Inhibition of Rac1-Derived Reactive Oxygen Species in Nucleus Tractus Solitarius Decreases Blood Pressure and Heart Rate in Stroke-Prone Spontaneously Hypertensive Rats

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Abstract—Reactive oxygen species (ROS) in the brain are thought to contribute to the neuropathogenesis of hypertension by enhancing sympathetic nervous system activity. The nucleus tractus solitarius (NTS), which receives afferent input from baroreceptors, has an important role in cardiovascular regulation. Reduced nicotinamide adenine dinucleotide phosphate oxidase is thought to be a major source of ROS in the NTS. Rac1 is a small G protein and a key component of reduced nicotinamide adenine dinucleotide phosphate oxidase. The role of Rac1-derived ROS in the NTS in cardiovascular regulation of hypertension is unknown. Therefore, we examined whether inhibition of Rac1 in the NTS decreases ROS generation, thereby reducing blood pressure in stroke-prone spontaneously hypertensive rats (SHRSPs). The basal Rac1 activity level in the NTS was greater in SHRSPs than in Wistar-Kyoto rats. Inhibition of Rac1, induced by transfecting adenovirus vectors encoding dominant-negative Rac1 into the NTS, decreased blood pressure, heart rate, and urinary norepinephrine excretion in SHRSPs but not in Wistar-Kyoto rats. Inhibition of Rac1 also reduced nicotinamide adenine dinucleotide phosphate oxidase activity and ROS generation. In addition, Cu/Zn-superoxide dismutase activity in the NTS of SHRSPs was decreased compared with that of Wistar-Kyoto rats, despite the increased ROS generation. Overexpression of Cu/Zn-superoxide dismutase in the NTS decreased blood pressure and heart rate in SHRSPs. These results indicate that the activation of Rac1 in the NTS generates ROS via reduced nicotinamide adenine dinucleotide phosphate oxidase in SHRSPs, and this mechanism might be important for the neuropathogenesis of hypertension in SHRSPs. (Hypertension. 2007;50:62-68.)

Key Words: blood pressure ■ heart rate ■ sympathetic nervous system ■ hypertension ■ brain

There is accumulating evidence that reactive oxygen species (ROS) in the cardiovascular regulatory nuclei in the brain have a crucial role in blood pressure regulation in hypertension via modulating the sympathetic nervous system.1-5 Reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase is a major source of ROS in hypertension6 and has a critical role in generating ROS in the brain.2,5-7 Rac1 is a small G protein that is an important signaling molecule involved in integrating intracellular transduction pathways toward NAD(P)H oxidase activation.2,8,9 Rac1 requires lipid modifications to migrate from the cytosol to the plasma membrane, which is a necessary step for activating the ROS-generating NAD(P)H oxidase enzyme system.8,9

The nucleus tractus solitarius (NTS) in the brain stem has an important role in cardiovascular regulation.10-16 The NTS receives afferent input from baroreceptors and chemoreceptors12 and has reciprocal interconnections with other nuclei involved in central autonomic regulation.17 In addition, the essential NAD(P)H oxidase subunit gp91phox is present in somatodendritic and axonal profiles that contain angiotensin II (Ang II) subtype 1 receptors in the NTS, and Ang II increases ROS generation via NAD(P)H oxidase in NTS neurons in vitro.7 The role of Rac1 and its derived ROS in the NTS in cardiovascular regulation of hypertension in vivo, however, is not known. Therefore, the aim of the present study was to determine the effects of the inhibition of Rac1 in the NTS on cardiovascular regulation of hypertension in the awake state. For this purpose, we transfected an adenovirus vector dominant-negative Rac1 into the NTS of stroke-prone spontaneously hypertensive rats (SHRSPs) and compared the effects with those in normotensive Wistar-Kyoto rats (WKYs).

Methods
An expanded Methods section is available in the online data supplement at http://hyper.ahajournals.org.
Rac1 and NAD(P)H oxidase activities are elevated in SHRSPs but not in WKYs. A, Rac1 activity in the NTS. The top panel shows a representative Western blot of Rac1-GTP bound to glutathione S-transferase–Pak1. Quantification of Rac1-GTP activity expressed as the relative ratio to control (WKYs), which was assigned a value of 1 (bottom). Rac1 activity in the NTS of SHRSPs was significantly increased compared with that of WKYs. Furthermore, dominant-negative Rac1 gene transfer significantly attenuated Rac1 activity in the NTS of SHRSPs. Lysates incubated with GDP or γ-GTP served as negative and positive controls, respectively (n=4 for each; *P<0.01 vs WKY; #P<0.05 vs SHRSP). B, NAD(P)H oxidase activity evaluated by lucigenin chemiluminescence in the NTS. NAD(P)H-dependent superoxide production was significantly higher in SHRSPs than in WKYs. Furthermore, dominant-negative Rac1 gene transfer significantly attenuated NAD(P)H oxidase activity in the NTS of SHRSPs (WKY rats=34.2±4.1; SHRSP=59.6±1.5; AdN17Rac1-transfected SHRSP=39.2±5.8 relative fluorescence units/mg/s; n=5 for each; *P<0.05 vs WKY; #P<0.05 vs SHRSP).

Animals and General Procedures
Male SHRSPs and WKYs (280 to 340 g; 14 to 18 weeks old) were obtained from SLC Japan (Hamamatsu, Japan). The 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.

Rac1 Activation Assays
Rac1 activity can be monitored by its interaction with p21-activated kinase (PAK), which only occurs when Rac1 is active.2,3 We used a Rac1 Activation kit (Upstate Biotechnology) to evaluate Rac1 activity in the NTS.

NAD(P)H-Dependent Superoxide Production
NAD(P)H-dependent superoxide production in the NTS was measured by lucigenin luminescence.8,18,19 A luminescence assay was performed in a balanced salt solution buffer containing 5 μmol/L of lucigenin (Sigma) using a luminescence reader (PerkinElmer). The reaction was started by adding 100 μmol/L of β-NAD(P)H (Sigma) as the substrate.

In Vivo Gene Transfer Into the NTS
We used adenoviral vectors encoding dominant-negative HA-tagged Rac1 (AdN17Rac1),2 human Cu/Zn-superoxide dismutase (SOD; AdCu/ZnSOD),20,21 and β-galactosidase (AdLacZ). The vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa. We transfected AdN17Rac1, AdCu/ZnSOD, and AdLacZ into the NTS as described previously.10,11 A telemetry system (DATA Sciences International) was used to measure mean blood pressure (MBP) and heart rate (HR).1,3,10,11 On day 7 after gene transfer, we calculated the 24-hour urinary norepinephrine excretion as an indicator of sympathetic nerve activity.1,3,10,11

Analysis of Gene Expression
To confirm the expression and localization of gene transfer in the NTS, we performed immunohistochemical staining for human Cu/Zn-SOD and β-galactosidase. To identify the cell types that were transfected by the adenovirus used in the present study, we performed double immunohistochemical staining for β-galactosidase and a neuronal marker (NeuN; Chemicon International Inc).22 Western blot analysis was performed using rabbit anti-SOD-1 polyclonal IgG (1:10 000, Santa Cruz Biotechnology), mouse anti-hemagglutinin (HA) monoclonal IgG (1:10 000, Sigma), or rabbit anti-β tubulin polyclonal IgG (1:10 000, Santa Cruz Biotechnology).

In Situ Detection of Superoxide
Brain superoxide anion levels were estimated by dihydroethidium (DHE) staining.3,5 Coronal sections (10 μm) were incubated for 10 minutes with the O2·− specific fluorogenic probe DHE (1 μmol/L, Sigma) at 37°C.

Thiobarbituric Acid–Reactive Substances
We examined thiobarbituric acid-reactive substance (TBARS) levels in the NTS as an indicator of oxidative stress, as described previously.1,3

Measurement of SOD Activity
Cu/Zn-SOD activity was assayed by monitoring the inhibition of the rate of xanthine/xanthine oxidase–mediated reduction of cytochrome c, as described previously.1

Statistical Analysis
All of the values were expressed as the mean±SEM. P<0.05 was considered significant.

Results
Rac1/NAD(P)H Oxidase Pathway Is Activated in the NTS of SHRSPs
Rac1-GTP levels were assessed as an index of Rac1 activation using a glutathione S-transferase–PAK pull-down assay. These studies revealed that Rac1 activity in the NTS of SHRSPs was significantly higher than in the NTS of WKYs (Figure 1A). Consistent with increased Rac1 activation, NAD(P)H-dependent superoxide production was also significantly higher in the NTS from SHRSPs than in the NTS from WKYs (Figure 1B). Gene transfer of AdN17Rac1 into the NTS of SHRSPs suppressed both Rac1/PKA binding (Figure 1A) and NAD(P)H oxidase activity (Figure 1B).

Effect of Rac1 Inhibition and Cu/Zn-SOD by Adenovirus-Mediated Gene Transfer
Western blot analysis of HA-tag, a marker of AdN17Rac1, was performed on tissue samples taken from rats on days 0,
3, 5, 7, 10, and 14 after gene transfer (n=3 per day), and representative images are shown in Figure 2A. The HA-tag expression level was significantly increased and peaked on day 7 after AdN17Rac1 transfection. We performed immunochemistry to examine the localization and distribution of adenoviral-mediated gene transfer. Immunohistochemical analysis on day 7 after gene transfer revealed localized human Cu/Zn-SOD (Figure 2B) or β-galactosidase gene expression (Figure 2C) in the NTS. Double staining of β-galactosidase and NeuN confirmed that some NeuN-positive cells expressed β-galactosidase protein, although NeuN-negative cells also expressed β-galactosidase protein (Figure 2D). AdN17Rac1-transfected SHRSPs exhibited a significant decrease in MBP and HR (Figure 3A). MBP and HR did not change in AdLacZ-transfected SHRSPs (Figure 3C). Urinary norepinephrine excretion measured on day 7 after gene transfer was significantly decreased in AdN17Rac1-transfected SHRSPs relative to that in non-treated SHRSPs (Figure 4A). In addition, overexpression of Cu/Zn-SOD in the NTS of SHRSPs decreased MBP, HR (Figure 3B), and urinary norepinephrine excretion (Figure 4A). In contrast, AdN17Rac1 and AdCu/ZnSOD transfection into the NTS of WKYs did not affect MBP, HR (Figure S1), or urinary norepinephrine excretion (Figure 4B).

Oxidative Stress in the NTS
Confocal analysis of DHE fluorescence was used to estimate superoxide levels in the NTS. We examined 4 groups (WKY, SHRSP, AdN17Rac1-transfected SHRSP, and AdCu/ZnSOD-transfected SHRSP; n=5 for each), and representative images are shown in Figure 5A. There was a significant increase in DHE fluorescence in sections that contained the NTS of SHRSPs compared with sections of the NTS of WKYs. Furthermore, DHE fluorescence in the NTS was significantly attenuated in both AdN17Rac1-transfected SHRSPs and AdCu/ZnSOD-transfected SHRSPs (Figure 5A). TBARS levels were also significantly higher in the NTS of SHRSPs than in the NTS of WKYs (Figure 5B). Gene transfer of either AdN17Rac1 or AdCu/ZnSOD suppressed TBARS levels in the NTS (Figure 5B), suggesting that the TBARS increase was the result of enhanced superoxide generation.

Expression and Activity of Cu/Zn-SOD in the NTS of SHRSPs
Western blot analysis revealed that the expression of a \( \approx 17\)-kDa isoform of rat Cu/Zn-SOD protein in the NTS was decreased in SHRSPs compared with WKYs (Figure 6A). There was also significantly less total SOD activity (5.9±0.3 versus 4.9±0.1 U/mg; \( P < 0.05; n=5 \)) and Cu/Zn-SOD activ-
ity in the NTS of SHRSPs compared with the NTS of WKYs (3.0±0.1 versus 2.5±0.2 U/mg; *P<0.05; n=5 for each; Figure 6B). The human Cu/Zn-SOD gene, which we used in the present study, produces a ~19-kDa isoform of human Cu/Zn-SOD protein. The NTS tissues from AdCu/ZnSOD-transfected SHRSPs on day 7 after transfection had a clear band representing human Cu/Zn-SOD. Human HeLa cells served as a positive control. The bands representing the expression of endogenous Cu/Zn-SOD at ~17 kDa were identical to those in AdCu/ZnSOD-transfected SHRSPs. AdLacZ-transfected SHRSPs did not produce human protein. We examined 5 individual AdCu/ZnSOD-transfected SHRSPs, and representative images are shown in Figure 6C. The increased Cu/Zn-SOD activity in the NTS of AdCu/ZnSOD-

**Figure 3.** Rac1-dependent superoxide production elevates MBP and HR in SHRSP. Time course of MBP and HR in AdN17Rac1-transfected SHRSPs (A), AdCu/ZnSOD-transfected SHRSPs (B), and AdLacZ-transfected SHRSPs (C) before and after gene transfer (AdN17Rac1-transfected SHRSP, n=5; AdCu/ZnSOD-transfected SHRSP, n=6; AdLacZ-transfected SHRSP, n=6; *P<0.05 vs before gene transfer).

**Figure 4.** Rac1-dependent superoxide production elevates urinary norepinephrine excretion in SHRSPs but not in WKYs. A, 24-hour urinary norepinephrine excretion as an indicator of sympathetic nerve activity. Urinary norepinephrine excretion on day 7 after gene transfer was significantly decreased in AdN17Rac1-transfected SHRSPs and AdCu/ZnSOD-transfected SHRSPs (SHRSP=1.7±0.1 μg per day; AdN17Rac1-treated SHRSP=1.4±0.1 μg per day; AdCu/ZnSOD-transfected SHRSP=1.2±0.2 μg per day; n=6 for each; *P<0.05 vs SHRSP). B, 24-hour urinary norepinephrine excretion in WKY rats. We did not detect any changes among WKY rats, AdN17Rac1-transfected WKY rats, and AdCu/ZnSOD-transfected WKY rats (WKY rats=0.8±0.1 μg per day; AdN17Rac1-transfected WKY rats=1.0±0.1 μg per day; AdCu/ZnSOD-transfected WKY rats=0.9±0.2 μg per day; n=6 for each).
transfected SHRSPs (2.5±0.2 versus 3.5±0.4 U/mg; *P<0.05; n=5 for each; Figure 6D) indicated that human Cu/Zn-SOD was bioactive in rat tissues in vivo.

Discussion

The major findings of the present study are that inhibition of Rac1 expression in the NTS decreased blood pressure, HR, and urinary norepinephrine excretion in awake SHRSPs. These effects were not observed in normotensive WKYs. Rac1 activity was increased in the NTS of SHRSPs compared with WKYs. Subsequent activation of NAD(P)H oxidase and ROS production in the NTS were also increased in SHRSPs compared with those in WKYs. These results indicate that activation of Rac1 in the NTS leads to ROS generation via NAD(P)H oxidase activation in awake SHRSPs. The present study provides the first evidence that Rac1 is activated in the NTS of SHRSPs and results in enhanced NAD(P)H oxidase activity. More importantly, the subsequent ROS generation leads to increases in blood pressure and HR via the sympathetic nervous system in awake SHRSPs.

Transfection of AdN17Rac1 into the NTS successfully decreased Rac1 activity and NAD(P)H oxidase activity in the NTS of SHRSPs. It also attenuated the subsequent ROS generation, as evaluated by the DHE staining and TBARS levels. In addition, transfection of Cu/Zn-SOD into the NTS, which scavenges ROS generation, decreased blood pressure, HR, and urinary norepinephrine excretion. Taken together, these results suggest that activation of Rac1 in the NTS leads to ROS generation via NAD(P)H oxidase activity, and this mechanism contributes to the neural mechanisms of hypertension in SHRSPs.

Recent studies demonstrated the importance of ROS generation in the NTS.7,23 Nox2-containing NAD(P)H oxidase in the NTS is the source of the Ang II–induced ROS generation in vitro.23 Consistent with those studies, our results indicate that the Rac1/NAD(P)H pathway is involved in neuronal activation in the NTS and further indicate that activation of this pathway and the subsequent ROS generation in the NTS occur in SHRSPs. More importantly, we demonstrated that the inhibition of Rac1 or overexpression of Cu/Zn-SOD in the NTS decreased blood pressure and HR in SHRSPs but not in WKYs. Localized human Cu/Zn-SOD or β-galactosidase gene expression in the NTS after gene transfer was confirmed by immunohistochemical staining. Gene transfer of either AdN17Rac1 or human Cu/Zn-SOD in adjacent regions not involved in cardiovascular regulation (anteroposterior angle 10°, 2.5-mm lateral, 2.5-mm deeper, to the calamus scriptorius) did not elicit any changes in MBP or HR (data not shown). The time course of the MBP and HR changes was similar to those induced by transgene expression, as shown using Western blot analysis, and was consistent with the results of our previous studies using adenovirus-mediated gene transfer.3,10,11 These results confirmed successful gene transfer into the NTS in the present study.

The degree of oxidative stress is determined by the balance between ROS generation and antioxidant enzymatic activity. NAD(P)H oxidase has a crucial role in generating ROS in the brain.2 In particular, most studies have been performed using Ang II infusion models to examine the role of NAD(P)H oxidase and the subsequent ROS generation in the brain and blood vessels.2,7,23 We used SHRSPs as a hypertensive model that resembles human essential hypertension with enhanced
sympathetic nerve activity.\textsuperscript{1,24} Interestingly, we found that Cu/Zn-SOD activity in the NTS was also decreased in SHRSPs compared with WKYs, which indicates that antioxidant enzymatic activity is attenuated in the NTS of SHRSPs.\textsuperscript{1,24} The results of the present study, though we reported previously poor neuronal selectivity.\textsuperscript{25,26} Therefore, we performed double immunohistochemical staining for β-galactosidase and a NeuN (Figure 2D). The transfected β-galactosidase gene was expressed in almost all cell types, including neurons. Therefore, we believe that neurons, as well as glia, in the NTS were transfected with the adenovirus gene. Because Nox2 is present in the NTS neurons,\textsuperscript{7,23,27} it is conceivable that transfection of AdN17Rac1 inhibits the NAD(P)H oxidase activity in those neurons.

Some studies examined the role of glia in cardiovascular regulation.\textsuperscript{26,28} Indeed, in the NTS, NADPH oxidase subunits are also present in astrocytes\textsuperscript{27}; therefore, we cannot exclude the possibility that inhibition of Rac1/NAD(P)H oxidase in glia also decreased blood pressure in the present study. The precise role of glia in cardiovascular regulation is unknown,\textsuperscript{29} ROS, such as NO produced by neurons, as well as glial cells, are diffusive gaseous molecules and are thereby considered to influence surrounding cells. The present study did not differentiate whether the ROS was produced by neurons or glia. Further studies using neuron-specific or glia-specific gene transfer techniques are required for this specific purpose.

We did not address the precise mechanisms by which ROS in the NTS alter blood pressure via the sympathetic nervous system. Extensive evidence supports the idea that Ang II signaling mediates ROS generation,\textsuperscript{2,5,30} and the NTS contains a high density of Ang II subtype 1 receptors located on both vagal and carotid sinus afferent terminals presynaptically and on neurons postsynaptically.\textsuperscript{1,31} The role of Ang II in the NTS, however, is complicated. Depending on the dose, microinjection of Ang II into the NTS elicits an increase or decrease in blood pressure.\textsuperscript{32–34} Although those studies were performed under anesthesia, there might be a connection between the renin–angiotensin system and ROS in the NTS.\textsuperscript{35} Another possibility is that reduced availability of NO is involved in the mechanisms. In the NTS, an increase in NO elicits sympathoinhibition by facilitating the release of excitatory amino acids, such as L-glutamate.\textsuperscript{10,36–39}

In the present study, HR was decreased in SHRSPs after transfection of AdNRac1 or AdCu/ZnSOD. There was also a decrease in urinary norepinephrine excretion. Therefore, we suggest that the effects of gene transfer-induced ROS inhibition are mediated by inhibition of the sympathetic nervous system. We cannot, however, exclude the possibility that vagal outflow is also modulated. We did not examine baroreflex control of HR and vagal outflow to the heart in the present study. It would be interesting to examine whether the bradycardic response induced by gene transfer is atropine sensitive. Further studies are needed to clarify these issues.

In conclusion, our findings indicate that inhibition of Rac1-derived ROS in the NTS decreases blood pressure, HR, and urinary norepinephrine excretion in awake SHRSPs. Activation of the Rac1/NAD(P)H oxidase pathway in the NTS might contribute to ROS generation and thereby enhanced sympathetic drive in SHRSPs.

Perspectives

The NTS regulates the baroreflex and chemoreflex functions and has an important role in cardiovascular regulation.\textsuperscript{12,16} ROS in the brain are thought to contribute to the neuropathogenesis of hypertension by enhancing sympathetic nervous system activity. NAD(P)H oxidase is the source of ROS in the brain. The present study demonstrated that the inhibition of Rac1, which is a key component of NAD(P)H oxidase, decreased sympathetic nerve activity in a rat model of hypertension. These findings have broad implications for the development of therapeutics for human essential hypertension.
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Disclosures
None.

References
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