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Angiotensin II Type 1 Receptor–Activated Caspase-3 Through Ras/Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase in the Rostral Ventrolateral Medulla Is Involved in Sympathoexcitation in Stroke-Prone Spontaneously Hypertensive Rats

Takuya Kishi, Yoshitaka Hirooka, Satomi Konno, Kiyohiro Ogawa, Kenji Sunagawa

Abstract—In the rostral ventrolateral medulla (RVLM), angiotensin II-derived superoxide anions, which increase sympathetic nerve activity, induce a pressor response by activating the p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK) pathway. The small G protein Ras mediates a caspase-3–dependent apoptotic pathway through p38 MAPK, ERK, and c-Jun N-terminal kinase. We hypothesized that angiotensin II type 1 receptors activate caspase-3 through the Ras/p38 MAPK/ERK/c-Jun N-terminal kinase pathway in the RVLM and that this pathway is involved in sympathoexcitation in stroke-prone spontaneously hypertensive rats (SHRSP), a model of human hypertension. The activities of Ras, p38 MAPK, ERK, and caspase-3 in the RVLM were significantly higher in SHRSP (14 to 16 weeks old) than in age-matched Wistar-Kyoto rats (WKY). The mitochondrial apoptotic proteins Bax and Bad in the RVLM were significantly increased in SHRSP compared with WKY. c-Jun N-terminal kinase activity did not differ between SHRSP and WKY. In SHRSP, intracerebroventricular infusion of a Ras inhibitor significantly reduced sympathetic nerve activity and improved baroreflex sensitivity, partially because of inhibition of the Ras/p38 MAPK/ERK, Bax, Bad, and caspase-3 pathway in the RVLM. Intracerebroventricular infusion of a caspase-3 inhibitor also inhibited sympathetic nerve activity and improved baroreflex sensitivity in SHRSP. Intracerebroventricular infusion of an angiotensin II type 1 receptor blocker in SHRSP partially inhibited the Ras/p38 MAPK/ERK, Bax, Bad, and caspase-3 pathway in the RVLM. These findings suggest that in SHRSP, angiotensin II type 1 receptor-activated caspase-3 acting through the Ras/p38 MAPK/ERK pathway in the RVLM might be involved in sympathoexcitation, which in turn plays a crucial role in the pathogenesis of hypertension. (***Hypertension*. 2010;55:291-297.**)

Key Words: angiotensin II ■ apoptosis ■ sympathetic nerve activity ■ brain ■ hypertension

Neuronal apoptosis in the brain is involved in regulating synaptic plasticity and neural function[1–3] and is mainly caused by reactive oxygen species (ROS).[4–8] Ras is a member of a superfamily of related small GTPases implicated in cellular proliferation and transformation, growth arrest, senescence, and apoptosis.[9–13] In cultured tumor cells or endothelial cells, the proapoptotic effects of Ras are mediated by the p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathway through phosphorylation of the proapoptotic proteins Bax and Bad and the antiapoptotic protein Bcl-2, which releases cytochrome *c* in the mitochondria.[14–17] Neuronal apoptosis is characterized by the release of cytochrome *c*, which activates caspase-3, the major executioner caspase in neurons.[18,19] Thus, neuronal apoptosis may be mainly mediated by caspase-3 through the Ras, p38 MAPK, ERK pathway. We previously demonstrated that ROS in a cardiovascular center of the brain stem increase sympathetic nerve activity (SNA) in hypertensive rats.[20] Accumulating evidence suggests that ROS in the brain are involved in the neural mechanisms of hypertension.[21,22] Although ROS are increased in the brain in a hypertensive state, it is not known whether a pivotal signaling pathway (such as the Ras, p38 MAPK, ERK pathway) and caspase-3, activated by ROS in the brain, are chronically activated in the hypertensive state or whether this pathway activates SNA.

The rostral ventrolateral medulla (RVLM) in the brain stem is a major vasomotor center, and it regulates SNA.[23,24] We previously demonstrated that ROS in the RVLM activates SNA and that ROS are increased in the RVLM of stroke-prone spontaneously hypertensive rats (SHRSP), a model of human hypertension,[25] with activation of SNA.[20] In the brain, ROS are produced by activation of the angiotensin II type 1 receptor (AT_1R), which in turn activates nicotinamide-

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adenine dinucleotide phosphate (NAD[P]H) oxidase.[26] NAD(P)H oxidase-derived superoxide anions mediate the angiotensin II-induced pressor effect via the activation of p38 MAPK and ERK in the RVLM.[27] Furthermore, in experimental endotoxemia, the proapoptotic protein Bax and caspase-3–dependent apoptosis in the RVLM mediate cardiovascular responses.[28] The mechanisms by which ROS in the RVLM regulate SNA have not been fully examined, especially the pivotal signaling pathway of ROS.

The aims of the present study were to determine whether stimulation of endogenous AT_1R activates caspase-3 through the Ras/p38 MAPK/ERK/c-Jun N-terminal kinase (JNK) pathway in the RVLM and, if so, to determine whether activation of this pathway is involved in the increased sympathoexcitation in SHRSP. Toward this end, we examined the activity of Ras, p38 MAPK, ERK, JNK, proapoptotic proteins Bax and Bad, antiapoptotic protein Bcl-2, and caspase-3 in the RVLM of SHRSP and normotensive rats. In addition, we performed intracerebroventricular (ICV) injections of a Ras inhibitor, a caspase-3 inhibitor, and an angiotensin receptor blocker (ARB), and examined the changes in blood pressure, heart rate (HR), SNA, and baroreflex sensitivity (BRS). To determine whether ICV injection of a Ras inhibitor, a caspase-3 inhibitor, or an ARB inhibits the pivotal signaling pathway in the RVLM, we also examined the changes in blood pressure, HR, and SNA evoked by microinjection of angiotensin II into the RVLM.

Methods

This study 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. Details of the methods are available in the online Data Supplement at http://hyper.ahajournals.org.

Animals and General Procedures

Male SHRSP/Izm rats and age-matched Wistar-Kyoto rats (WKY) (14 to 16 weeks old), fed standard feed, were divided into 7 groups (SHRSP treated with Ras inhibitor [S-RI], SHRSP treated with caspase-3 inhibitor [S-CI], SHRSP treated with ARB [S-ARB], SHRSP treated with vehicle [S-Veh], WKY treated with Ras inhibitor [W-RI], WKY treated with caspase-3 inhibitor [W-CI], and WKY with vehicle [W-Veh]; n=5/group). In the S-RI, W-RI, S-CI, W-CI, S-Veh, W-Veh, and S-ARB groups, we measured blood pressure and HR using a radiotelemetry system as described previously.[20] Urinary norepinephrine excretion (uNE) for 24 hours was calculated as an indicator of SNA, as described previously.[20,22] Furthermore, in the S-RI, W-RI, S-CI, W-CI, S-Veh, and W-Veh groups, spectral analysis was performed to provide power spectra for systolic blood pressure.

Activity of Ras, p38 MAPK, ERK, JNK, and Caspase-3 and Expression of Bax, Bad, and Bcl-2 in the RVLM

The activity of Ras, p38 MAPK, ERK, JNK, and caspase-3 and the expression of Bax, Bad, and Bcl-2 in the RVLM were measured as described previously.[29]

ICV Injection of Ras Inhibitor, Caspase-3 Inhibitor, and AT_1R Blocker

S-Farnesylthiosalicylic acid (1 mmol/L), a specific Ras inhibitor[30]; *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe) fluoromethyl ketone (Z-DEVD-FMK, 1 μmol/L), a specific caspase-3 inhibitor[31]; candesartan (1 μg/μL); or vehicle was administered by ICV infusion for 14 days with an osmotic minipump (Alzet 1003D). We also determined the changes in blood pressure and HR of SHRSP after terminating the 14-day ICV infusion of the Ras inhibitor (n=4). The candesartan dose was selected as described previously.[32]

Statistical Analysis

Normally distributed variables are expressed as mean±SE. Unpaired *t* and Mann-Whitney U tests were used to compare the differences in normally distributed and nonnormally distributed variables, respectively. Data were also analyzed by a 2-factor repeated-measures analysis of variances. Differences were considered to be statistically significant at $P<0.05$.

Results

Blood Pressure, HR, SNA, and BRS

The Ras inhibitor *S*-farnesylthiosalicylic acid was infused ICV for 14 days. Mean blood pressure (MBP), HR, uNE, and normalized unit of the low-frequency component of systolic blood pressure (LFnuSBP) at day 14 were significantly higher in S-Veh than in W-Veh (Figure 1A through 1D). MBP, HR, and LFnuSBP in SHRSP returned to control levels 4 days after terminating the ICV infusion of *S*-farnesylthiosalicylic acid (data not shown). BRS at day 14 was significantly lower in S-Veh than in W-Veh (Figure 2). At days 2 to 14, MBP and HR were significantly lower in S-RI than in S-Veh (Figure 1A and 1B), and at day 14, uNE and LFnuSBP were significantly lower in S-RI than in S-Veh (Figure 1C and 1D). BRS at day 14 was significantly higher in S-RI than in S-Veh (Figure 2). MBP, HR, LFnuSBP, uNE, and BRS, however, did not differ between W-RI and W-Veh (Figures 1A through 1D and 2).

The caspase-3 inhibitor Z-DEVD-FMK was infused ICV for 14 days. At days 4 to 14, MBP and HR were significantly lower in S-CI than in S-Veh (Figure 1A and 1B), and at day 14, uNE and LFnuSBP were also significantly lower in S-CI than in S-Veh (Figure 1C and 1D). BRS at day 14 was significantly higher in S-CI than in S-Veh (Figure 2). MBP, HR, LFnuSBP, uNE, and BRS did not differ between W-CI and W-Veh (Figures 1A through 1D and 2).

On day 14 of the ICV infusion of candesartan in SHRSP, the systolic blood pressure, HR, uNE, and LFnuSBP were significantly lower in S-ARB than in S-Veh (Figures 1A through 1D).

Ras, p38 MAPK, ERK, and JNK Activity in the RVLM

Ras, p38 MAPK, and ERK activities were significantly higher in S-Veh than in W-Veh and significantly lower in S-RI than in S-Veh (Figure 3A through 3C). Furthermore, Ras, p38 MAPK, and ERK activity was significantly lower in S-ARB than in S-Veh (Figure 3A through 3C). Ras, p38 MAPK, and ERK activity in SHRSP did not differ between S-CI and S-Veh (Figure 3A through 3C) or between W-Veh and W-CI (Figure 3A through 3C). JNK activity did not differ between S-Veh and W-Veh (Figure 3D).

Caspase-3 Activity and Expression of Bax, Bad, and Bcl-2 in the RVLM

Caspase-3 activity in the cytosolic fraction of the RVLM and the expression of Bax and Bad in the mitochondrial fraction of the RVLM were significantly higher in S-Veh than in W-Veh (Figure 4A through 4C) and significantly lower in

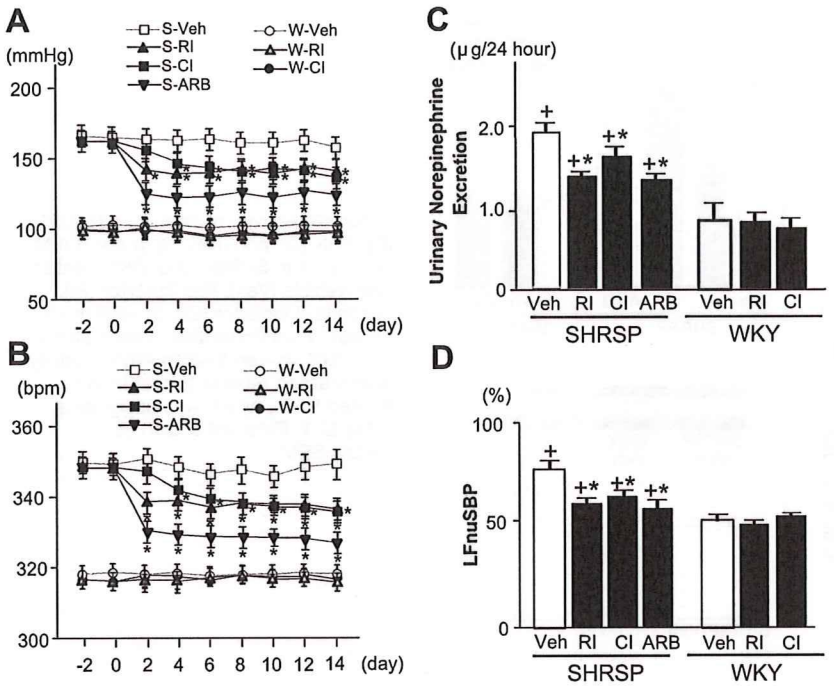

Figure 1. Time course of MBP (A, mm Hg) and HR (B, bpm) in S-RI (n=5), S-CI (n=5), S-ARB (n=5), S-Veh (n=5), W-RI (n=5), W-CI (n=5), and W-Veh (n=5). *P<0.05 for Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB vs vehicle (Veh) values in each strain. C and D, 24-hour uNE (μg) (C) and LFnuSBP (%) (D) on day 14 in SHRSP treated with RI, caspase-3 inhibitor (CI), ARB, or vehicle (Veh) and WKY treated with RI, caspase-3 inhibitor (CI), or Veh (n=5 for each). *P<0.05 for RI, CI, or ARB vs Veh values in each strain. +P<0.05 vs W-Veh. Data are shown as mean±SEM.

S-RI than in S-Veh (Figure 4A through 4C). ICV infusion of Z-DEVD-FMK significantly inhibited caspase-3 activity in both SHRSP and WKY (Figure 4A). In WKY, however, neither caspase-3 activity nor the expression of Bax and Bad differed between W-Veh and W-RI (Figure 4A through 4C). ICV infusion of candesartan in SHRSP significantly decreased caspase-3 activity and the expression of Bax and Bad (Figure 4A through 4C).

The expression of Bcl-2 was significantly lower in S-Veh than in W-Veh (Figure 4D) and significantly higher in S-RI than in S-Veh (Figure 4D). In WKY, however, the expression of Bcl-2 did not differ between W-Veh and W-RI (Figure 4D). ICV infusion of candesartan in SHRSP significantly increased Bcl-2 expression (Figure 4D).

Microinjection of Angiotensin II into the RVLM

The changes in MBP, HR, and LFnuSBP evoked by microinjection of angiotensin II into the bilateral RVLM were significantly smaller in S-RI than in S-Veh (MBP, 8±5 mm Hg versus 14±3 mm Hg; HR, 7±8 bpm versus 22±9 bpm; LFnuSBP, 3±3% versus 8±2%; n=5 for each; P<0.01).

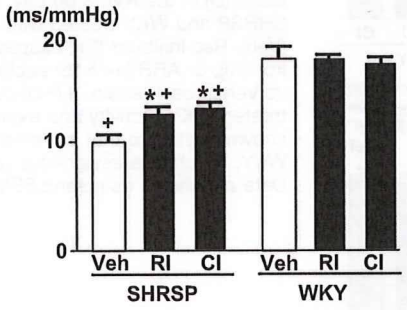

Figure 2. BRS (ms/mm Hg) in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), or caspase-3 inhibitor (CI) (n=5 for each). *P<0.05 vs Veh in each strain. +P<0.05 vs W-Veh. Data are shown as mean±SEM.

Discussion

The novel findings in the present study are as follows: (1) Ras, p38 MAPK, ERK, mitochondrial apoptotic proteins Bax and Bad, and caspase-3 in the RVLM are activated in SHRSP; (2) ICV infusion of a Ras inhibitor decreases MBP, HR, and SNA and increases BRS through the partial inhibition of p38 MAPK, ERK, Bax, Bad, and caspase-3 in the RVLM of SHRSP; (3) ICV infusion of a caspase-3 inhibitor decreases MBP, HR, and SNA and increases BRS through the partial inhibition of caspase-3 in the RVLM of SHRSP; (4) ICV infusion of candesartan decreases systolic blood pressure, HR, and SNA through the partial inhibition of Ras, p38 MAPK, ERK, Bax, Bad, and caspase-3 in the RVLM of SHRSP; and (5) ICV infusion of the Ras inhibitor in SHRSP abolishes the pressor effect evoked by the microinjection of angiotensin II into the RVLM. These findings indicate that AT_1R-induced activation of caspase-3 through the Ras/p38 MAPK/ERK pathway in the RVLM might increase MBP, HR, and SNA and decrease BRS (Figure 5).

The present findings are the first to demonstrate that Ras, p38 MAPK, and ERK activity is increased in the RVLM of SHRSP. A previous study suggested that an acute injection of angiotensin II induced AT_1R-dependent ROS production and phosphorylation of p38 MAPK and ERK in the RVLM.[27] Activation of p38 MAPK and ERK by angiotensin II is also reported in mesenteric smooth muscle cells[33,34] and aorta.[35,36] In the forebrain, MAPK is activated in a model of heart failure in which the brain renin-angiotensin system is upregulated.[37] ROS activates Ras,[38] and Ras activates caspase-3 through p38 MAPK and ERK.[4–7,39] Previously, we demon-

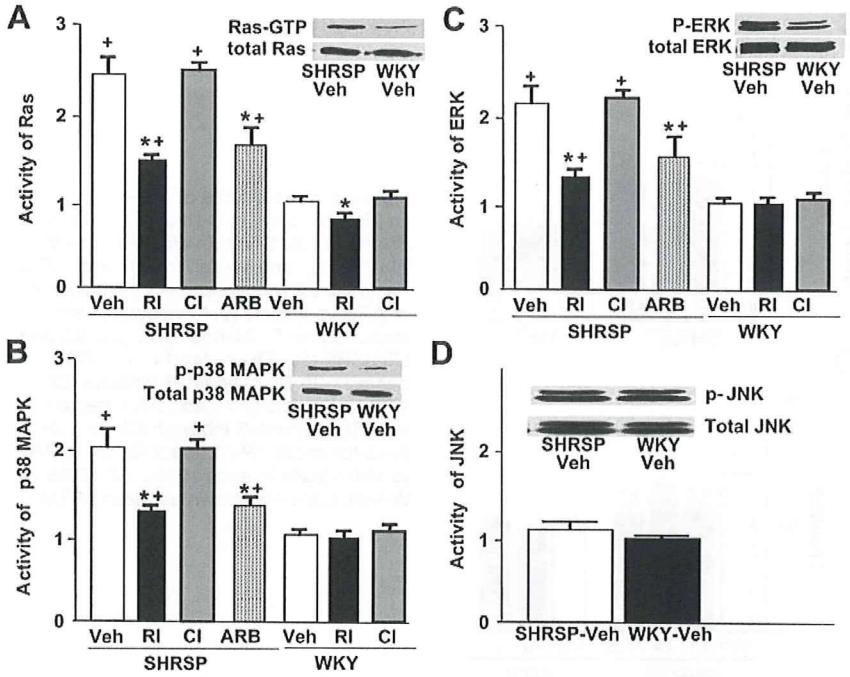

Figure 3. Activity of Ras (A), p38 MAPK (B), ERK (C), and JNK (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5/group). *$P<0.05$ vs Veh in each strain. +$P<0.05$ vs Veh-treated WKY. Activity is expressed relative to that in Veh-treated WKY, which was assigned a value of 1. Data are shown as mean±SEM.

strated that ROS in the RVLM increases SNA,[20,22] and ROS is produced in the brain by angiotensin II and NAD(P)H oxidase.[25] In the present study, ICV infusion of the Ras inhibitor decreased MBP, HR, and SNA and increased BRS because of the partial inhibition of Ras, p38 MAPK, ERK, and caspase-3 in the RVLM of SHRSP, and it abolished the pressor effect evoked by the microinjection of angiotensin II into the RVLM. ICV infusion of the caspase-3 inhibitor also inhibited MBP, HR, and SNA and increased BRS through the partial inhibition of caspase-3 activity in the RVLM of SHRSP. Furthermore, ICV infusion of candesartan decreased MBP, HR, and SNA, consistent with previous reports.[32] In the present study, ICV infusion of candesartan also partially inhibited Ras, p38 MAPK, ERK, and caspase-3 in the RVLM of SHRSP. The degree of the depressor effect of the Ras inhibitor on MBP in SHRSP was almost half that in WKY. These results suggest that AT_1R-activated caspase-3 acting through the Ras/MAPK/ERK pathway in the RVLM is one of the major pathways through which MBP, HR, and SNA are increased and BRS is decreased in SHRSP.

Another intriguing finding of the present study is that the apoptotic proteins Bax and Bad were activated, and the

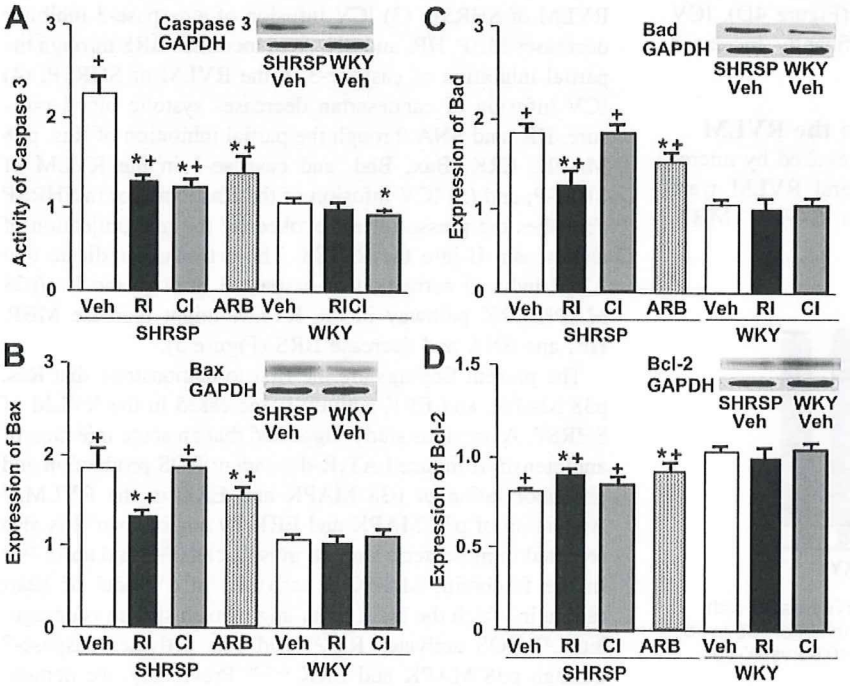

Figure 4. Activity of caspase-3 (A) and expression of Bax (B), Bad (C), and Bcl-2 (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5 for each). *$P<0.05$ vs Veh in each strain. +$P<0.05$ vs Veh-treated WKY. Activity and expression are shown relative to that in Veh-treated WKY, which was assigned a value of 1. Data are shown as mean±SEM.

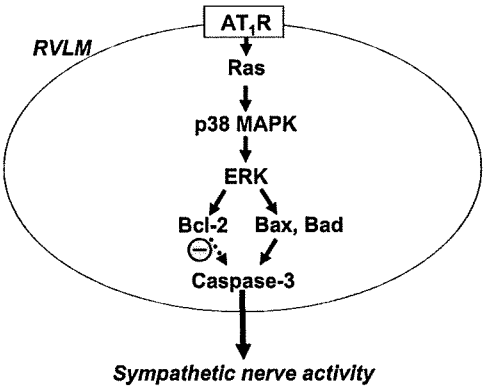

Figure 5. Illustration showing the major findings suggested by the results of the present study.

antiapoptotic protein Bcl-2 was inhibited in the RVLM of SHRSP. Neuronal apoptosis is mediated by caspase-3 activated by Bax and Bad and inhibited by Bcl-2 in mitochondria.[1] Activation of caspase-3 induces neuronal apoptosis.[18,19] Other reports indicate that p38 MAPK and ERK activate caspase-3–dependent neuronal apoptosis.[8] We previously demonstrated that mitochondria-derived ROS mediate sympathoexcitation induced by angiotensin II in the RVLM,[40] and these results suggest that mitochondrial dysfunction in the RVLM causes sympathoexcitation via ROS production. We hypothesized that Ras, p38 MAPK, and ERK activate the mitochondrial apoptotic pathway and inhibit the mitochondrial antiapoptotic pathway and that caspase-3–dependent neuronal apoptosis is activated in the RVLM of SHRSP. The possibility of caspase-3–independent neuronal apoptosis in the RVLM or of a direct link between ROS and caspase-3 activation was not examined in the present study. A previous report suggested that neural apoptosis in the RVLM leads to a reduction of sympathetic outflow.[40] Further study is necessary to determine the reasons for this discrepancy.

In the present study, we determined the ICV infusion dose of the Ras or caspase-3 inhibitor that inhibits blood pressure, HR, and SNA. There were dose-dependent effects of the Ras and caspase-3 inhibitors on blood pressure and HR (data not shown). Furthermore, the doses of Ras or caspase-3 inhibitor used in the present study did not change blood pressure or HR when injected intravenously (data not shown). In addition, Ras and caspase-3 activity were significantly higher in SHRSP than in WKY, and the depressor and sympathoinhibitory effects of Ras and caspase-3 inhibitors were also significantly greater in SHRSP than in WKY. Thus, we consider that the doses of Ras and caspase-3 inhibitor used in the present study were reasonable to inhibit Ras or caspase-3 activity in the RVLM. Future studies, however, are needed to investigate the effects of inhibiting Ras or caspase-3 activity specifically in the RVLM.

Interestingly, JNK was not altered in the RVLM of SHRSP. JNK is an upstream activator of apoptosis. In a heart failure model, JNK is upregulated in the RVLM.[41] Angiotensin II and NAD(P)H oxidase-derived superoxide anions, however, do not activate JNK in the RVLM,[27] and these findings are consistent with the present results. We did not explore the mechanisms of this discrepancy in the present study and are therefore not able to exclude the importance of JNK in the RVLM for cardiovascular regulation. JNK in the RVLM might be significantly activated in heart failure progressing to hypertension. Furthermore, we did not examine the protein kinase C–dependent pathway in the RVLM. A previous report indicates that protein kinase C–dependent translocation of Bax in the RVLM initiates caspase-3–dependent apoptosis during experimental endotoxemia.[28] It is possible that this pathway is also a major pathway involved in the increase in SNA in SHRSP.

The present study has some limitations. Ras activity in the RVLM was inhibited by ICV infusion of the Ras inhibitor, and the inhibition of Ras activity was not limited to the RVLM; therefore, we cannot exclude the possible effects of Ras inhibition in other brain sites, and our results do not suggest that the AT_1R/Ras/caspase-3 pathway in the RVLM is the only major pathway of the sympathetic control. Moreover, none of the ICV antagonists completely normalized BP, HR, and SNA in SHRSP. Many factors in the RVLM may be involved in changing SNA. Nevertheless, Ras activity was inhibited in the RVLM, and, therefore, the neural activity of the RVLM directly influenced SNA.[23,24] Furthermore, we found that the pressor effect evoked by microinjection of angiotensin II into the RVLM was attenuated in SHRSP treated with ICV infusion of the Ras inhibitor. Previous reports suggest that activation of the brain angiotensin system contributes to the neural mechanisms of hypertension.[23,24,42–45] In addition, a renin-angiotensin system also exists inside the blood-brain barrier.[42,46] All components of the renin-angiotensin system are present in the brain, such as renin, angiotensinogen, angiotensin-converting enzyme, angiotensin II, and AT_1 and angiotensin type 2 (AT_2) receptors.[45] Importantly, AT_1 receptors are richly distributed in the paraventricular nucleus of the hypothalamus, nucleus tractus solitarius, and RVLM, which are involved in autonomic cardiovascular regulation.[42,44–46] Therefore, it is conceivable that alteration of a signaling pathway in the RVLM influences central sympathetic outflow via AT_1R in the RVLM of SHRSP, although we cannot exclude the possible interaction of other autonomic nuclei, such as the paraventricular nucleus of the hypothalamus. The findings of the present study do not exclude the possibility that similar effects might occur in other nuclei or that these findings are indirect effects. In this regard, further study is necessary to determine the role of other autonomic nuclei in neural control of blood pressure. It would be interesting if we could examine the direct effect of chronic infusion of a Ras inhibitor and/or a caspase inhibitor directly into the RVLM. In addition, we did not measure SNA directly in the present study because chronic direct measurement of SNA is technically difficult. We examined SNA by measuring 24-hour uNE and spectral analysis of systolic blood pressure. uNE is considered to be a measure of SNA,[20,47] and measurement of uNE is often used to assess SNA in small awake animals.[47] We consider that uNE and LFnuSBP are appropriate parameters for assessing SNA.

In conclusion, AT_1R-induced activation of caspase-3 through Ras/p38 MAPK/ERK and the mitochondrial apoptotic pathway in the RVLM of SHRSP increases blood pressure, HR, and SNA

and decreases BRS in SHRSP. Inhibition of this pathway by ARB in the RVLM may be a novel therapeutic approach to sympathoexcitation in hypertension.

Perspectives

Our results suggest that Ras-activated caspase-3, acting through the p38 MAPK, ERK, and mitochondrial apoptotic pathways in the RVLM, increases SNA. Previous studies indicate that angiotensin II and ROS produced by NAD(P)H oxidase are upstream of Ras. In the RVLM, angiotensin II and ROS are important modulating factors regulating SNA, which is involved in cardiovascular disease, such as hypertension and heart failure. We consider that neural apoptosis in the RVLM is a novel target for the treatment of cardiovascular diseases exhibiting increased SNA.

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Disclosures

None.

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Sympathoinhibition Induced by Centrally Administered Atorvastatin Is Associated With Alteration of NAD(P)H and Mn Superoxide Dismutase Activity in Rostral Ventrolateral Medulla of Stroke-Prone Spontaneously Hypertensive Rats

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Abstract: Oxidative stress in the rostral ventrolateral medulla (RVLM) increases sympathetic nervous system activity (SNA). Oral treatment with atorvastatin decreases SNA through antioxidant effects in the RVLM of stroke-prone spontaneously hypertensive rats (SHRSP). We aimed to examine whether centrally administered atorvastain reduces SNA in SHRSP and, if so, to determine whether it is associated with the reduction of oxidative stress induced by alteration of activities of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase and superoxide dismutase (SOD) in the RVLM of SHRSP. SHRSP received atorvastatin (S-ATOR) or vehicle (S-VEH) by continuous intracerebroventricular infusion for 14 days. Mean blood pressure, heart rate, and SNA were significantly lower in S-ATOR than in S-VEH. Oxidative stress, Rac1 activity, NAD(P)H oxidase activity, Rac1, gp91^{phox} and p22^{phox} expression in the membrane fraction, and p47^{phox} and p40^{phox} expression in the cytosolic fraction in the RVLM were significantly lower in S-ATOR than in S-VEH. Rac1 expression in the cytosolic fraction and Mn-SOD activity, however, were significantly higher in S-ATOR than in S-VEH. Our findings suggest that centrally administered atorvastatin decreases SNA and is associated with decreasing NAD(P)H oxidase activity and upregulation of Mn-SOD activity in the RVLM of SHRSP, leading to suppressing oxidative stress.

Key Words: hypertension, sympathetic nerve activity, atorvastatin, oxidative stress, brain

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INTRODUCTION

In the brainstem, the rostral ventrolateral medulla (RVLM) is known as one of the vasomotor centers that regulates sympathetic nervous system activity (SNA).[1,2] Previously, we reported that the levels of reactive oxygen species (ROS) in the RVLM are increased in stroke-prone spontaneously hypertensive rats (SHRSP), which is a hypertensive rat model exhibiting increased SNA. We also demonstrated that the increase in SNA was due to ROS activation,[3] consistent with the findings of other studies.[4–6] Furthermore, oral administration of atorvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, suppresses SNA probably through the inhibition of ROS in the RVLM of SHRSP.[7] Other studies suggest that central infusion of simvastatin suppresses SNA in heart failure models.[8–10] Our previous study was based on the oral administration of atorvastatin, however, and it is not known whether atorvastatin directly and chronically administered into the brain reduces the central sympathetic outflow via its effects on oxidative stress in the brain, particularly in the RVLM of hypertensive models.

In the brain, ROS are produced mainly through the activation of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase by the small G protein Rac1.[11,12] NAD(P)H oxidase is a multicomponent enzyme complex that comprises a membrane-bound heterodimer of gp91^{phox} (phagocytic oxidase) and p22^{phox}, and the cytosolic regulatory subunits p40^{phox}, p47^{phox}, p67^{phox}, and Rac1.[13–15] Transfection of dominant-negative Rac1 in the nucleus tractus solitarius decreases ROS and SNA.[12] Atorvastatin is also suggested to inhibit NAD(P)H oxidase activity in the vasculature,[16] the quadriceps muscle of diabetic rats,[17] and cardiomyocytes.[18] Furthermore, atorvastatin inhibits membrane translocation of Rac1, which is required for the activation of NAD(P)H oxidase in the vasculature.[16] In the kidney, rosuvastatin attenuates NAD(P)H oxidase activity through the inhibition of Rac1 and p22^{phox}.[18,19] In the brain, however, the contribution of atorvastatin to reducing ROS and its involvement in the inhibition of the membrane translocation of Rac1 and NAD(P)H oxidase activity is unknown. We previously demonstrated that Mn superoxide dismutase (SOD) activity is decreased in the RVLM of SHRSP, and the decrease contributes to the increase

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in ROS.[3] A number of reports suggest that statins upregulate SOD in the vasculature.[20–23] Furthermore, the upregulation of Rac1 and NAD(P)H oxidase and the inhibition of SOD in the RVLM and nucleus tractus solitarius have major roles in increasing SNA and blood pressure (BP).[3,24] However, the mechanisms involved by which atorvastatin reduces ROS in the RVLM of SHRSP are not evaluated. The aim of the present study was thus to determine whether the sympathoinhibitory effect of atorvastatin due to the reduction of ROS in the RVLM is caused by the inhibition of Rac1-NAD(P)H oxidase activity and upregulation of Mn-SOD and Cu/Zn-SOD in the RVLM of SHRSP. Therefore, the aim of the present study was to examine the effects of atorvastatin administered into the brain and evaluate the changes in BP and SNA in SHRSP and to evaluate the oxidative stress and the NAD(P)H oxidase activity in the RVLM as the ROS generation. For this purpose, we determined the expression of Rac1, gp91^{phox}, and p22^{phox} in the membrane fraction and the expression of Rac1 and p40^{phox} in the cytosolic fraction of the RVLM. In addition, the activity of Cu/Zn-SOD, and Mn-SOD as scavenging enzymes of ROS was measured in the RVLM of intracerebroventricular (ICV) atorvastatin–treated and vehicle-infused SHRSP and Wistar Kyoto (WKY) rats.

MATERIALS AND METHODS

Animals and General Procedures

Male SHRSP/Izm rats and age-matched WKY rats (14–16 weeks old) were obtained from SLC Japan, Hamamatsu, Japan. Rats were fed a standard diet, and each strain was divided into 4 groups (SHRSP treated with atorvastatin, S-ATOR; SHRSP treated with vehicle, S-VEH; WKY treated with atorvastatin, W-ATOR; and WKY treated with vehicle, W-VEH; n = 5 per group). Atorvastatin (Pfizer, Inc, New York, NY) was dissolved in dimethyl sulfoxide and further diluted in artificial cerebrospinal fluid for a final concentration of 40 μg/mL. Atorvastatin or dimethyl sulfoxide in artificial cerebrospinal fluid was infused at 1 μL/h for 14 days with an osmotic minipump (Alzet 1003D; Alza Scientific Products, Palo Alto, CA) into the left lateral ventricle of the brain (from bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; and depth, 3.5 mm). The flow rate of agents in ICV methods was determined to have the significant effect in brainstem.[25] In a preliminary experiment, this dose of atorvastatin did not affect BP and heart rate (HR) when administered intravenously. Food and tap water were available ad libitum throughout the study. BP and HR were measured using the UA-10 radio-telemetry system (Data Science International, Dallas, TX) as described previously.[3,26–28] Urinary norepinephrine excretion (uNE) for 24 hours was calculated as an indicator of SNA, as described previously.[3,25–27] In addition, spectral analysis was performed using an adaptive autoregressive model to provide power spectra for systolic BP (SBP). Low frequency power of SBP was computed by integrating the spectra between 0.04 and 0.15 Hz, and SNA is presented as the normalized unit of the low frequency component of SBP (LFnuSBP).[29–31] Baroreflex sensitivity (BRS) was measured using the spontaneous sequence method as a parameter of autonomic control. Sequence analysis was performed to detect sequences of 3 or more beats in which there was either an increase in SBP and pulse interval (up sequence) or a decrease in SBP and pulse interval (down sequence). BRS was estimated as the mean slope of the up and down sequences.[32–34] The RVLM was defined according to a rat brain atlas as described previously.[3,26–28] 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.

Measurement of TBARS

The RVLM tissues were homogenized, and thiobarbituric acid (0.3%) was added to the homogenate. The mixture was extracted with a mixture of distilled water and *n*-butanolpyridine (15:1) and centrifuged at 1600*g* for 10 minutes. The amount of thiobarbituric acid reactive substances (TBARS) was determined by absorbance measured at 532 nm, as described previously.[3,7]

Expression of Rac1, gp91^{phox}, and p22^{phox} in the Membrane Fraction and Rac1, p47^{phox} and p40^{phox} in the Cytosolic Fraction

Western blot analysis was used to determine the expression of Rac1 (Upstate Biotechnology, Lake Placid, NY),[12] gp91^{phox}, and p22^{phox} in the membrane fraction (Santa Cruz Biotechnology, Santa Cruz, CA), and the expression of Rac1, p47^{phox}, and p40^{phox} in the cytosolic fraction (Santa Cruz Biotechnology, Santa Cruz, CA) of the RVLM.

Activity of Rac1 in the RVLM

Rac1 activity can be monitored by its interaction with p21-activated kinase, which only occurs when Rac1 is active. We used a Rac1 Activation kit (Upstate Biotechnology, Lake Placid, NY) to evaluate Rac1 activity in the RVLM, as previously described.[12]

NAD(P)H Oxidase Activity

NAD(P)H-dependent superoxide production in the RVLM was measured using a lucigenin luminescence assay as described previously.[35,36] Quantification of NAD(P)H oxidase activity was expressed relative to that in WKY rats, which was assigned a value of 1.

Cu/Zn-SOD and Mn-SOD Activity in the RVLM

Cu/Zn-SOD or Mn-SOD activity was assayed by monitoring the inhibition of the rate of xanthine-mediated/xanthine oxidase–mediated reduction of cytochrome c (pH 7.4). To discriminate between Cu/Zn-SOD and Mn-SOD activities, the assay was also performed after incubation in the presence of KCN, which selectively inhibits the Cu/Zn-SOD isoform.[37] Cu/Zn- and Mn-SOD activities were expressed relative to those in vehicle-treated WKY rats, which were assigned a value of 1.

Microinjection of Apocynin Into the Bilateral RVLM

In other S-ATOR and S-VEH, (n = 5 for each) on day 14, the NAD(P)H oxidase inhibitor apocynin (1 nmol) was microinjected bilaterally into the RVLM, as described previously.[3]

Statistical Analysis

Normally distributed variables were expressed as mean ± SD. An unpaired *t* test was used to compare the differences between groups of normally distributed variables, and the Mann–Whitney *U* test was used to compare differences between groups of non–normally distributed variables. A 2-factor repeated-measures analysis of variance was used to compare differences between groups. Differences were considered to be statistically significant with a *P* value of less than 0.05.

RESULTS

BP, HR, SNA, and BRS

Mean BP (MBP) and HR were significantly decreased on day 4 after the administration of atorvastatin in S-ATOR. On day 14, MBP, HR, 24-hour uNE, and LFnuSBP were significantly higher in S-VEH than in W-VEH and lower in S-ATOR than in S-VEH (Fig. 1A–D). BRS was significantly lower in S-VEH than in W-VEH (12.8 ± 2.3 vs. 19.7 ± 1.8 ms/mm Hg, n = 5 for each; $P < 0.05$) and significantly higher in S-ATOR than in S-VEH (16.4 ± 1.6 vs. 12.8 ± 2.3 ms/mm Hg, n = 5 for each; $P < 0.05$). Mean BP, HR, 24-hour uNE, LFnuSBP, and BRS values did not significantly differ between W-ATOR and W-VEH (Fig. 1A–D).

Oxidative Stress Measured by TBARS Methods in the RVLM

Oxidative stress in the RVLM measured by the TBARS method was significantly lower in S-ATOR than in S-VEH (Fig. 2). Oxidative stress did not differ significantly between W-ATOR and W-VEH (Fig. 2).

Activity of NAD(P)H Oxidase and Rac1 in the RVLM

The activity of NAD(P)H oxidase was significantly lower in S-ATOR than in S-VEH (Fig. 3A). The activity of Rac1 was also significantly lower in S-ATOR than in S-VEH (Fig. 3B). NAD(P)H oxidase activity and Rac1 activity did not significantly differ between W-ATOR and W-VEH (Fig. 3A, B).

Expression of Rac1, gp91^{phox}, and p22^{phox} in the Membrane Fraction and Rac1, p47^{phox}, and p40^{phox} in the Cytosolic Fraction

The expression of Rac1, gp91^{phox}, and p22^{phox} in the membrane fraction was significantly lower in S-ATOR than in S-VEH (Fig. 4A–C). The expression of p47^{phox} and p40^{phox} in the cytosolic fraction was also significantly lower in S-ATOR than in S-VEH (Fig. 5B, C). The expression of Rac1 in the cytosolic fraction was significantly higher, however, in S-ATOR than in S-VEH (Fig. 5A). The expression of Rac1, gp91^{phox}, and p22^{phox} in the membrane fraction and the expression of Rac1, p47^{phox}, and p40^{phox} in cytosolic fraction did not differ significantly between W-ATOR and W-VEH (Figs. 4A–C, 5A–C).

Cu/Zn- and Mn-SOD Activity in the RVLM

Mn-SOD activity in the RVLM was significantly higher in S-ATOR than in S-VEH, but Cu/Zn-SOD activity did not significantly differ between S-ATOR and S-VEH (Fig. 6A, B).

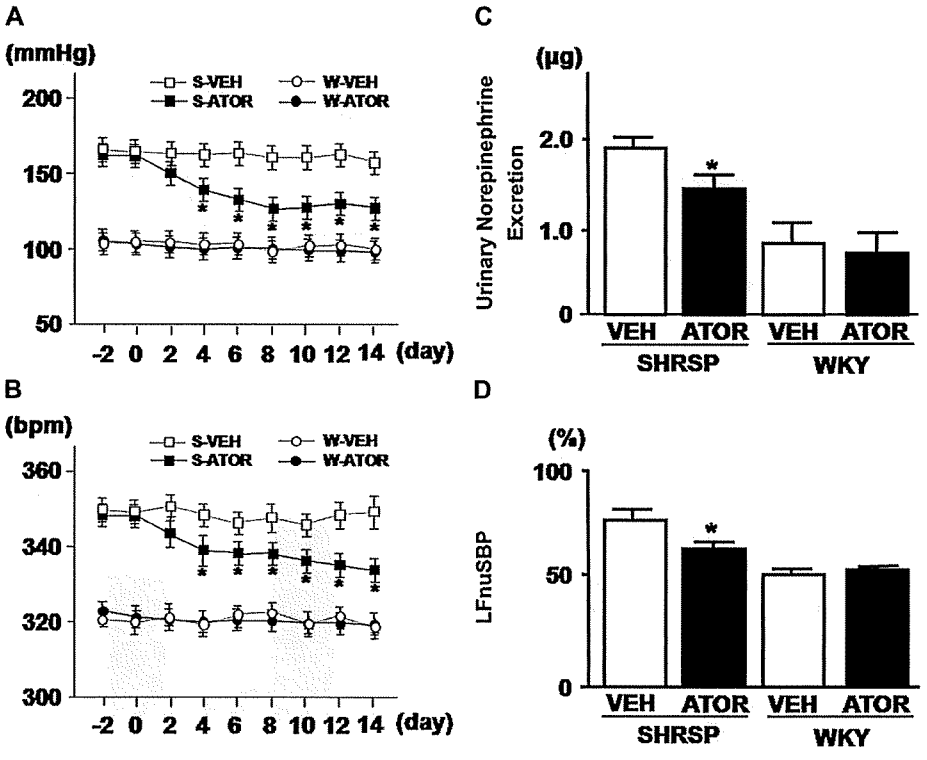

FIGURE 1. Time course of MBP (in mm Hg) (A) and HR (in beats per minute) (B) in S-ATOR (n = 5), S-VEH (n = 5), W-ATOR (n = 5), and W-VEH (n = 5). *$P < 0.05$ for ATOR versus VEH values in each strain. C, D, Urinary norepinephrine excretion for 24 hours (in micrograms) (C) and LFnuSBP (percentage) (D) at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 for each). *$P < 0.05$ for ATOR versus VEH values in each strain. †$P < 0.05$ compared with VEH-treated WKY. Data are shown as mean ± standard error of the mean.

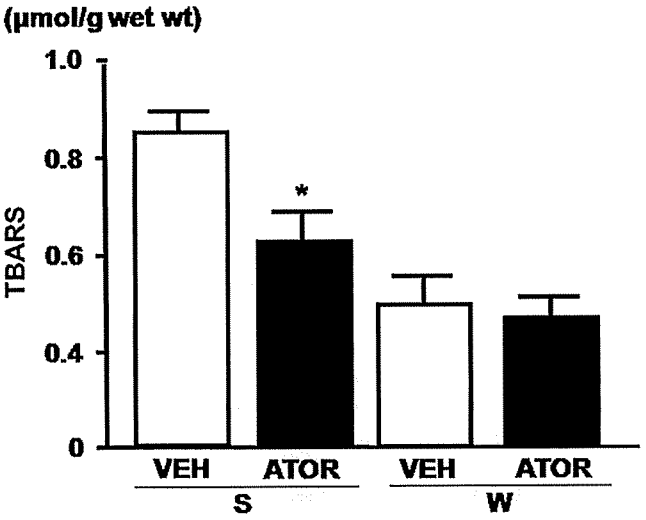

FIGURE 2. TBARS levels (in micromolars per gram wet weight) in the RVLM at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 for each). *$P < 0.05$ for ATOR versus VEH in each strain. †$P < 0.05$ compared with VEH-treated WKY. Data are shown as mean ± standard error of the mean.

Cu/Zn- and Mn-SOD activity did not significantly differ between W-ATOR and W-VEH (Fig. 6A, B).

Microinjection of Apocynin Into the RVLM

The degree of the change in MBP induced by the microinjection of apocynin into the bilateral RVLM was significantly smaller in S-ATOR than in S-VEH (−9.4 ± 1.9 vs. −26.4 ± 3.7 mm Hg; n = 5; $P < 0.05$).

DISCUSSION

The novel finding of the present study was that atorvastatin administered chronically into the brain in SHRSP reduced BP and SNA in SHRSP and that it was associated with reduced oxidative stress, probably due to the inhibition of NAD(P)H oxidase and the activation of Mn-SOD in the RVLM of SHRSP. This is supported by the following findings: (1) ICV injection of atorvastatin for 14 days decreased MBP, HR, SNA, and TBARS in the RVLM of SHRSP; (2) ICV injection of atorvastatin decreased NAD(P)H oxidase activity through the inhibition of Rac1 membrane translocation in the RVLM of SHRSP; (3) ICV injection of atorvastatin activated Mn-SOD in the RVLM of SHRSP; and (4) changes in MBP induced by microinjection of NAD(P)H oxidase inhibitor into the RVLM were significantly smaller in SHRSP treated with atorvastatin than in SHRSP treated with vehicle. Thus, atorvastatin inhibits Rac1 membrane translocation and Rac1 activity in the RVLM of SHRSP.

Atorvastatin decreased the expression of NAD(P)H membrane-bound subunits $gp91^{phox}$ and $p22^{phox}$ and the cytosolic regulatory subunit $p47^{phox}$ and $p40^{phox}$ and inhibited NAD(P)H oxidase activity in the RVLM of SHRSP. Oral administration of atorvastatin decreases ROS in the RVLM of SHRSP.[3] In the brain, ROS is produced mainly by NAD(P)H oxidase, which is activated through Rac1 membrane translocation.[11] In another area of the brainstem, the nucleus tractus solitarius, the inhibition of Rac1 decreases NAD(P)H oxidase activity and ROS formation.[12] Previous reports suggest that atorvastatin inhibits Rac1 membrane translocation and NAD(P)H oxidase activity in the vasculature of hypertensive rats.[13] We found that the depressor response elicited by apocynin into the RVLM was attenuated in SHRSP treated with ICV atorvastatin in the present study. Based on these findings, we suggest that the atorvastatin-induced reduction of ROS in the RVLM of SHRSP is caused by a decrease in NAD(P)H oxidase activity linked to the inhibition of Rac1 membrane translocation.

Atorvastatin activated Mn-SOD activity in the RVLM of SHRSP but not Cu/Zn-SOD. In the RVLM of SHRSP, Mn-SOD activity is decreased, and overexpression of Mn-SOD in the RVLM of SHRSP decreases ROS.[3] A number of reports suggest that statins activate total SOD[20–23] and Cu/Zn-SOD in the vasculature.[26,27] In the present study, however, atorvastatin did not activate Cu/Zn-SOD in the RVLM of SHRSP. In the nucleus tractus solitarius, Cu/Zn-SOD expression is decreased in SHRSP.[26] It is not clear why atorvastatin did not activate Cu/Zn-SOD in the present study. Recently, we reported that angiotensin II increases the intracellular Ca^{2+} concentration and that the increase in mitochondrial Ca^{2+} uptake leads to mitochondrial ROS production in the RVLM.[24] Therefore, it is possible that atorvastatin-induced activation of Mn-SOD in the RVLM of SHRSP contributes to inhibit ROS to an even greater extent than Cu/Zn-SOD.

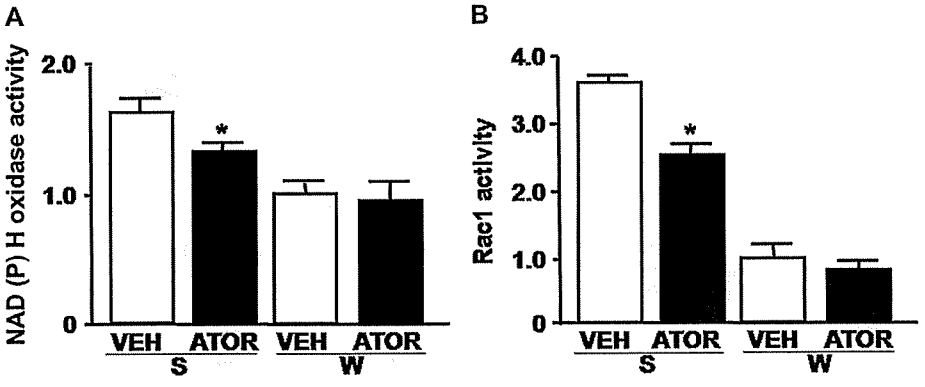

FIGURE 3. NAD(P)H oxidase activity (A) and Rac1 activity (B), in the RVLM at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 for each). *$P < 0.05$ for ATOR versus VEH in each strain. †$P < 0.05$ compared with VEH-treated WKY. NAD(P)H oxidase or Rac1 activity was expressed relative to that in W-VEH, which was assigned a value of 1. Data are shown as mean ± standard error of the mean.

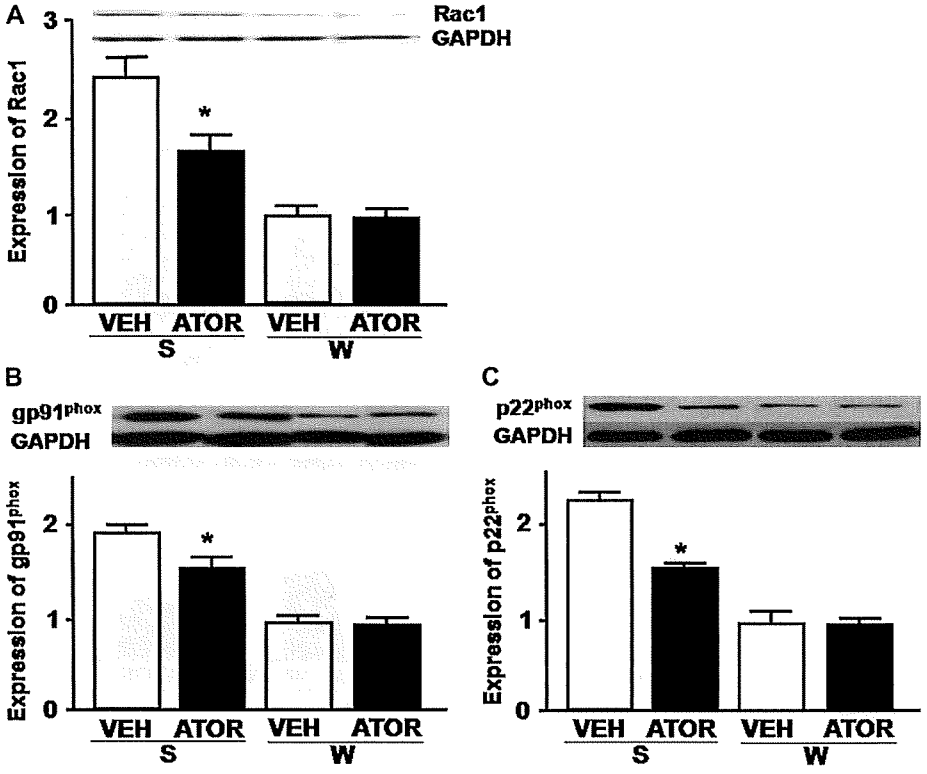

FIGURE 4. Western blot analysis showing the level of expression of Rac1 (A), gp91^{phox} (B), and p22^{phox} (C) in the membrane fraction of the RVLM at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 for each). *$P < 0.05$ for ATOR versus VEH in each strain. †$P < 0.05$ compared with VEH-treated WKY. The expression level of Rac1, gp91^{phox}, and p22^{phox} was expressed relative to that in W-VEH, which was assigned a value of 1. Data are shown as mean ± standard error of the mean.

In the present study, we measured SNA by spectral analysis. Low frequency power of SBP was computed by integrating the spectra between 0.04 and 0.15 Hz, and SNA is presented as LFnuSBP, as described in previous reports.[29–31] On day 14, the LFnuSBP values were comparable to those of uNE. Therefore, this method seems to be useful for measuring SNA in awake animals. Furthermore, atorvastatin improved the impaired baroreflex control in the SHRSP in the present study. Whereas we did not measure cardiac output in the present study and the reduction of BP and HR due to atorvastatin might cause a potential fall in cardiac output, the effects of atorvastatin are due to the decrease in sympathetic nerve activity. It is generally accepted that SNA is enhanced in SHRSP,[3,5,26–28,40] and atorvastatin attenuates the enhanced central sympathetic outflow to various organs including heart, kidney, and vasculature. At least, atorvastatin did not induce heart failure due to low cardiac output. We consider that the decrease in central sympathetic outflow reduced the peripheral vascular resistance by which cardiac output keep constant instead of the reduction of sympathetic outflow to the heart.

Another intriguing finding of the present study is that the BP-lowering and sympathoinhibitory effects are comparable between oral administration (50 mg/kg^{-1}/day^{-1})[7] and ICV injection (2 μg/kg^{-1}/day^{-1}) of atorvastatin. We confirmed the direct effects of atorvastatin administered into the brain on BP, SNA, and baroreflex function in SHRSP as one of the hypertensive models in the present study. The changes in TBARS levels are also similar between oral administration and ICV injection of atorvastatin. In SHRSP, the blood–brain barrier might be disrupted[38] and oral administration of atorvastatin is considered to affect the brain directly.[39] The present findings suggest that orally administered atorvastatin crosses the blood–brain barrier and affects the brain of SHRSP. The abnormal activation of sympathetic nervous system causes hypertension, heart failure, and ischemic heart diseases, and we consider that oral administration of atorvastatin has a potential to treat cardiovascular diseases due to the sympathoinhibition through the antioxidant effect in the RVLM.

We previously demonstrated that oral administration of atorvastatin increases the expression of endothelial nitric oxide synthase (eNOS) in the brainstem.[40] Overexpression of eNOS in the RVLM decreases SNA in WKY and SHRSP.[26–28] In the present study, we did not investigate whether an increase in NO production in the RVLM is involved in the reduction of BP and oxidative stress. It is possible, however, that ICV injection of atorvastatin increases eNOS in the RVLM of SHRSP and that an increase in eNOS contributes to the sympathoinhibitory effect. Further study is needed to clarify this issue.

In WKY rats, atorvastatin does not alter SNA and oxidative stress in the RVLM; these results are compatible with our previous report.[7] Moreover, atorvastatin also does not alter Rac1-induced NAD(P)H oxidase activity and Mn-SOD activity in the RVLM of WKY rats. In the present study, the mechanisms by which atorvastatin affected Rac1-induced NAD(P)H oxidase activity and Mn-SOD activity in SHRSP, but not in WKY, were not determined. It may be that there are thresholds for the induction of Rac1-induced NAD(P)H oxidase activity and Mn-SOD activity in the RVLM, which

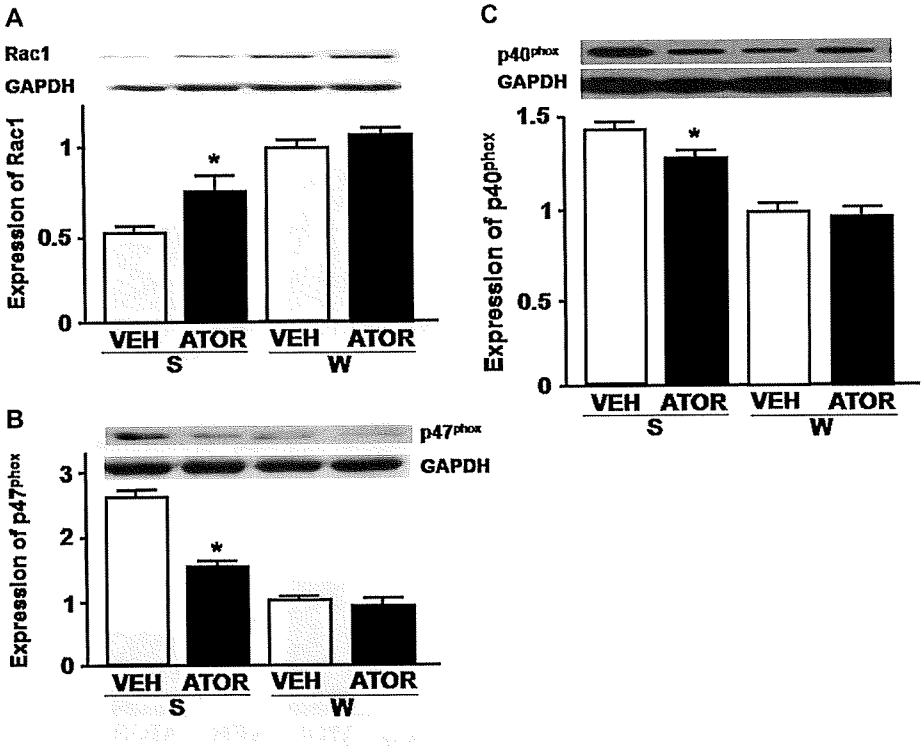

FIGURE 5. Western blot analysis showing the level of expression of Rac1 (A), $p47^{phox}$ (B), and $p40^{phox}$ (C) in the cytosolic fraction of the RVLM at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 per group). $*P < 0.05$ for ATOR versus VEH in each strain. $\dagger P < 0.05$ compared with VEH-treated WKY. The expression level of Rac1, $p47^{phox}$, and $p40^{phox}$ was expressed relative to that in W-VEH, which was assigned a value of 1. Data are shown as mean ± standard error of the mean.

are differently affected by atorvastatin between SHRSP and WKY rats.

STUDY LIMITATIONS

The present study has several limitations. First, we examined the effects of atorvastatin specifically in only the RVLM, and its effects in other brain areas cannot be excluded at this time. Nevertheless, neural activity in the RVLM has a direct influence on SNA,[1,2] and the present results identified an antioxidant effect of atorvastatin and its mechanisms in the RVLM. Angiotensin II type 1 receptors (AT_1R) are abundantly distributed in the RVLM, and there is a close link between AT_1R stimulation and NAD(P)H oxidase activation.[41] Therefore, in the present study, we focused on the RVLM, although other brain regions related to central autonomic control also contain AT_1R and NAD(P)H oxidase. Second, among all statins, we only studied the effect of atorvastatin, which is a lipophilic statin.[42] Our previous studies suggested that oral atorvastatin also reduces oxidative stress in the RVLM.[7] Further study is needed to clarify whether our results in the present study are broad class effects or are specific for atorvastatin. Finally, a recent study suggests that statins reduce BP in patients with hypertension.[43] It will be important to determine whether atorvastatin has this beneficial effect caused by the mechanism related to our suggestion in the present study, although we understand that this is difficult to examine in humans.

CONCLUSIONS

In conclusion, atorvastatin administered directly into the brain of SHRSP decreases BP, SNA, and baroreflex function. The findings of the present study suggest that these effects are associated with inhibition of oxidative stress in the RVLM, probably resulting from a decrease in NAD(P)H oxidase activity and the upregulation of Mn-SOD activity in the RVLM.

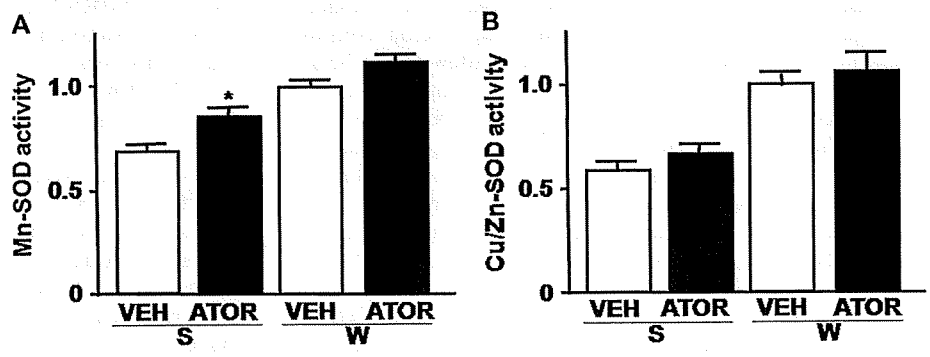

FIGURE 6. The activities of Mn-SOD (A) and Cu/Zn-SOD (B) in the RVLM at day 14 in ATOR- or VEH-treated SHRSP or WKY (n = 5 for each). $*P < 0.05$ for ATOR versus VEH in each strain. $\dagger P < 0.05$ compared with VEH-treated WKY. The activities of Mn-SOD and Cu/Zn-SOD were expressed relative to that in W-VEH, which was assigned a value of 1. Data are shown as mean ± standard error of the mean.

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Atorvastatin Improves the Impaired Baroreflex Sensitivity via Anti-Oxidant Effect in the Rostral Ventrolateral Medulla of SHRSP

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We have demonstrated that oxidative stress in the rostral ventrolateral medulla (RVLM), a vasomotor center in brainstem, increases sympathetic nerve activity (SNA) and that oral administration of atorvastatin inhibited SNA via anti-oxidant effect in the RVLM of stroke-prone spontaneously hypertensive rats (SHRSPs). The impairment of baroreflex sensitivity (BRS) is known as the predictive factor of mortality in the hypertension and BRS is impaired in SHRSP. The aim of the present study was to determine whether oral administration of atorvastatin improved the impaired BRS via anti-oxidant effect in the RVLM in SHRSP. Atorvastatin (20 mg/kg/day) or vehicle was orally administered for 28 days in SHRSPs. Systolic blood pressure (SBP), heart rate, and 24-h urinary norepinephrine excretion as an indicator of SNA were comparable between atorvastatin- and control-SHRSP. Thiobarbituric acid-reactive substance (TBARS) levels as a marker of oxidative stress was significantly lower in atorvastatin-SHRSP than in control-SHRSP. Baroreflex sensitivity measured by the spontaneous sequence method was significantly higher in atorvastatin-SHRSP than in control-SHRSP. These results suggest that atorvastatin improves the impaired BRS in SHRSP via its anti-oxidant effect in the RVLM of SHRSP.

Keywords statin, oxidative stress, brain, hypertension, baroreflex

Introduction

Rostral ventrolateral medulla (RVLM) in the brainstem is the vasomotor center that determines basal sympathetic nerve activity, and the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone (1–3). We have demonstrated that oxidative stress in the RVLM increases the sympathetic nerve activity (4), and that nitric oxide (NO) in the RVLM decreases the sympathetic nerve activity (5,6). Previously, we also demonstrated that overexpression of endothelial NO synthase in the RVLM of Stroke-prone

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spontaneously hypertensive rats (SHRSPs) improved the baroreflex control of heart rate due to the sympatho-inhibition caused by the increase in NO production in the RVLM (7). However, it has not been determined whether the inhibition of oxidative stress in the RVLM improves the impaired baroreflex control of the heart rate of SHRSP or not.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis, and statins have reported to have an anti-oxidant effect (8). Previously, we have demonstrated that orally atorvastatin increases the expression of NO synthase in the brain of SHRSPs (9), and that NO in the RVLM improves the impaired baroreflex control of heart rate in SHRSPs (7). These results suggested that orally atorvastatin might have the potential to improve the baroreflex control of heart rate in SHRSPs. Moreover, orally atorvastatin also inhibited the sympathetic nerve activity through the decrease in oxidative stress in the RVLM of SHRSPs (10).

Therefore, the aim of the present study was to investigate the effect of oral-administered atorvastatin on the baroreflex control of heart rate through its anti-oxidative stress in the RVLM of SHRSPs.

Materials and Methods

Animals and General Procedures

Twelve-week-old male SHRSPs/Izm and Wistar-Kyoto (WKY) rats (280 to 340g; SLC Japan, Hamamatsu, Japan) were fed a standard rodent diet. Food and tap water were available *ad libitum* throughout the study. The rats were kept in a room maintained at a constant temperature and humidity under a 12-h light period between 8:00 AM and 8:00 PM. After adaptation to these conditions over at least 2 weeks, SHRSPs were divided into two groups: 1) atorvastatin-treated SHRSP, treated with atorvastatin of 20mg/kg/day for 28 days, and 2) control-SHRSPs, treated with vehicle (0.5% methyl cellulose). All drugs were dissolved in 0.5% methyl cellulose and administered by gastric gavage everyday. Systolic blood pressure (SBP) and heart rate were measured using the tail-cuff method (BP-98A; Softron, Tokyo, Japan). We calculated the urinary norepinephrine excretion for 24 h as an indicator of sympathetic nerve activity, as described previously (4–7,10). To obtain the RVLM tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with phosphate buffer saline (PBS) (150 mol/L NaCl, 3 mmol/L KCl, and 5 nmol/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 was defined according to a rat brain atlas as described previously (4–7,10), and obtained by a punch-out technique. This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University.

Measurement of TBARS

The RVLM 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 thiobarbituric acid-reactive substances (TBARS) was determined by absorbance, as described previously (4,10).

Measurement of Baroreflex Sensitivity by Spontaneous Sequence Method

Rats were initially anesthetized with sodium pentobarbital (50 mg/kg IP followed by 20 mg · kg^{-1} · h^{-1} IV). A catheter was inserted into the femoral artery to record arterial blood pressure, and a heart rate (HR) was derived from the blood pressure recording. The other catheter was also inserted into the femoral vein to allow for intravenous infusion of sodium pentobarbital. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. Sequence analysis detected sequences of three or more beats in which there was an increase both in SBP and pulse interval (up sequence) or a decrease both in SBP and pulse interval (down sequence). Baroreflex sensitivity (BRS) was estimated as the mean slope of the up sequences (up BRS), the down sequences (down BRS), and also the mean slope of all sequences (sequence BRS) (11,12).

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. ANOVA was used to compare the blood pressure, HR, baroreflex sensitivity, and TBARS level in atorvastatin- or control-SHRSP and WKY. Differences were considered to be statistically significant at a P value of < 0.05.

Results

BP, HR, and Urinary Norepinephrine Excretion

Systolic blood pressure was significantly higher in atorvastatin-SHRSP and control-SHRSP than in WKY, and atorvastatin did not alter SBP in SHRSP (Figure 1A). Heart rate was significantly higher in atorvastatin-SHRSP and control-SHRSP than in WKY, and atorvastatin also did not alter HR in SHRSP (Figure 1B). Urinary norepinephrine excretion was significantly higher in atorvastatin- and control-SHRSP than in WKY, and was not different between control- and atorvastatin-SHRSP (Figure 2).

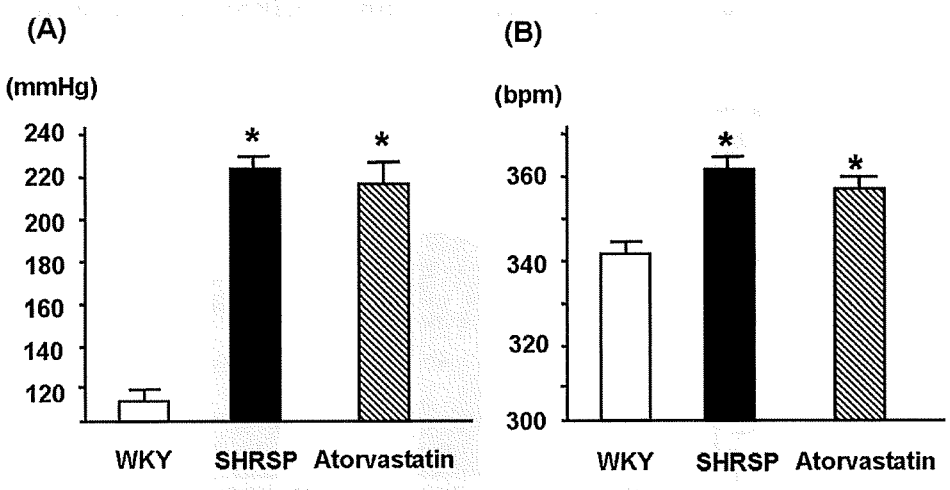

Figure 1. (A) Effects of the treatment with atorvastatin for 28 days on systolic blood pressure (SBP) of SHRSP and WKY. Data are shown as mean ± SEM (n = 5 for each group). *P < 0.05 vs. WKY. (B) Effects of the treatment with atorvastatin for 28 days on heart rate of SHRSP and WKY. Data are shown as mean ± SEM (n = 5 for each group). *P < 0.05 vs. WKY.

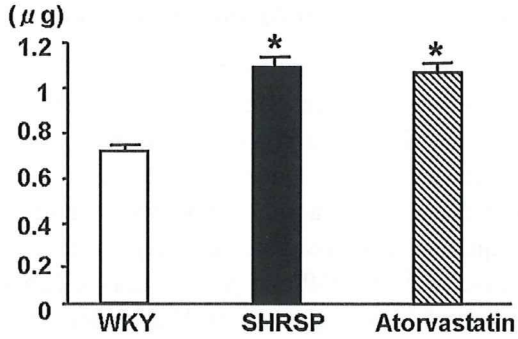

Figure 2. Effects of the treatment with atorvastatin for 28 days on 24-h urinary norepinephrine excretion of SHRSP and WKY. Data are shown as mean ± SEM (n = 5 for each group). *P < 0.05 vs. WKY.

TBARS Levels in the RVLM Tissues

Thiobarbituric acid-reactive substance levels in the RVLM were significantly higher in control- and atorvastatin-SHRSP than in WKY, and those of atorvastatin-SHRSP were significantly lower than those of control-SHRSP (0.70 ± 0.05 μmol/g wet wt vs. 0.91 ± 0.06 μmol/g wet wt, n = 5 for each, $P < 0.05$; Figure 3).

Baroreflex Sensitivity

Baroreflex sensitivity of control-SHRSP was significantly lower than that of WKY (9.2 ± 0.7 ms/mmHg vs. 19.1 ± 0.5 ms/mmHg, n = 5 for each, $P < 0.01$), and that of atorvastatin-SHRSP was significantly higher than that of control-SHRSP (14.8 ± 0.7 ms/mmHg vs. 9.2 ± 0.7 ms/mmHg, n = 5 for each, $P < 0.01$) (Figure 4).

Discussion

In the present study, we demonstrated for the first time that oral administration of atorvastatin improved the impaired baroreflex control of HR in SHRSP, and the improvement

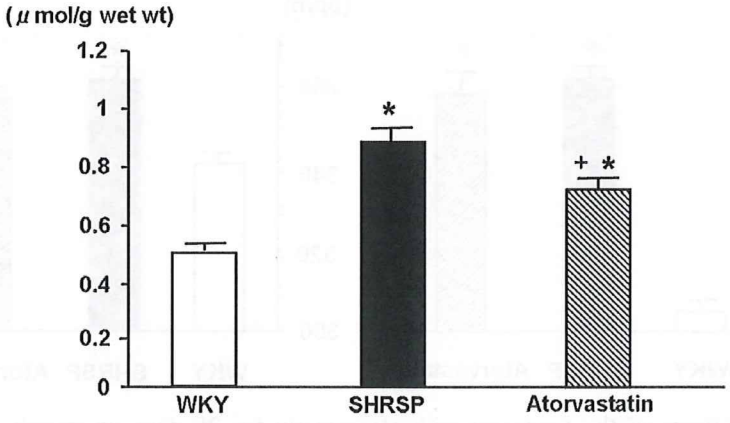

Figure 3. Effects of the treatment with atorvastatin for 28 days on TBARS levels in the RVLM of SHRSP and WKY. Data are shown as mean ± SEM (n = 5 for each group). *P < 0.05 vs. WKY; $^{+}$P < 0.05 vs. control-SHRSP.

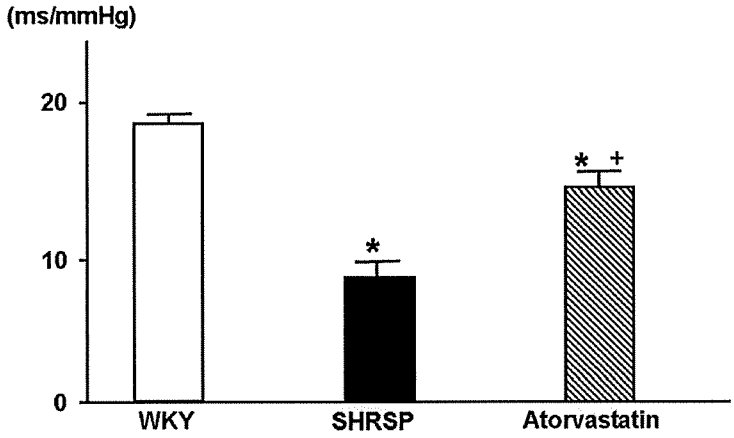

Figure 4. Effects of the treatment with atorvastatin for 28 days on baroreflex control of heart rate of SHRSP and WKY. Data are shown as mean ± SEM (n = 5 for each group). *P < 0.05 vs. WKY; $^{+}$P < 0.05 vs. control-SHRSP.

might be due in part to the inhibition of oxidative stress in the RVLM. Moreover, the improvement of baroreflex control of HR was independent of sympathetic nerve activity or blood pressure. We consider that these effects of atorvastatin benefit the treatment of the cardiovascular diseases with the disorder of baroreflex control.

In the present study, we demonstrated that atorvastatin improved the impaired baroreflex control without the reduction of BP or sympathetic nerve activity. Previously we reported that high-dose orally atorvastatin decreased BP and sympathetic nerve activity through the inhibition of oxidative stress in the RVLM (10). However, in the present study, low-dose atorvastatin did not decrease BP or sympathetic nerve activity, whereas oxidative stress in the RVLM was inhibited. We consider that this discrepancy is due to the smaller reduction of oxidative stress in the RVLM measure by TBARS compared to our previous study (10). We selected the lower dose of atorvastatin, because the effect of atorvastatin on baroreflex control should be examined in the condition excluded by BP and sympathetic nerve activity lowering effects. Moreover, baroreflex control is one of the key mechanisms responsible for the short-term control of BP. Impairment of this reflex has been found in a number of conditions, such as aging (13), heart failure (14), post-myocardial infarction (15), and the impairment of baroreflex sensitivity is known as the predictive factor of mortality in the hypertension (16). From the results in the present study, we consider that atorvastatin benefits the treatment for cardiovascular diseases.

The mechanisms in which atorvastatin improved the baroreflex control have not been determined in the present study. We consider that one of the possibilities in the mechanisms was the inhibition of oxidative stress in the RVLM, because the oxidative stress is the important modulator on the sympathetic nerve activity (4,10). Moreover, NO in the RVLM of SHRSPs improved the baroreflex control of HR (7). The inhibition of oxidative stress due to atorvastatin will contribute to the increase in NO in the RVLM of SHRSP and to the improvement of baroreflex control of HR.

A recent study suggests that the reduction of BP by clinical doses of statin is small but significant (17). However, the change in BP in the previous clinical study is significantly smaller than that in the present and previous animal study (10). Moreover, we are not able to determine the oxidative stress in the brain of human *in vivo* now and to determine whether the clinical doses of atorvastatin have the anti-oxidant effect in the