Cardiovascular Effects of Overexpression of Endothelial Nitric oxide Synthase in the Rostral Ventrolateral Medulla in Stroke-Prone Spontaneously Hypertensive Rats

Takuya Kishi, Yoshitaka Hirooka, Koji Ito, Koji Sakai, Hiroaki Shimokawa, Akira Takeshita

Abstract—We have previously demonstrated that the overexpression of endothelial NO synthase (eNOS) in the rostral ventrolateral medulla (RVLM) decreases blood pressure, heart rate, and sympathetic nerve activity via an increase in γ-amino butyric acid release in normotensive Wistar-Kyoto rats (WKY). Stroke-prone spontaneously hypertensive rats (SHRSP) appear to have reductions of NO production and GABA release in the RVLM. The aim of this study was to determine whether the effects of the increase in NO production in the RVLM in SHRSP are different from those in WKY. We transfected adenovirus vectors encoding either eNOS (AdNOS) or β-galactosidase (Adβgal) into the RVLM of both strains. In the AdNOS-treated group, mean arterial blood pressure and heart rate in the conscious state were significantly decreased at day 7 after the gene transfer in both strains. The decreases in mean arterial blood pressure and heart rate were significantly greater in SHRSP than in WKY. Urinary norepinephrine excretion was also decreased to a greater degree in SHRSP than in WKY after the gene transfer. The pressor response evoked by bicuculline into the RVLM of WKY was greater than that of SHRSP in the nontransfected group. However, in the AdNOS-treated group, the pressor response did not differ between SHRSP and WKY after the gene transfer. These results indicate that the increase in NO production evoked by the overexpression of eNOS in the RVLM causes greater depressor and sympathoinhibitory responses in SHRSP than in WKY by improving an inhibitory action of GABA on the RVLM neurons. (Hypertension. 2002;39:264-268.)

Key Words: genes • nitric oxide • cardiovascular diseases • brain • sympathetic nervous system

The rostral ventrolateral medulla (RVLM) contains sympathetic premotor neurons responsible for maintaining the tonic excitation of sympathetic preganglionic neurons involved in cardiovascular regulation, and the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone.5 Recently, we developed a technique of endothelial NO synthase (eNOS) gene transfer bilaterally into the nucleus tractus solitarii2,3 and RVLM4 of rats in vivo, and we demonstrated that the increase in NO production caused by the overexpression of eNOS in the RVLM decreased blood pressure, heart rate (HR), and sympathetic nerve activity in conscious rats.4

It has been shown that spontaneously hypertensive rats (SHR) or stroke-prone SHR (SHRSP) exhibit increased sympathetic activity during development of hypertension and that a positive correlation exists between mean blood pressure and sympathetic nerve activity.5-10 Some studies have also reported that there is a disorder of the L-arginine–NO pathway in SHR and SHRSP.11-18 The pressor responses to intravenous administration of the NOS inhibitors Nω-nitro-L-arginine methyl ester (L-NAME)11 or Nω-monomethyl-L-arginine (L-NMMA)12 have been demonstrated to be smaller in SHR than in WKY. Cabrera et al13 showed that the depressor responses to intracerebroventricular injection of an NO donor, Et3N[N(O)NO]Na (NOC 18), were greater, whereas the pressor responses to intracerebroventricular injection of L-NAME were smaller in SHRSP than in normotensive control rats. The depressor responses of the microinjection of NOC 18 into the RVLM were enhanced in SHR compared with Wistar-Kyoto rats (WKY).14 However, these studies were performed in acute experiments with anesthetized animals. It would be important to determine the effect of NO in the RVLM of hypertensive rats in the conscious state. In SHR, it has been reported that there is a disinhibition of the GABA-mediated inhibition of neuronal activity in the RVLM.15-17 GABA has been implicated as a putative inhibitory neurotransmitter in the RVLM that is important to the regulation of tonic vasomotor control in the RVLM. Injection of GABA into the RVLM has been shown to elicit a vasodepressor response.15,16 Conversely, the microinjection of bicuculline, a GABA-A receptor antagonist, into the RVLM caused a pressor response.4,15-18 It has also been reported that inhibition of the caudal ventrolateral medulla or blockade of its GABAergic inhibitory input to the RVLM by
injection of bicuculline into the RVLM caused a smaller increase in blood pressure in SHR than in normotensive WKY.15–18 These results indicate that blood pressure in SHR may be elevated because of a disinhibition of RVLM, leading to a relative excess of excitatory activity of RVLM vasomotor neurons. We have previously reported that NO in the RVLM increased the release of GABA in the RVLM2 and propose that a disorder of l-arginine–NO pathway in the RVLM may cause a decrease in the release of GABA in SHRSP.

In this study, our goal was to determine whether the effects of an increase in NO production in the RVLM in SHR caused by the overexpression of eNOS are different from those in normotensive rats. We transfected adenovirus vectors encoding either eNOS (AdeNOS) or β-galactosidase (Adβgal) into the RVLM of rats in vivo. Furthermore, to explore the mechanisms by which the overexpression of eNOS in the RVLM affects the mean arterial blood pressure (MAP) and HR in SHRSP, we examined the effect of GABA in the RVLM by the bilateral microinjection of bicuculline into the RVLM.

**Methods**

This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, Japan.

**In Vivo Gene Transfer Into the RVLM**

Adenoviral vectors encoding either the bacterial β-galactosidase gene or the bovine eNOS gene were used as described previously.2,4 These adenoviral vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa, Iowa City. Adult (16 to 20 weeks old) male WKY and SHRSP (Japan SLC, Hamamatsu, Japan), weighing 240 to 300 g, were used. The procedures for the microinjection of vectors into the RVLM have been described in detail.4

**Analysis of Gene Expression for β-Galactosidase or eNOS**

At day 7 after gene transfer, the expression of β-galactosidase was confirmed by X-Gal staining. The analysis was performed at day 7 after the gene transfer because the expression of β-galactosidase has been shown to peak at day 7.2–4 We performed immunohistochemical staining for eNOS as described previously.2,4 To confirm the local overexpression of eNOS and expression of neuronal NOS (nNOS) protein in the RVLM, Western blot analysis for eNOS and nNOS protein from the tissue containing the injected sites of the RVLM obtained by punch-out technique was performed at day 7 after the gene transfer.2,4 In the Western blot analysis for nNOS protein, we used mouse IgG monoclonal antibody to nNOS (1:2500, Transduction Laboratories).

**Radio-Telemetry Monitoring of Blood Pressure and HR**

The UA-10 telemetry system (Data Sciences International) was used to measure MAP and HR, as described previously.2–4

**Measurement of Urinary Norepinephrine Excretion**

We measured the urinary norepinephrine concentration before the gene transfer and at day 7 after the gene transfer by high-performance liquid chromatography, and we calculated the urinary norepinephrine excretion for 24 hours as an indicator of sympathetic nerve activity.2,4

**Microinjections Into the RVLM**

To confirm that changes of MAP and HR resulted from the increase in NO production in the RVLM caused by the AdeNOS transfection, we microinjected L-NMMA (100 nmol) bilaterally into the RVLM at day 7 after the gene transfer in both AdeNOS-treated and nontreated groups of SHRSP and WKY. To explore the mechanisms by which the increase in NO production caused by the overexpression of eNOS in the RVLM affects MAP and HR, we microinjected bicuculline (200 pmol) bilaterally into the RVLM at day 7 after the gene transfer in all groups. Because the baseline MAP before the microinjection is different in SHRSP and WKY, we expressed changes in MAP/basal MAP as a measure of increases in MAP.

**Statistical Analysis**

All values are expressed as mean±SEM. Two-way ANOVA was used to compare the MAP and HR between the Adβgal-treated and AdeNOS-treated groups and between SHRSP and WKY. Comparisons between any 2 mean values were performed with the application of Bonferroni’s procedure. A paired t test was used to compare the urinary norepinephrine excretion before and after the gene transfer. Differences were considered statistically significant when P<0.05.

**Results**

**Analysis of Gene Expression of β-Galactosidase or eNOS**

Figure 1A shows β-galactosidase staining in a section of the rat brain medulla at day 7 after the gene transfer. The presence of β-galactosidase staining was observed locally in the RVLM, where Adβgal had been microinjected. Similarly, in the AdeNOS-treated rats, the expression of eNOS protein was observed locally in the RVLM, where the AdeNOS had been injected (Figure 1B). No eNOS-positive cells were found in the other regions of the brain, such as the nucleus.
tractus solitarii, caudal ventrolateral medulla, or hypothalamus. The expression of eNOS peaked at day 7 after the gene transfer and, thereafter, declined over time as detected by immunohistochemistry. At day 21 or 30 after the eNOS gene transfer, we were unable to demonstrate eNOS-positive cells immunohistochemically. Western blot analysis revealed that the expression of eNOS protein was significantly increased in tissue from the RVLM of AdeNOS-treated rats compared with that of Ad\textgreek{beta}gal-treated and nontreated rats at day 7 after gene transfer (Figure 1C). Both in SHRSP and WKY, the intensity quantified by densitometric analysis was significantly greater in AdeNOS-treated rats than in Ad\textgreek{beta}gal-treated rats, and the increase in the intensity in the AdeNOS-treated group was not different between SHRSP and WKY (n=5 for each). The expression of nNOS protein was not different among all groups (Figure 1C, n=5 for each).

**MAP, HR, and Urinary Norepinephrine Excretion**

Figure 2A and 2B show the time courses in MAP and HR before and after the eNOS gene transfer in both animals (n=5 for each). MAP and HR significantly decreased between day 5 to day 10 after the eNOS gene transfer in both groups. Figure 2C and 2D show the changes in MAP and HR at day 7 after the eNOS gene transfer compared with those before the gene transfer in SHRSP and WKY. The magnitudes of the decreases in MAP and HR were significantly greater in SHRSP than in WKY (MAP, $-39\pm 4$ versus $-20\pm 5$ mm Hg; HR, $-94\pm 4$ versus $-79\pm 8$ bpm; n=5, P<0.01 for each). In contrast, in the Ad\textgreek{beta}gal-treated rats, these variables did not change in either strain. As shown in Figure 2E, in the AdeNOS-treated group, 24-hour urinary norepinephrine excretion was significantly higher in SHRSP than in WKY before the eNOS gene transfer (1.34\pm 0.15 versus 0.90\pm 0.10 g; n=5, P<0.01). At day 7 after the eNOS gene transfer, urinary norepinephrine excretion was decreased both in SHRSP and WKY, and the magnitude of the decrease was significantly greater in SHRSP than in WKY ($-0.63\pm 0.11$ versus $-0.34\pm 0.10$ g; n=5, P<0.01). In contrast, in the Ad\textgreek{beta}gal-treated rats, urinary norepinephrine excretion was not changed in either SHRSP or WKY.

**Microinjection of L-NMMA Into the RVLM**

The microinjection of L-NMMA bilaterally into the RVLM at day 7 after the gene transfer produced a gradual increase in MAP. As shown in Figure 3A, in both SHRSP and WKY, the increase in MAP caused by L-NMMA was significantly greater in the AdeNOS-treated than in the nontreated rats. In
the nontransfected rats, the increase in MAP was not different between SHRSP and WKY (changes in MAP/basal MAP, 8±1% versus 9±2%; n=5 for each). However, in the AdeNOS-treated rats, the increase in MAP was significantly greater in SHRSP than in WKY (changes in MAP/basal MAP, 27±3% versus 21±2%; n=5, P<0.01). Microinjection of L-NMMA increased MAP to the same level in nontreated WKY and AdeNOS-treated WKY. Moreover, microinjection of L-NMMA increased MAP to the same level in nontreated SHRSP and AdeNOS-treated SHRSP.

Microinjection of Bicuculline Into the RVLM

As shown in Figure 3B, the microinjection of bicuculline bilaterally into the RVLM at day 7 after gene transfer also produced a gradual increase in MAP. In the nontransfected rats, the increase in MAP was significantly smaller in SHRSP than in WKY (changes in MAP/basal MAP, 21±2% versus 39±3%; n=5, P<0.01). However, in the AdeNOS-treated rats, the pressor response evoked by microinjection of bicuculline was significantly greater than that in nontreated rats and did not differ between SHRSP and WKY (changes in MAP/basal MAP, 54±3% versus 56±2%; n=5 for each).

**Discussion**

The major findings of this study were (1) the decrease in MAP, HR, and urinary norepinephrine excretion caused by the overexpression of eNOS in the RVLM were greater in SHRSP compared with WKY, and (2) these decreases were mediated by an increase in GABA release. These findings suggest that a dysfunction of the NO pathway and the resulting disinhibition of RVLM contribute to activation of RVLM sympathetic premotor neurons and increase sympathetic nerve activity in SHRSP. We used eNOS instead of nNOS, which is normally present in the central nervous system. However, the purpose of this study was to increase the NO production locally in the RVLM for a much longer period in the rats treated with AdeNOS in the conscious state. In the rats treated with AdeNOS, eNOS was expressed in neurons, glia, and other tissues in the RVLM, where we transfected genes in this study as reported previously.2–4

An important finding of our study is that the sympathoinhibitory response caused by the overexpression of eNOS in the RVLM was enhanced in SHRSP compared with normotensive WKY. In this study, at day 7 after the gene transfer in the AdeNOS-treated rats, the magnitude of the decreases in MAP and HR was significantly greater in SHRSP than in WKY. Furthermore, urinary norepinephrine excretion was decreased at day 7 after eNOS gene transfer into the RVLM, and the magnitude of the decrease in urinary norepinephrine excretion was significantly greater in AdeNOS-treated SHRSP than in AdeNOS-treated WKY. Moreover, the microinjection of L-NMMA into the RVLM after the gene transfer increased MAP to the same level in AdeNOS-treated SHRSP and nontreated SHRSP. These results suggest that the increase in NO production caused by eNOS gene transfer into the RVLM decreases sympathetic nerve activity in conscious rats, and the cardiovascular responses were greater in SHRSP than in WKY. Previous studies investigating the expression of NO in the brain of the hypertensive rats have reported inconsistent results.19–22 In our study, the expression of eNOS and nNOS protein in the RVLM was not different between nontreated SHRSP and nontreated WKY. The expression of eNOS protein in the RVLM caused by the eNOS gene transfer was also not different between AdeNOS-treated SHRSP and AdeNOS-treated WKY. However, the depressor response of the overexpression of eNOS in the RVLM was greater in SHRSP. These results suggest that NO in the RVLM of nontreated SHRSP is not increased sufficiently to compensate for the high blood pressure resulting from the increased sympathetic nervous activity and that the bioactivity of NO in the RVLM of SHRSP may be decreased. We speculate that one of the mechanisms of the dysfunction of NO in the RVLM in the SHRSP may be mediated by an increase in oxidative stress in SHRSP, which in turn destroys NO. In fact, it has been suggested that chronic oxidative stress in the RVLM modulates sympathetic nerve activity in pigs.23

In this study, we transfected adenovirus vectors encoding either the β-galactosidase gene or the eNOS gene into the RVLM of rats in vivo. As described previously, this method is useful to examine the effect of NO in the RVLM on cardiovascular regulation in the conscious state, and these hypotensive and bradycardiac responses did not result from
either inflammation or cytotoxicity.\textsuperscript{2,4} In the AdeNOS-treated rats, the expression of eNOS protein was not different between SHRSP and WKY. Furthermore, we performed the microinjection of L-NMMA bilaterally into the RVLM to confirm that the decrease in MAP is caused by transfected eNOS activity. These results suggest that the NO production was increased in the RVLM of AdeNOS-treated rats and that the cardiovascular responses that occurred after the eNOS gene transfer were indeed mediated by NO.

Another important finding of our study is that the increase in NO production in the RVLM caused by the overexpression of eNOS reduces the disinhibition of the RVLM neurons by a GABA-mediated mechanism. The majority of GABAergic neuronal terminals in the RVLM come from the caudal ventrolateral medulla and inhibit neuronal excitability of the RVLM neurons.\textsuperscript{1,17} In SHR, it has been reported that there is a disinhibition of RVLM and that this disinhibition contributes to the increase in sympathetic nerve activity.\textsuperscript{15,17} In our study, the increase in MAP and HR caused by the microinjection of bicuculline into the RVLM were significantly smaller in the nontreated SHRSP than in nontreated WKY. These results support the conclusion that a disinhibition of the RVLM in SHRSP contributes to an increase in sympathetic nerve activity in these animals. In the AdeNOS-treated SHRSP, the changes in MAP caused by microinjection of bicuculline were significantly greater compared with those in the nontreated rats and were not different between AdeNOS-treated SHRSP and AdeNOS-treated WKY. These results suggest that NO in the RVLM of SHRSP increases the release of GABA in the RVLM and that the increase in NO production caused by the overexpression of eNOS in the RVLM reduces the disinhibition of RVLM of SHRSP through this increase in GABA release. The reduction of the disinhibition of RVLM via GABA may contribute to the greater depressor response caused by the overexpression of eNOS in the RVLM in SHRSP.

In summary, the increase in NO production caused by the overexpression of eNOS in the RVLM led to decreases in MAP, HR, and sympathetic nerve activity in SHRSP, and the sympathoinhibitory effects were enhanced in SHRSP compared with WKY. These effects resulted from a GABA-mediated reduction of the disinhibition of the RVLM in SHRSP. Our results suggest that a dysfunction of NO and the resulting disinhibition of RVLM contribute to the increase in sympathetic nerve activity in SHRSP.

Acknowledgments

This work was supported by the Grant-in-Aid for Scientific Research (C11670689) from the Ministry of Education, Science, Sports, and Culture and from the Japan Society for the Promotion of Science and by a Grant for Research on Autonomic Nervous System and Hypertension from Kimura Memorial Heart Foundation/Pfizer Pharmaceuticals. The authors thank Drs. Donald D Heistad and Beverly L Davidson (The University of Iowa Gene Transfer Vector Core, supported by National Institutes of Health grants and the Carver Foundation) for the preparation of vectors.

References

10. Yang TL, Chai CY, Yen CT. Enhanced sympathetic reactivity to gluta
12. Fozard JR, Part ML. Haemodynamic response to \textsuperscript{N}\textsuperscript{-}monomethyl-l-
15. Smith JK, Barron KW. GABAergic responses in ventrolateral medulla in sponta
16. Smith JK, Barron KW. Cardiovascular effects of \textsuperscript{L}-glutamate and tetro
18. Muratani H, Ferrario CM, Averill DB. Ventrolateral medulla in spontane
Cardiovascular Effects of Overexpression of Endothelial Nitric Oxide Synthase in the Rostral Ventrolateral Medulla in Stroke-Prone Spontaneously Hypertensive Rats
Takuya Kishi, Yoshitaka Hirooka, Koji Ito, Koji Sakai, Hiroaki Shimokawa and Akira Takeshita

Hypertension. 2002;39:264-268
doi: 10.1161/hy0202.102701

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/39/2/264

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/