring the mean arterial pressure (MAP) after injection of a small dose of L-glutamate. The identification of the RVLM was confirmed as described previously.

In Vivo Gene Transfer of MnSOD into the RVLM
To confirm the role of ROS in the RVLM in the regulation of blood pressure in a conscious state, we transfected adenovirus vectors encoding the MnSOD gene (AdMnSOD) into the bilateral RVLM. The vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa. The method of transfection was described previously. An adenoviral suspension containing 1 × 10⁹ plaque-forming units (pfu)/mL was injected into each injection site over a period of 15 minutes (500 nL/site). The UA-10 telemetry system (Data Sciences International) was used to measure MAP and heart rate (HR), as described previously. Urinary norepinephrine concentration was measured before the gene transfer and at day 10 after the gene transfer, and we calculated the urinary norepinephrine excretion for 24 hours as an indicator of sympathetic nerve activity, as described previously. Urinary norepinephrine excretion was measured before the gene transfer and at day 10 after the gene transfer. In the Western blot analysis for MnSOD protein from tissues containing the injected sites of the RVLM and other sites obtained by the punch-out technique were performed before and at day 10 after the gene transfer. In the Western blot analysis for MnSOD protein, we used mouse IgG monoclonal antibody to MnSOD (1:2500, Transduction Laboratories).

Statistical Analysis
All values were expressed as the mean ± SEM. One-way ANOVA was used to compare the ESR signal decay rate in WKY and SHRSP in mannose in conjunction with a post hoc test with Scheffé’s correction. An unpaired t test was used to compare the magnitude of changes in MAP caused by bilateral microinjection of tempol into the RVLM and by MnSOD overexpression in the RVLM between WKY and SHRSP and to compare the urine norepinephrine excretion before and after gene transfer. Values of P < 0.05 were considered significant.

Results
TBARS Levels in the Brain Tissues
TBARS levels were significantly higher in the whole brain, NTS, and RVLM of SHRSP compared with WKY (whole brain, 0.83 ± 0.05 versus 0.38 ± 0.07 μmol/g wet wt, P < 0.05, n = 5; RVLM, 0.74 ± 0.04 versus 0.23 ± 0.05 μmol/g wet wt, P < 0.05, n = 5; NTS, 0.48 ± 0.02 versus 0.19 ± 0.06 μmol/g wet wt, P < 0.05, n = 5; Figure 1).
ROS Production in the RVLM

The intensity of ESR signals decreased more rapidly in SHRSP than in WKY (P<0.05, n=7 for each) (Figure 2A). There was a linear relation of the semilogarithmic plot of peak signal intensity versus time (Figure 2B). The rate of signal decay, calculated from the slope of this line, was significantly higher in SHRSP than in WKY (0.051±0.004 versus 0.018±0.003, P<0.05, n=7 for each) (Figure 2C). In the absence of RVLM tissues, ESR signals did not decline during the study period. DMTU abolished the increase in the signal decay rate in SHRSP, confirming an enhanced generation of hydroxyl radicals (0.023±0.003 versus 0.051±0.004, P<0.05, n=7 for each) (Figure 2C). DFO inhibited the increase of signal decay in SHRSP (0.022±0.003 versus 0.051±0.004, P<0.05, n=7 for each) (Figure 2C). DFO inhibited the increase of signal decay in SHRSP (0.022±0.003 versus 0.051±0.004, P<0.05, n=7 for each) (Figure 2C). Catalase also attenuated the increased signal decay rate in SHRSP (0.028±0.002 versus 0.051±0.004, P<0.05, n=7 for each) (Figure 2C). In SHRSP, Tiron was similarly effective in attenuating the increase of the signal decay (0.025±0.003 versus 0.051±0.004, P<0.05, n=7 for each) (Figure 2C). In WKY, DMTU, catalase, DFO, and Tiron had no effect on the rate of signal decay.

Total SOD Activity in the RVLM

There was significantly less total SOD activity in the RVLM of SHRSP compared with WKY (2.8±0.5 versus 4.4±0.3 U/mg, P<0.05, n=5).

Microinjection of Tempol Into the RVLM

Basal MAP and HR were significantly higher in SHRSP than in WKY (170±8 versus 112±7 mm Hg, 371±10 versus 338±12 bpm, P<0.05, n=5 for each). Microinjection of tempol (100 pmol) into the RVLM decreased MAP (−37±3 mm Hg, n=5) and HR (−40±7 bpm, n=5) in SHRSP but not in WKY (−7±6 mm Hg, −4±5 bpm, n=5 for each). These responses were dose-dependent in SHRSP. Even after normalization of MAP and HR, the magnitude of the decrease in these variables was significantly greater in SHRSP than in WKY (Figure 3). Because the MAP and HR baseline values before microinjection were different in SHRSP and WKY, the changes in MAP/basal MAP and HR/basal HR were expressed as a measure of changes in MAP and HR.

MnSOD Overexpression in the RVLM

Western blot analysis revealed that MnSOD expression was significantly increased in the tissue from the RVLM of the AdMnSOD-treated SHRSP to the same level as WKY at day 10 after the gene transfer (Figure 4A). Peak MnSOD expression occurred at day 10 after the gene transfer. MnSOD activity was also increased in the RVLM tissue of AdMnSOD-transfected SHRSP at day 10 after the gene transfer (Figure 4B). TBARS levels were significantly decreased in the RVLM of AdMnSOD-transfected SHRSP compared with nontreated SHRSP (0.38±0.06 versus 0.74±0.04 μmol/g wet wt, P<0.05, n=5, Figure 4C). TBARS levels were not changed significantly in the RVLM of AdMnSOD-transfected WKY compared with nontreated WKY (Figure 4C). At day 10 after the gene transfer, MAP and HR of AdMnSOD-transfected SHRSP were significantly decreased compared with nontreated SHRSP but not WKY (Figure 5A). Urinary norepinephrine excretion was significantly higher in SHRSP than in WKY before the gene transfer. At day 10 after the gene transfer, urinary norepi-

Figure 1. Lipid peroxidation as indicated by TBARS levels in whole brain, RVLM, and NTS tissues from WKY and SHRSP (n=5 for each). *P<0.01 vs WKY.

**Figure 2.** ESR analysis of hydroxy-TEMPO in RVLM tissues. A, Sequential sample of ESR spectra of hydroxy-TEMPO in RVLM tissues from WKY (lower spectra) and SHRSP (upper spectra). B, Semilogarithmic plots of peak heights of ESR spectra of hydroxy-TEMPO in RVLM tissues from WKY (open circle) and SHRSP (closed circle). C, Summary data for signal decay rate in RVLM tissues from WKY and SHRSP and effects of an oxygen radical scavenger on signal decay rate in SHRSP. *P<0.01 vs WKY. #P<0.01 vs SHRSP without scavengers.
nephrine excretion was significantly decreased in AdMnSOD-transfected SHRSP but not in WKY (Figure 5B).

**Discussion**

The present study examined ROS levels in the RVLM, a vasomotor center, in a rodent model of hypertension. The results indicated that there was an increased generation of superoxide anions in the RVLM of SHRSP compared with WKY. SOD activity in the RVLM was significantly decreased in SHRSP compared with WKY. Tempol-induced inhibition of superoxide anion production in the RVLM decreased blood pressure in SHRSP but not in WKY. Furthermore, MnSOD overexpression in the bilateral RVLM decreased TBARS levels in the RVLM, MAP, HR, and sympathetic nerve activity in the SHRSP but not in WKY in a conscious state. These results suggest that increases in oxidative stress in the RVLM contribute to central nervous system mechanisms of hypertension in SHRSP.

TBARS measurement and ESR spectroscopy with hydroxy-TEMPO were used to quantify ROS in the RVLM. TBARS levels are widely used as an indirect marker of oxidative stress. In the present study, TBARS levels were significantly increased in the RVLM of SHRSP compared with WKY. TBARS levels were also increased in whole brain and NTS, indicating that the increase in oxidative stress in hypertension was not specific to the RVLM. Previous studies reported that intracerebroventricular infusion of tempol did not alter blood pressure in SHR. Zanazinger et al., however, demonstrated that chronic oxidative stress in the RVLM increases sympathetic nerve activity, suggesting that this site plays an important role in modulating sympathetic nerve activity by an increase in ROS.

The results of ESR spectroscopy suggested that the generation of superoxide anions and then hydroxyl radicals within the RVLM was enhanced in the RVLM of SHRSP compared with that of WKY. DMTU abolished the increase in the signal decay rate in SHRSP, confirming an enhanced generation of superoxide anions. DFO inhibited the increase of signal decay in SHRSP, suggesting that highly reactive hydroxyl radicals are generated by the interaction of superoxide anions and hydrogen peroxide through the iron-catalyzed Harber-Weiss reaction and/or by the interaction of hydrogen peroxide and iron through the Fenton reaction. Catalase also attenuated the increased signal decay rate in SHRSP, indicating that hydrogen peroxide is involved in the production of hydroxyl radicals. Furthermore, Tiron and catalase were similarly effective in attenuating the increase of the signal decay, suggesting that superoxide anions contributed to the production of hydrogen peroxide. DMTU, catalase, DFO, and Tiron had no effect on the signal decay rate in WKY. Taken together, these results suggest that the production of superoxide anions in the RVLM is enhanced in SHRSP compared with WKY and that superoxide anions are converted into hydrogen peroxide and hydroxyl radicals are formed via an iron-catalyzed reaction.

Microinjections of tempol into the RVLM decreased blood pressure in a dose-dependent manner in SHRSP but not in WKY. It is possible that the depressor response in SHRSP is caused by structural changes of the blood vessels in hypertension. This possibility is unlikely, however, because other studies that examined the effect of microinjections of kynurenic acid (a glutamate receptor antagonist), muscimol, or glycine into the RVLM of SHR or WKY reported specific responses evoked by several agonists or antagonists. Importantly, tempol reduced MAP and HR only in SHRSP. In addition, even after normalization of the changes in blood pressure to the baseline blood pressure, the depressor response observed in SHRSP was still greater than in WKY (Figure 3, C and D). Therefore, our observations suggest that the tempol-induced decrease in blood pressure and HR is mediated by a decrease in sympathetic nerve activity.

MnSOD overexpression in the RVLM increased MnSOD expression and activity and decreased TBARS levels in the RVLM of SHRSP. Moreover, MAP, HR, and urinary norepi-
nephrine excretion were decreased in SHRSP by MnSOD overexpression in the RVLM. These results strongly suggest that the inhibition of oxidative stress caused by MnSOD overexpression inhibits sympathetic nerve activity and that the increase in oxidative stress in the RVLM contributes to the increase in sympathetic nerve activity in SHRSP. A previous study reported that MnSOD and Cu/ZnSOD are present in the rat brain.\textsuperscript{15} The purpose of the present experiment was to examine the effect of the inhibition of the production of superoxide anions in RVLM in a conscious state and whether MnSOD overexpression in the RVLM of SHRSP decreases TBARS levels in the RVLM to the same level as WKY. Inhibition of oxidative stress by MnSOD overexpression in the RVLM of SHRSP decreased MAP, HR, and sympathetic nerve activity, suggesting that an increase in oxidative stress in the RVLM of SHRSP increases sympathetic nerve activity in a conscious state.

The mechanisms by which the increase in ROS in the RVLM increased sympathetic nerve activity and blood pressure are not known. These responses might be mediated by an interaction between superoxide and NO. Superoxide anions react rapidly with NO, forming peroxynitrite and decreasing the bioavailability of NO.\textsuperscript{28} Consistent with these observations, NO in the RVLM causes hypotension and sympathoinhibition.\textsuperscript{18,20} Thus, the increase in superoxide anion levels in the RVLM might decrease the bioavailability of NO in the RVLM. This mechanism might contribute to the increase in sympathetic nerve activity and hypertension.

It remains to be determined whether the increased ROS in the RVLM in the present study is cause or effect. The sources of increased superoxide anions in the RVLM of SHRSP were not addressed in the present study. Many complex pathways produce superoxide anions in the brain.\textsuperscript{29} The decrease in SOD activity in the RVLM of SHRSP compared with WKY might contribute to the increased superoxide anions. We also did not examine whether oxidative stress increases the neuronal activity of RVLM neurons. Further study is needed to clarify these important questions.

In conclusion, the present study demonstrates that generation of superoxide anions and then hydroxyl radicals is enhanced in the RVLM of SHRSP and that inhibition of the production of superoxide in the RVLM markedly decreases arterial blood pressure in SHRSP in an anesthetized or conscious state. Taken together, these results suggest that oxidative stress in the RVLM increases blood pressure, which might be caused by an increase in sympathetic nerve activity.

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