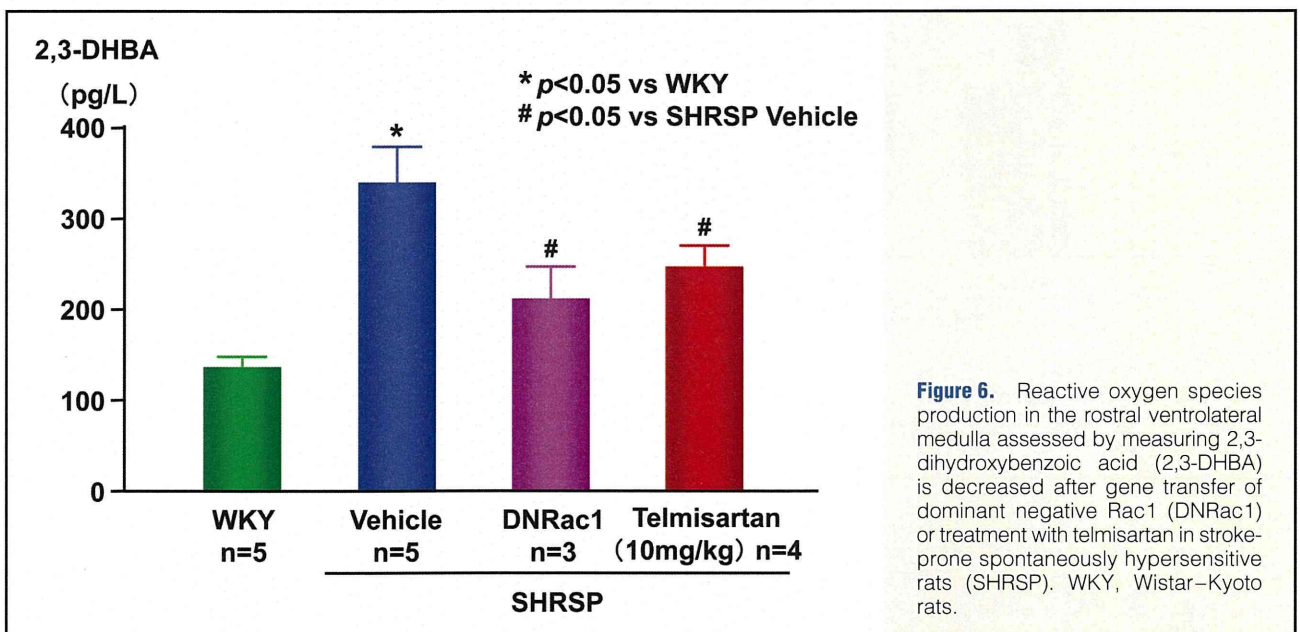
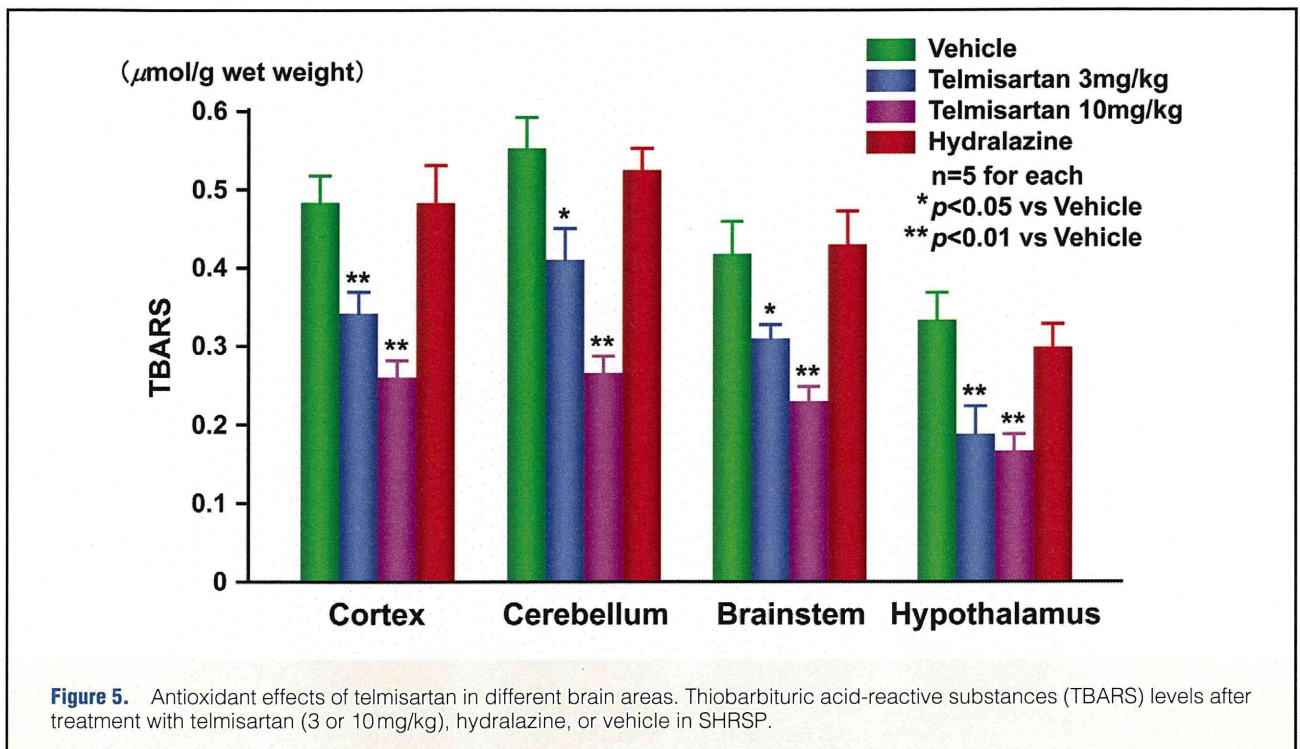


Figure 4. (A) Time course of changes in systolic blood pressure (SBP) and heart rate (HR) in SHRSP treated with telmisartan. (B) Telmisartan decreases 24-h urinary norepinephrine excretion at day 30, and hydralazine increases 24-h urinary norepinephrine excretion.

effect of treatment with telmisartan at either a high dose ($10\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or a low dose ($3\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), or hydralazine for 30 days on hypertension.⁴⁵ Systolic blood pressure (SBP) and heart rate were measured using the tail-cuff method. Urinary norepinephrine excretion was measured as a marker of the sympathetic nervous system activity. We evaluated ROS in the brain (cortex, cerebellum, hypothalamus, and brainstem) of SHRSP on ESR spectroscopy and TBARS. Oral treatment with telmisartan reduced SBP dose-dependently and hydralazine reduced SBP to a similar level to the high dose of telmisartan (Figure 4). Telmisartan reduced, while hydralazine increased, urinary norepinephrine excretion (Figure 4). TBARS levels were significantly increased in each area of the brain of SHRSP compared with WKY rats (Figure 5). Oral treatment with telmisartan reduced the TBARS levels, but hydralazine did not (Figure 5). These findings suggest that (1) anti-hypertensive treatment with

telmisartan reduces ROS in the brain of SHRSP; (2) telmisartan decreases blood pressure, at least in part, via a reduction of the sympathetic nervous system activity in SHRSP; and (3) these effects induced by telmisartan might be associated with protection of the brain of SHRSP from oxidative stress. We also measured the concentration of hydroxyl radicals using a modified procedure based on the hydroxylation of sodium salicylate by hydroxyl radicals,⁴⁶ leading to the production of 2,3-dihydroxybenzoic acid (2,3-DHBA).^{29,47} Inhibition of Rac1 in the RVLM and oral treatment with telmisartan significantly decreased the production of hydroxyl radicals in the RVLM (Figure 6).⁴⁷

Recently, we used *in vivo* ESR to assess oxidative stress in the brain, and found that oral treatment with another ARB, olmesartan, reduces oxidative stress in the brain of SHRSP without inducing reflex activation of the sympathetic nervous system.⁴⁸ In that study we evaluated the *in vivo* ESR signal



decay rates of the brain using methoxycarbonyl-PROXYL, a nitroxyl radical species, as a blood-brain barrier-permeable spin probe.⁴⁹ Oral treatment with olmesartan attenuated the exaggerated pressor response to an excitatory amino acid, L-glutamate, in the RVLM of SHR compared to WKY rats.⁵⁰ Further, the pressor response to microinjection of Ang II into the RVLM was diminished in SHR treated with olmesartan.⁵⁰ Thus, the importance of oxidative stress in the brain and hypertension is supported by our studies as well as those of others.¹¹

Several questions, however, remain to be answered. A

recent study suggested that systemic administration of candesartan reduces brain Ang II levels because it attenuates the mRNA expression of both angiotensinogen and angiotensin-converting enzyme in Ang II-infused rats.⁵¹ Whether systemic treatment with ARBs indirectly regulates brain Ang II remains to be determined.⁵²

Effects of Other Cardiovascular Drugs on Brain Oxidative Stress

Considering that ARBs act to inhibit NAD(P)H oxidase activ-

ity, it is reasonable that ARBs have an antioxidant effect, although there are some unresolved questions, as mentioned previously. Calcium channel blockers, azelnidipine and amlodipine, but not nicardipine, which also have antioxidant properties, have a sympatho-inhibitory effect on the brain.^{53,54} In particular, treatment with azelnidipine reduces oxidative stress in the RVLM associated with a decrease in the activity of NAD(P)H oxidase, Cu/Zn-SOD, and Mn-SOD.⁵³ These effects might be related to an improvement in NO production,⁵⁵ because we also demonstrated that overexpression of endothelial NOS in the NTS or RVLM decreases blood pressure and heart rate via the inhibition of sympathetic nervous system activity.^{56–59} Surprisingly, we also found that atorvastatin inhibits the sympathetic nervous system as a result of upregulating NO activity and reducing oxidative stress.^{60–63} Further studies are needed to determine if this mechanism is also applicable in humans.

Salt-Sensitive Hypertension and Brain Oxidative Stress

Activation of the sympathetic nervous system, in particular, an increase in central sympathetic outflow, plays an important role in the pathogenesis of salt-sensitive hypertension as well as that of kidney diseases.^{64,65} Recent studies suggest that oxidative stress in the brain contributes to blood pressure elevation in salt-sensitive hypertension.^{66,67} We demonstrated that high salt intake exacerbates blood pressure elevation and sympathetic nervous system activity during the development of hypertension in SHR, and these responses are mediated by increased ROS generation, probably because of an upregulation of AT1 receptors and NAD(P)H oxidase in the RVLM.⁶⁶ The findings of a recent study from Kyushu University Graduate School of Medical Sciences indicate that mice with pressure overload acquired brain salt-sensitivity.⁶⁸ This means that high salt intake increases the transport from the blood to the cerebrospinal fluid and the response of the sympathetic nerve activity to salt administered into the brain. These results suggest that pressure overload affects salt sensitivity, thereby enhancing central sympathetic outflow and cardiac function.⁶⁸ Left ventricular hypertrophy is an independent risk of cardiovascular event and high salt intake is an important environmental factor of hypertension, both of which increased ROS, and sympathoexcitation may be involved in the pathogenesis of the development of hypertension. A recent clinical trial suggested that left ventricular hypertrophy is related to cardiovascular events in Japanese high-risk hypertensive patients.⁶⁹

Summary and Future Perspectives

Currently in Japan, many patients with hypertension also have metabolic syndrome. Importantly, the prevalence of metabolic syndrome increases linearly with an increase in heart rate among Japanese men and women,⁷⁰ suggesting that activation of the sympathetic nervous system is involved in the pathogenesis of hypertension.⁷¹ The prevalence of obstructive sleep apnea has increased as a result of the increase in the number of obese patients with hypertension. Obese patients with sleep apnea have enhanced central sympathetic outflow, which worsens hypertension and leads to cardiovascular events.⁷² Further, there is considerable evidence that psychological stress is a major risk factor for cardiovascular diseases and events associated with hypertension.⁷³ Another therapeutic target for the treatment of hypertension is heart

failure with a preserved ejection fraction.⁷⁴ As suggested here, salt-sensitivity might also be enhanced in these patients, thereby further enhancing central sympathetic outflow.⁶⁸ Oxidative stress in the brain as well as other organs might underlie these mechanisms. Future studies of the effects of oxidative stress in the brain are warranted and will provide useful information for the treatment of hypertension.

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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

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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 (AT₁R), 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 AT₁R 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 AT₁R Blocker

S-Farnesylthiosalicylic acid (1 mmol/L), a specific Ras inhibitor³⁰; N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe) fluoromethyl ketone (Z-DEVD-FMK, 1 μmol/L), a specific caspase-3 inhibitor³¹; 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

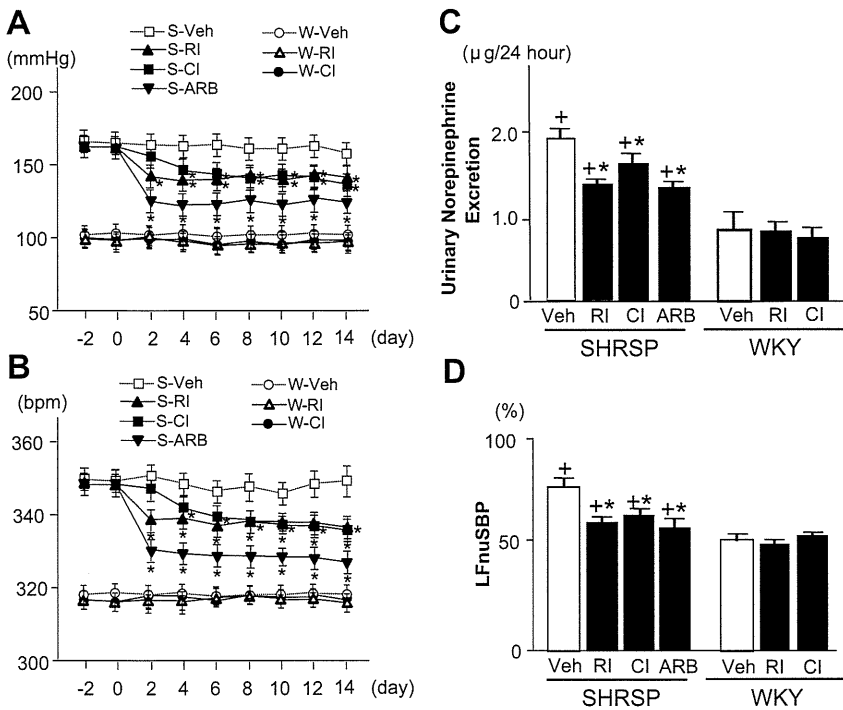


Figure 1. Time course of MBP (A, mm Hg) and HR (B, bpm) in S-RI (n=5), S-CI (n=5), S-ARB (n=5), S-Veh (n=5), W-RI (n=5), W-CI (n=5), and W-Veh (n=5). **P*<0.05 for Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB vs vehicle (Veh) values in each strain. C and D, 24-hour uNE (µg) (C) and LFnuSBP (%) (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).

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 AT₁R-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 AT₁R-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-

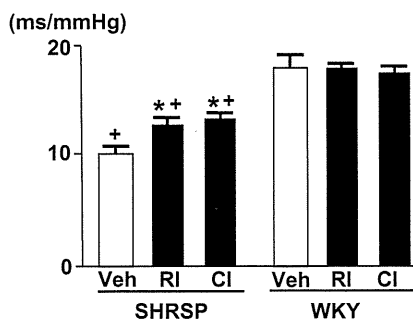


Figure 2. BRS (ms/mm Hg) in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), or caspase-3 inhibitor (CI) (n=5 for each). **P*<0.05 vs Veh in each strain. +*P*<0.05 vs W-Veh. Data are shown as mean±SEM.

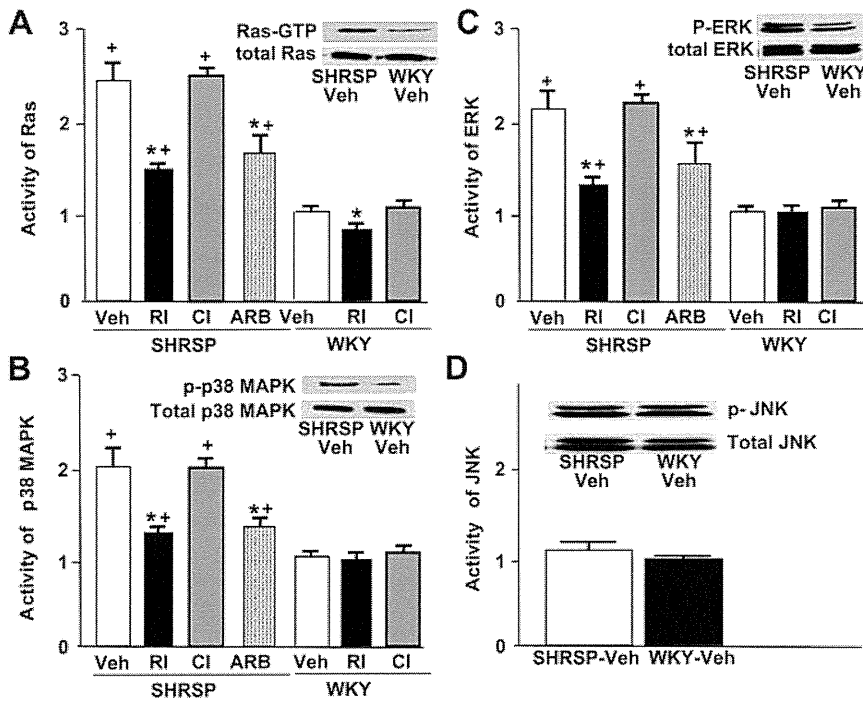


Figure 3. Activity of Ras (A), p38 MAPK (B), ERK (C), and JNK (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5/group). **P*<0.05 vs Veh in each strain. +*P*<0.05 vs Veh-treated WKY. Activity is expressed relative to that 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 AT₁R-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

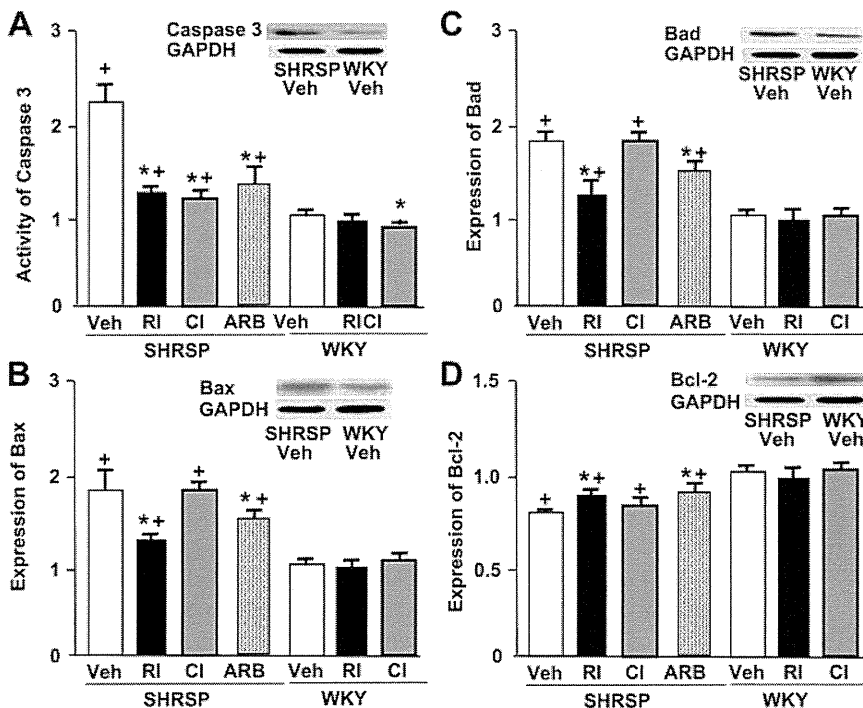


Figure 4. Activity of caspase-3 (A) and expression of Bax (B), Bad (C), and Bcl-2 (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5 for each). **P*<0.05 vs Veh in each strain. +*P*<0.05 vs Veh-treated WKY. Activity and expression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

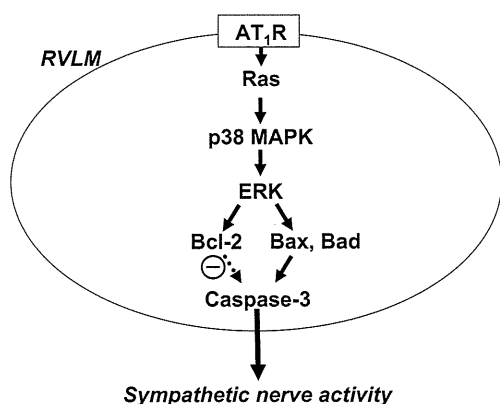


Figure 5. Illustration showing the major findings suggested by the results of the present study.

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 AT₁R/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 AT₁ and angiotensin type 2 (AT₂) receptors.⁴⁵ Importantly, AT₁ 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 AT₁R 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, AT₁R-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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ONLINE SUPPLEMENT

AT₁ Receptor-Activated Caspase-3 through Ras/MAPK/ERK in the RVLM Is Involved in the Sympathoexcitation in SHRSP

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Short title: Ras and Apoptosis in Brain Increases SNA

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Supplemental 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.

Animals and General Procedures

Male SHRSP/Izm rats and age-matched Wistar-Kyoto (WKY) rats (14-16 weeks-old; SLC Japan, Hamamatsu, Japan), fed standard feed, were divided into seven groups (SHRSP treated with Ras inhibitor, S-RI; SHRSP treated with caspase-3 inhibitor, S-CI; SHRSP treated with an angiotensin receptor blocker [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 for each). In the S-RI, W-RI, S-CI, W-CI, S-Veh, W-Veh, and S-ARB groups, we measured blood pressure, and heart rate (HR) using the UA-10 radiotelemetry system (Data Science International) as described previously.¹ Urinary norepinephrine concentrations were measured, and urinary norepinephrine excretion (uNE) for 24 hours was calculated as an indicator of sympathetic nerve activity (SNA), as described previously.^{1,2} Furthermore, in the S-RI, W-RI, S-CI, W-CI, S-Veh, and W-Veh groups, spectral analysis was performed using an adaptive auto-regressive model to provide power spectra for systolic blood pressure (SBP). Blood pressure was recorded for 5 minutes between 9AM and 12 PM every day, and we then determined the total power of SBP and the total spectral density of the variables. The relative value of each spectral power component was also measured and expressed in normalized units. The low frequency (LF) power of SBP was computed by integrating the spectra between 0.04 and 0.15 Hz, and SNA was calculated using the normalized unit of the LF component of SBP (LFnuSBP).³⁻⁶ Baroreflex sensitivity (BRS) was measured using a spontaneous sequence method as a parameter of autonomic control. Sequence analysis detected sequences of three or more beats in which there was either an increase in SBP and pulse interval (PI; Up-Sequence) or a decrease in SBP and PI (Down-Sequence). BRS was estimated as the mean slope of the Up- and Down-Sequences.^{7,8}

To obtain the RVLM tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with phosphate-buffered 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 1-mm thick sections were cut using a cryostat at $-7\pm 1^\circ\text{C}$. The RVLM was defined according to a rat brain atlas, as described previously.¹

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

The Ras activity was determined by measuring the expression of Ras-GTP per total Ras.⁹ The activities of p38 MAPK, ERK, and JNK were determined by measuring the expression of the phosphorylated form of each protein per total Ras, p38 MAPK, ERK, and JNK, respectively. The expression of phosphorylated or total RAS, p38 MAPK, ERK, and JNK in the RVLM tissue was determined by Western blot analysis.

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

The caspase-3 activity in the cytosolic fraction of the RVLM tissues was measured using the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4 methyl coumarin (Ac-DEVD-AMC), as described previously.¹⁰ The reactions were incubated at 37°C and the release of the fluorescent

product was monitored with a spectrofluorometer using excitation and emission wavelengths of 380 and 440 nm, respectively. The expression of Bax, Bad, and Bcl-2 in the mitochondrial fraction of RVLM tissues was determined by Western blot analysis.

Intracerebroventricular Injection of Ras Inhibitor, Caspase-3 Inhibitor, and Angiotensin II Type 1 Receptor Blocker

S-Farnesylthiosalicylic acid (FTS), a specific Ras inhibitor (Calbiochem, La Jolla, CA),¹¹ was dissolved in dimethylsulfoxide (DMSO) and further diluted in artificial cerebrospinal fluid (aCSF) at a concentration of 1 mmol/L. *N*-Benzyloxycarbonyl-Asp (OMe)-Glu (OMe)-Val-Asp (OMe)-fluoro-methylketone (Z-DEVD-FMK), a specific caspase-3 inhibitor (Calbiochem), was also dissolved in DMSO and further diluted in aCSF to a concentration of 750 μ mol/L.¹² FTS, Z-DEVD-FMK, candesartan (1 μ g/ μ l), or DMSO in aCSF as vehicle was infused at 0.5 μ l/h for 14 days with an osmotic minipump (Alzet 1003D; Alza Scientific Products), the cannula of which was placed in the left ventricle (from bregma: anteroposterior, -0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) of SHRSP and WKY. These doses of FTS and Z-DEVD-FMK were determined to decrease blood pressure, HR, and SNA in SHRSP. Changes in blood pressure and HR were measured in SHRSP after terminating the 14-day ICV infusion of the Ras inhibitor (n=4). The dose of candesartan used has no centrally mediated antihypertensive effect in SHR and SHRSP and blocks changes in blood pressure and HR in response to ICV infusion of angiotensin II.¹³

Microinjection of Angiotensin II into the RVLM

Telemetry was used to monitor the changes in mean blood pressure (MBP), HR, and LFnuSBP evoked by the bilateral microinjection of angiotensin II (25 pmol in 50 nL of PBS) into the RVLM of S-RI or S-VEH in anesthetized rats 14 days after beginning the ICV infusion. The microinjection procedures and the method used to verify cannula placement in the RVLM were described previously.¹

Statistical Analysis

Normally distributed variables are expressed as mean \pm SE. Unpaired *t* and Mann-Whitney U tests were used to compare the differences in normally distributed and non-normally distributed variables, respectively. Data were also analyzed by a two-factor repeated-measures analysis of variance. Differences were considered to be statistically significant at a *P* value of less than 0.05.

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Cross-Sectional Characterization of all Classes of Antihypertensives in Terms of Central Blood Pressure in Japanese Hypertensive Patients

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BACKGROUND

Central blood pressure (CBP) has been reported to be superior to brachial blood pressure (BP) as a cardiovascular risk predictor in hypertensive patients; however, the effects of antihypertensives on CBP have not been fully examined. This cross-sectional hypothesis-generating study aimed to tentatively characterize all classes of antihypertensives in relation to CBP.

METHODS

Calibrated tonometric radial artery pressure waveforms were recorded using an automated device in 1,727 treated hypertensive patients and 848 nonhypertensive (non-HT) participants. Radial artery late systolic BP (SBP) has been reported to reflect central SBP. The difference between late and peak SBPs (Δ SBP2) was assessed with linear regression model-based adjustments. Separate regression models for Δ SBP2 were constructed for both participant groups as well as specified sub-populations.

RESULTS

Δ SBP2 was 3.3 mm Hg lower in patients treated with any single-vasodilating (VD) antihypertensive agent without significant interclass difference than with non-VD agents, and was 2.0 mm Hg

lower than estimated in nonhypertensive subjects. Combinations of two vasodilators were 6.6 and 2.9 mm Hg lower in Δ SBP2 than nonvasodilator combinations and nonhypertensive subjects, respectively ($P < 0.001$ for all comparisons). Nonvasodilators and their combination showed high Δ SBP2, 1.1 and 3.7 mm Hg higher than in nonhypertensive subjects ($P < 0.001$ for both). Additional adjustment of the pulse rate reduced high Δ SBP2 with β -blockers (β BLs).

CONCLUSIONS

This cross-sectional observation suggests that vasodilatory antihypertensives lower CBP independently of peripheral BP levels without evident class-specific differences, whereas nonvasodilators may raise CBP.

Keywords: angiotensin receptor blockers; angiotensin-converting enzyme inhibitors; antihypertensive agents; blood pressure; calcium channel blockers; central blood pressure; diuretics; hypertension; late systolic blood pressure; nonvasodilating antihypertensive agents; pulse waveform; radial artery tonometry; vasodilating antihypertensive agents; α -blockers; β -blockers

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From the physical viewpoint, central blood pressure (CBP) more directly imposes mechanical stress on the left ventricle, large arteries and the vital organ vasculature than brachial

blood pressure (BP). This impact of CBP was suggested by large-scale intervention trials and population-based studies, such as the Conduit Artery Function Evaluation (CAFE) study of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)¹ and Strong Heart Study (SHS).² In the CAFE study, only calcium channel blocker (CCB) and β -blocker (β BL)-based treatments were compared in estimated CBP. Prior to the study, several small-scale investigations assessing therapeutic alterations in CBP or aortic wave reflection had been reported.³⁻¹¹ Various theoretical explanations of the benefit of vasodilators to lower CBP have also been published,¹²⁻¹⁴ however, only limited classes of antihypertensive drugs, such as angiotensin-converting enzyme inhibitors (ACEI) and β BL, including nitrates, have been investigated comparatively or noncomparatively. Hence, the effects of various antihypertensives on CBP are not fully understood. Randomized intervention trials are necessary to assess the effects of each antihypertensive

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drug; however, it is practically difficult to directly compare all classes of antihypertensive agents at the same time in a single intervention trial. This cross-sectional observation therefore aimed to tentatively characterize all classes of antihypertensive agents commonly used in Japan in terms of CBP.

METHODS

Study design. This study was a cross-sectional observation, designed as an exploratory (or data-mining) study to generate rather than to test hypotheses.

Subjects. We enrolled 1,727 Japanese patients with essential hypertension (HT), who had been on stable antihypertensive medication for at least 3 months, and with medical data, including radial artery tonometry-derived parameters relating to CBP, from seven major centers and their related medical facilities participating in the Antihypertensives and Blood pressure of Central artery study in Japan (ABC-J). The subjects also included 1,094 participants receiving no antihypertensive therapy. From the untreated population, 848 nonhypertensive (non-HT) subjects were extracted based on BP (systolic BP (SBP) <140 mm Hg and diastolic BP (DBP) <90 mm Hg) measured when the radial artery pulse wave was recorded (Table 1).

The study protocol was approved by the institutional review board of each ABC-J center. Data were obtained from archived medical records of participants in whom the radial artery pulse wave had been recorded in accordance with the method described below. All participants were informed of this study procedure and gave consent to providing their data. The data were collected from January to December in 2007.

Radial artery pulse wave measurement and evaluation of CBP.

Radial artery pressure pulse waveform was recorded with an automated tonometric system, HEM-9000AI (Omron Healthcare, Kyoto, Japan) in a sitting position after at least 5 min of rest. The waveform was calibrated automatically using built-in oscillometric brachial sphygmomanometry. The peak and bottom of the radial pressure wave were adjusted to brachial SBP and DBP, respectively. The HEM-9000AI algorithm automatically performed online detection of the second peak (late systolic inflection) based on the second maxima of the fourth derivative of the radial pressure waveform to determine the radial augmentation index as well as the late or second SBP (SBP2), as shown in Figure 1. The outline of the built-in algorithm of this device has been reported elsewhere.¹⁵

In order to assess CBP-lowering effects selectively, we focused on central SBP levels relative to brachial SBP because absolute CBP levels largely depend on the mean BP level, which is nearly identical for both central and peripheral sites.¹⁶ Figure 1 shows the parameters derived from radial pulse waveform analysis. The height of the second peak corresponds to SBP2, which is reportedly an alternative¹⁷ to or is closely related¹⁸ to directly measured central aortic SBP. SBP2 obtained by the same device as in this study has also been reported to be comparable to central SBP estimated using a generalized aorto-radial transfer function.^{19,20} We created an

index, Δ SBP2, defined as “SBP2 – SBP” (Figure 1), to assess peak SBP reduction between peripheral and central sites.

Therapeutic drugs. Antihypertensive drugs being administered at the time of measurement were obtained from medical records together with coadministered antidyslipidemia and antidiabetic drugs, nitrates and/or nicorandil. All class names and antihypertensive abbreviations are in the footnote of Table 2.

Data analysis. All data are expressed as the mean \pm 1 s.d. unless otherwise specified. Intergroup comparisons of mean values and ratios of subjects' characteristics were tested by unpaired Student's *t*-test and Fisher's exact test, respectively. Multiple regression analysis (forced entry method) was employed to compare all classes of, as well as broadly grouped (vasodilating (VD) and non-VD), antihypertensive drugs in terms of the association with Δ SBP2, where VD included angiotensin receptor blockers (ARB), ACEI, CCB, and α -blocker; and non-VD included Diur and β BL. Categorical data, such as gender and drugs, were assessed as dummy variables (“0” or “1”) in regression models. For VD and non-VD, we made a dummy variable, “Drug group,” which was coded “1” if the patient took any one or several non-VD drugs without VD drugs in combination whereas code “0” indicated any others, including any one or several VD drugs and mixed combinations of VD plus non-VD drugs. In Model 1 in Table 3, where patients with mixed combination were excluded from the subject population, code “0” implied that a patient took

Table 1 | Subject characteristics

	Treated HT	Non-HT	P
N (pts)	1,727	848	
Male/female (pts)	884/843	468/380	0.059
Age (years)	66.5 \pm 11.5	50.3 \pm 16.7	<0.001
Height (cm)	157.6 \pm 9.3	162.4 \pm 9.6	<0.001
Weight (kg)	60.0 \pm 11.4	59.2 \pm 10.5	0.109
BMI (kg/m ²)	24.0 \pm 3.4	22.4 \pm 2.9	<0.001
PR (bpm)	69.2 \pm 12.0	69.1 \pm 10.4	0.949
<i>Calibrated radial artery tonometry</i>			
SBP (mm Hg)	137.7 \pm 17.2	118.4 \pm 11.6	<0.001
SBP2 (mm Hg)	127.6 \pm 18.3	106.7 \pm 14.3	<0.001
DBP (mm Hg)	74.8 \pm 11.8	70.0 \pm 8.9	<0.001
rAI (%)	85.5 \pm 14.0	76.7 \pm 17.1	<0.001
<i>Number of drugs in combination, pts (%)</i>			
1	596 (34.5%)		
2	632 (36.6%)		
3	390 (22.6%)		
4	97 (5.6%)		
5	12 (0.7%)		

BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; Diur, diuretics; HT, treated hypertensives; non-HT, nonhypertensives; PR, pulse rate; pts, number of patients; rAI, radial augmentation index; SBP, systolic blood pressure; SBP2, late systolic blood pressure.

any one or several VD drugs only. Multiple regression models were used for adjusted comparisons of individual classes or broadly divided groups of antihypertensive drugs and their combinations, in which determinants of Δ SBP2 other than a specified antihypertensive drug or a drug combination were adjusted. Because pulse rate is largely attributable to the drug effect itself, it was not adjusted unless otherwise specified. The adjusted comparisons were as follows:

1. A multiple regression model was constructed for Δ SBP2 forcing all possible independent variables, including all antihypertensive drugs either broadly grouped (VD vs. non-VD) or grouped by drug class, to enter the models. Interactive terms relating to drug combinations were examined and significant interactive terms were considered to be included in the model.
2. For the non-HT population, a separate model without the drug variable was constructed. Based on this model, Δ SBP2 was modified by adjusting common confounders (age, gender, height, BMI, DBP) to the mean value of HT. This provided the estimated physiological reference value of Δ SBP2 when the DBP level was comparable with treated HT.
3. We collected adjusted Δ SBP2 data for an individual group or class of antihypertensive agents by extracting cases given a specified type of drugs irrespective of monotherapy or combination. Likewise, data for each combination of two specified antihypertensive classes were collected, including combinations of ≥ 3 drugs.
4. In addition to adjusting for all common confounders and the use of nitrates (=“0”), further adjustments of Δ SBP2 were made for each specified group or class of drugs or their combination. For interdrug group or interclass comparisons, variables for irrelevant classes coadministered and interactive terms were set as “0” (i.e., not used). Similarly, for intercombination therapy comparisons,

variables for coadministered drugs not included in the specified combination as well as interactive terms (unless applicable) were set as “0.”

5. Interdrug group, interclass or intercombination therapy comparisons of CBP indexed by Δ SBP2 were made using the adjusted data, as described above. Adjusted Δ SBP2 was also compared between each drug group and non-HT. Two-group comparisons, including VD only vs. non-VD only and each drug group vs. non-HT, were tested by the Mann–Whitney U-test, whereas interclass and intercombination therapy as well as intertreatment group (i.e., VD vs. non-VD vs. Mixed) comparisons were tested by the Kruskal–Wallis test with multiple comparisons by the Games–Howell method.

All statistical analyses were performed with a commercially available statistical package (SPSS, version 11.0; SPSS, Chicago, IL) and spreadsheet calculation (Excel 2007; Microsoft, Washington, DC). *P* values < 0.05 were regarded as significant.

RESULTS

Subjects' characteristics and details of antihypertensive therapy are shown in **Tables 1** and **2**. Overall, $\geq 60\%$ patients were treated with CCB or ARB. Only one third of participants was treated with monotherapy (**Table 1**), and monotherapy with some classes of drugs was very rare (**Table 2**), which made it difficult to compare all individual antihypertensive classes directly. We therefore first examined broadly divided drug groups, i.e., VD and non-VD. As shown in **Table 3**, partial regression coefficient (*B*) estimates of “Drug group” in Models 1 and 2 consistently indicated that Δ SBP2 was 2.7 mm Hg higher with non-VD than with VD when all included confounders were adjusted.

In 510 participants who provided a detailed clinical data set, including laboratory data and comorbidities, none showed significant associations with Δ SBP2 by multiple regression

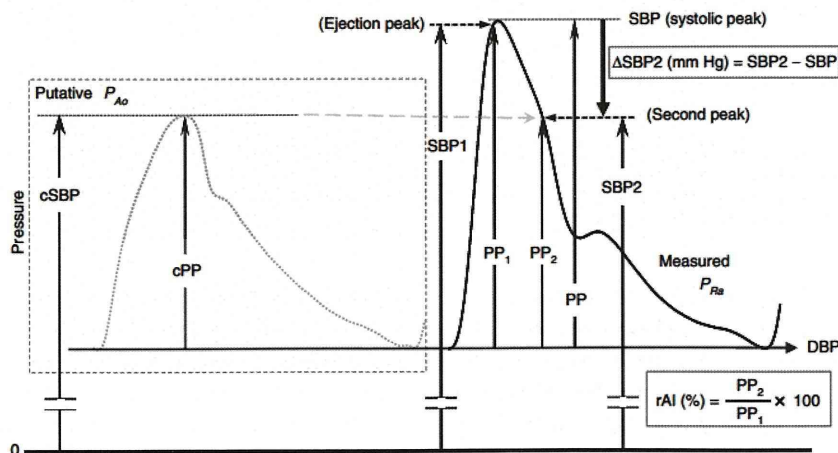


Figure 1 | Definitions of Δ SBP2 and radial augmentation index (rAI), and relationship between each parameter derived from calibrated tonometric radial pressure waveform (P_{Ra} = solid line; right) and corresponding putative aortic pressure waveform (P_{Ao} = dotted line; left). These definitions are expressed as formulas inside the Figure. cPP, central pulse pressure; cSBP, central systolic blood pressure; DBP, diastolic blood pressure; PP, radial pulse pressure; PP₁, ejection peak amplitude of P_{Ra} ; PP₂, second peak amplitude of P_{Ra} ; SBP, radial peak systolic blood pressure; SBP1, radial artery pressure at the ejection peak, which is not necessarily identical to the systolic peak of P_{Ra} ; SBP2, radial artery pressure at the second peak.

Table 2 | Details of antihypertensive therapy

	Class of antihypertensives ^a						
	ARB	ACEI	CCB	α BL	Diur	β BL	Nitro
N (pts (%))	1,019 (59.0)	207 (12.0)	1,178 (68.2)	161 (9.3)	422 (24.4)	373 (21.6)	121 (7.0)
Male/female (pts)	537/482	125/82	601/577	87/74	190/232	189/184	84/37
Age (years)	66.7 \pm 11.7	64.8 \pm 13.2	67.4 \pm 10.7	65.7 \pm 10.9	66.6 \pm 11.4	66.5 \pm 11.0	73.3 \pm 8.2
Height (cm)	157.7 \pm 9.5	158.7 \pm 9.7	157.1 \pm 9.3	157.7 \pm 8.0	157.0 \pm 9.4	158.4 \pm 9.0	157.5 \pm 8.4
Weight (kg)	60.6 \pm 11.8	60.3 \pm 11.5	60.1 \pm 11.5	61.9 \pm 11.2	61.7 \pm 11.6	61.5 \pm 11.4	57.8 \pm 9.2
BMI (kg/m ²)	24.2 \pm 3.5	23.8 \pm 3.2	24.2 \pm 3.4	24.8 \pm 3.7	25.0 \pm 3.6	24.4 \pm 3.5	23.2 \pm 2.7
PR (bpm)	69.1 \pm 11.8	70.9 \pm 13.1	69.1 \pm 12.2	68.1 \pm 12.9	68.2 \pm 12.2	63.7 \pm 11.2	69.0 \pm 13.0
<i>Calibrated radial artery tonometry</i>							
SBP (mm Hg)	138.5 \pm 17.3	137.4 \pm 17.1	138.2 \pm 16.9	138.9 \pm 17.3	134.4 \pm 18.1	134.5 \pm 17.6	139.2 \pm 16.4
SBP2 (mm Hg)	128.1 \pm 18.7	126.3 \pm 18.1	127.7 \pm 18.0	126.8 \pm 18.0	124.8 \pm 18.1	126.2 \pm 18.9	125.3 \pm 18.1
DBP (mm Hg)	74.9 \pm 12.0	75.0 \pm 11.5	74.6 \pm 11.6	73.7 \pm 12.3	71.7 \pm 12.6	73.2 \pm 11.8	72.2 \pm 10.9
rAI (%)	85.0 \pm 13.9	83.8 \pm 13.9	85.3 \pm 14.0	83.6 \pm 15.5	85.5 \pm 13.1	88.2 \pm 14.5	80.9 \pm 14.8
<i>Number of drugs prescribed:</i>							
1 (pts)	235	54	274	0	26	34	16
\geq 2 (pts)	784	153	904	161	396	339	105
<i>Drug classes used in combination^b</i>							
	ARB+CCB	ARB+Diur	ARB+ β BL	CCB+ACEI	CCB+Diur	CCB+ β BL	Diur+ β BL
N (pts (%))	633 (36.7)	274 (15.9)	201 (11.6)	120 (6.9)	267 (15.5)	257 (14.9)	116 (6.7)
Male/female (pts)	345/288	123/151	106/95	72/48	122/145	127/130	54/62
Age (years)	67.7 \pm 11.4	67.6 \pm 11.0	67.1 \pm 11.5	67.2 \pm 10.8	67.6 \pm 10.7	66.4 \pm 11.2	65.9 \pm 11.4
Height (cm)	157.4 \pm 9.6	156.5 \pm 9.5	158.2 \pm 9.3	157.5 \pm 9.9	156.3 \pm 9.1	158.2 \pm 9.2	158.2 \pm 9.3
Weight (kg)	61.0 \pm 12.2	61.7 \pm 12.0	62.4 \pm 12.4	60.2 \pm 11.7	61.4 \pm 11.1	62.0 \pm 11.3	64.5 \pm 11.9
BMI (kg/m ²)	24.5 \pm 3.6	25.1 \pm 3.7	24.8 \pm 3.9	24.1 \pm 3.3	25.1 \pm 3.6	24.7 \pm 3.4	25.7 \pm 3.9
PR (bpm)	69.0 \pm 12.1	68.2 \pm 12.1	63.0 \pm 11.0	71.9 \pm 13.9	67.4 \pm 11.9	63.4 \pm 11.1	62.8 \pm 10.7
<i>Calibrated radial artery tonometry</i>							
SBP (mm Hg)	139.3 \pm 17.1	135.1 \pm 18.3	135.6 \pm 18.2	140.1 \pm 15.3	135.2 \pm 17.8	134.8 \pm 16.9	130.3 \pm 18.7
SBP2 (mm Hg)	128.2 \pm 18.7	125.2 \pm 18.1	127.1 \pm 20.0	128.1 \pm 16.0	125.1 \pm 17.2	126.1 \pm 18.5	122.4 \pm 19.4
DBP (mm Hg)	74.2 \pm 12.0	71.7 \pm 12.4	72.8 \pm 12.6	75.4 \pm 11.1	72.0 \pm 11.8	72.8 \pm 11.7	70.8 \pm 12.1
rAI (%)	84.6 \pm 14.4	85.2 \pm 13.1	88.1 \pm 14.0	83.6 \pm 13.6	85.3 \pm 12.4	87.5 \pm 14.0	87.5 \pm 14.3
<i>Number of drugs prescribed</i>							
2 (pts)	309	66	28	62	40	67	2
\geq 3 (pts)	324	208	173	58	227	190	114

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; CCB, calcium channel blockers; DBP, diastolic blood pressure; Diur, diuretics; Nitro, nitrates or nicorandil; pts, number of patients; α BL, α -blockers; β BL, β -blockers.
^aBecause of its vasoactive action, Nitro is included although drugs in this class are not classified as antihypertensives. ^bThe number for each specified drug combination includes patients taking three or more antihypertensives other than the specified drugs.

analysis (Supplementary Table S1a online). We further examined the multiple regression models individually, including each clinical variable available in this study. Although the total cholesterol level ($N = 784$; $B = 0.02$ mm Hg-dl/mg; $P = 0.01$), serum creatinine ($N = 1,374$; $B = -0.27$ mm Hg-dl/mg; $P = 0.04$), and hemoglobin ($N = 868$; $B = 0.27$ mm Hg-dl/g; $P = 0.03$) reached a significant level, only modest influences on the B estimates of "Drug group" were observed (Supplementary Table S1b online).

Δ SBP2 data adjusted using Model 1 were compared between VD only and non-VD only as well as with non-HT, in which

adjusted Δ SBP2 was estimated based on Model 3 (area A in Figure 2). Although the difference between VD and non-VD was evident, N with actual non-VD only was far fewer than with VD only, which resulted in larger variance in this group. We then estimated Δ SBP2 with VD only or non-VD only in patients with mixed combination therapy based on Model 2, which increased the number of data compared. The results are shown with adjusted data of actual mixed combination within area B in Figure 2. Estimated Δ SBP2 with VD alone (-10.1 mm Hg) was lower than with non-VD alone (-6.6 mm Hg), and even lower than in non-HT (-7.7 mm Hg).