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Oxidative Stress and Central Cardiovascular Regulation

– Pathogenesis of Hypertension and Therapeutic Aspects –

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Oxidative stress is a key factor in the pathogenesis of hypertension and target organ damage, beginning in the earliest stages. Extensive evidence indicates that the pivotal role of oxidative stress in the pathogenesis of hypertension is due to its effects on the vasculature in relation to the development of atherosclerotic processes. It remains unclear, however, whether oxidative stress in the brain, particularly the autonomic nuclei (including the vasomotor center), has an important role in the occurrence and maintenance of hypertension via activation of the sympathetic nervous system. The aim of the present review is to describe the contribution of oxidative stress in the brain to the neural mechanisms that underlie hypertension, and discuss evidence that brain oxidative stress is a potential therapeutic target. (*Circ J* 2010; **74**: 827–835)

Key Words: Blood pressure; Brain; Heart rate; Hypertension; Sympathetic nervous system

Accumulating evidence indicates that the sympathetic nervous system plays an important role in the pathogenesis of hypertension.^{1–3} Activation of the sympathetic nervous system is involved in the stages, clinical forms, 24-h blood pressure patterns, end-organ damage, and metabolic abnormalities of hypertension.^{1–3} Although peripheral factors are also involved, the central nervous system (CNS) mechanisms are considered crucial.^{3–7} The results of recent studies strongly suggest that central sympathetic outflow is increased in hypertension.^{3–7} Increased oxidative stress is also involved in the pathogenesis of hypertension.⁸ Although there have been many studies regarding target organ damage in hypertension, relatively few studies have addressed the role of oxidative stress in sympathetic nervous system activation.^{9–11} Based on the role of angiotensin II (Ang II) in the generation of reactive oxygen species (ROS), the relationship between brain angiotensin and central sympathetic outflow has been examined.^{12,13} Our group was the first to report that increased ROS generation in the brainstem contributes to the neural mechanisms of hypertension in hypertensive rats,¹⁴ and we and other investigators have reported additional evidence to support this concept and the potential therapeutic aspects.^{9–11} This review focuses on the role of oxidative stress within the brain in the neural pathogenesis of hypertension.

Increased Oxidative Stress in the Brain in Hypertension

Among the target organs of hypertensive vascular diseases, the brain is most affected by aging and oxidative stress.^{15,16} Cell membranes in the brain contain a high concentration

of polyunsaturated fatty acids. These fatty acids are targeted by ROS, which elicit chain reactions of lipid peroxidation. Oxidative stress is determined by measuring levels of thiobarbituric acid-reactive substances (TBARS), end products of lipid peroxidation. The levels of TBARS reflect those of malondialdehyde, although the assay is not specific for malondialdehyde.^{15,17} There are some important points, however, for assessing the levels of TBARS.¹⁷ The medium used for tissue preparation needs to contain a chelating agent and an antioxidant, and conditions for the assay must be kept constant. Therefore, we used another method for assessing the ROS production, which is electron spin resonance (ESR) spectroscopy. The amount of ROS was quantified by monitoring the time-dependent decay of the amplitude of the ESR spectra produced by the nitroxide radical 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (hydroxyl-TEMPO) as a spin probe.^{9,14} The signal decay of ESR spectroscopy reflects oxidative stress more directly. Also, it has an advantage for in vivo study.¹⁸ We evaluated oxidative stress in the brains of stroke-prone spontaneously hypertensive rats (SHRSP) compared with normotensive Wistar-Kyoto (WKY) rats.^{9,14} The rostral ventrolateral medulla (RVLM) is the major vasomotor center that determines basal sympathetic nervous system activity and it is essential for the maintenance of basal vasomotor tone.^{3–7} Spontaneously hypertensive rats (SHR) or SHRSP exhibit increased sympathetic nervous system activity during the development of hypertension and are commonly used in experimental studies as models of human essential hypertension.^{3–7} We previously investigated whether ROS are increased in the RVLM of SHRSP.¹⁴ First, we found that ROS levels measured by TBARS and ESR spectroscopy were increased in the RVLM of SHRSP compared with WKY

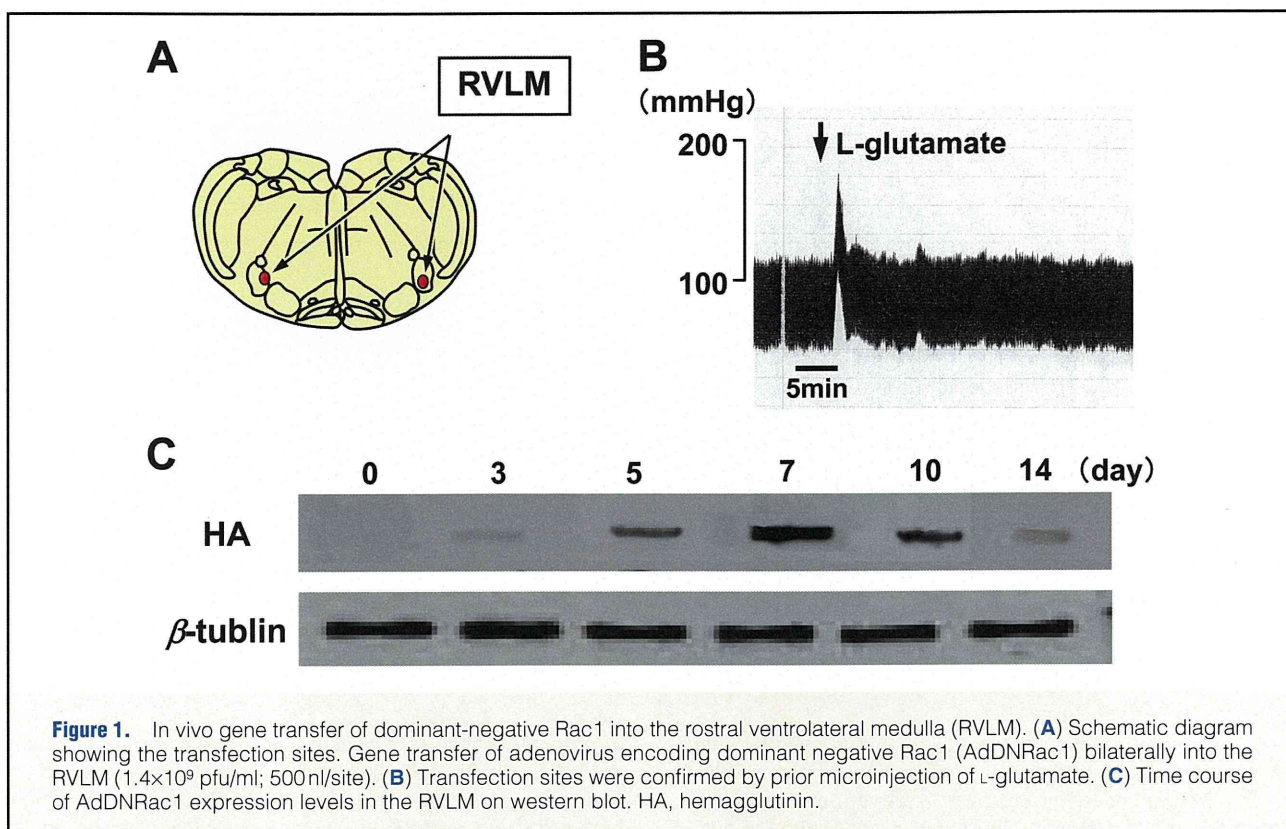
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rats. In addition, superoxide dismutase (SOD) expression and activity, which are ROS scavenging factors, were decreased in the RVLM of SHRSP compared with WKY rats. Functionally, microinjection of the membrane-permeable radical scavenger tempol into the RVLM decreased blood pressure, heart rate, and sympathetic nervous system activity in SHRSP but not in WKY rats. More importantly, overexpression of Mn-SOD, an antioxidant enzyme, in the RVLM of SHRSP decreased blood pressure and sympathetic nervous system activity. These findings strongly indicate that oxidative stress in the RVLM is increased in SHRSP and contributes to the neural mechanisms of hypertension. As described here, brain ROS is one of the results of generalized target organ damage, appearing earlier in the brain due to its susceptibility. The brain ROS would increase blood pressure via activation of the sympathetic nervous system and this would ultimately result in a vicious cycle. It would be possible, however, that brain ROS is involved in the early stage of hypertension in SHR or SHRSP, because we found that oxidative stress in the brain assessed on *in vivo* ESR was enhanced in young (6-week-old) SHR or SHRSP compared with age-matched WKY rats (unpublished data). The levels of TBARS were not different, probably because the levels of TBARS reflect lipid peroxidation caused by ROS. Other investigators also found that an increase in superoxide anions in the RVLM is associated with hypertension in SHR,¹⁹ and reduced expression and activity in Cu/Zn-SOD and Mn-SOD within the RVLM contribute to oxidative stress and neurogenic hypertension in SHR.²⁰ An increase in oxidative stress within the RVLM also plays an important role in maintaining high arterial blood pressure and sympathetic activation in 2-kidney 1-clip (2K-1C) hypertensive rats, which is a renovascular hypertension model.²¹ In that study, Oliveira-Sales et al

demonstrated that the mRNA expression of NAD(P)H oxidase subunits (p47^{phox} and gp91^{phox}) in the RVLM was greater in 2K-1C than in the control group. Interestingly, there were no differences in Cu/Zn-SOD expression between the two groups. TBARS levels in the RVLM were significantly greater in the 2K-1C than in the control group, suggesting enhanced oxidative stress. Functionally, microinjection of vitamin C into the RVLM decreased blood pressure and renal sympathetic nerve activity in 2K-1C but not in controls. Importantly, in a subsequent study, these authors suggested that the paraventricular nucleus of the hypothalamus is also involved.²² Notably, although 2K-1C is a model of renovascular hypertension, suggesting that circulating Ang II is increased, angiotensin type I (AT1) receptor gene expression levels within the RVLM and paraventricular nucleus were upregulated in this model, indicating that ROS was produced via the activation of nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase.

Sources of ROS Production in the Brain

As a source of ROS production in the CNS, NAD(P)H oxidase is a major player. NAD(P)H oxidase is composed of two membrane-bound subunits, gp91^{phox} and p22^{phox}; several cytoplasmic subunits, p47^{phox}, p40^{phox}, and p67^{phox}; and the small G-protein Rac1.^{23–26} Stimulation of AT1 receptors activates NAD(P)H oxidase by which the cytoplasmic subunits of Rac1/NAD(P)H oxidase such as Rac1 bind to the membrane subunits, thereby activating the enzyme leading to superoxide generation. Rac1 requires lipid modification to migrate from the cytosol to the plasma membrane, which is a necessary step for activating ROS-generating NAD(P)H oxidase. NAD(P)H oxidase activity is greater in the brainstem of SHRSP than in that of WKY.^{27,28} We transfected adenovirus

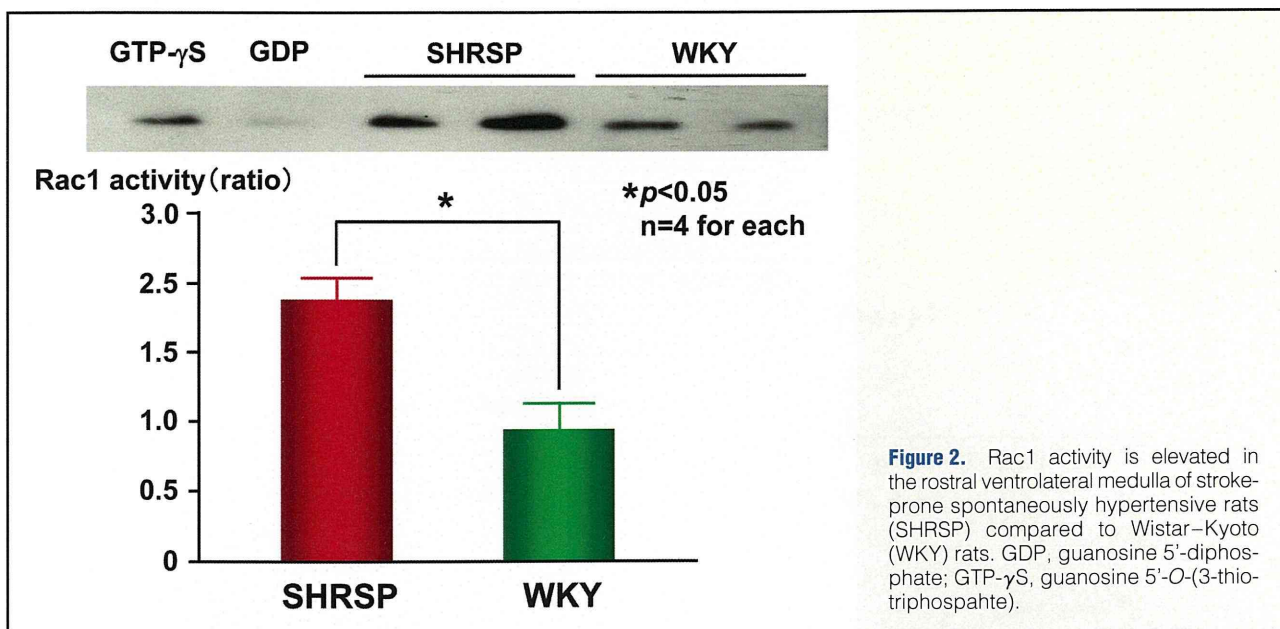


Figure 2. Rac1 activity is elevated in the rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats (SHRSP) compared to Wistar-Kyoto (WKY) rats. GDP, guanosine 5'-diphosphate; GTP- γ S, guanosine 5'-O-(3-thio-triphosphate).

encoding dominant-negative Rac1 into the RVLM of SHRSP and WKY rats (Figure 1).²⁷ Rac1 activity in the RVLM tissue was increased in SHRSP compared to WKY rats (Figure 2).²⁷ Importantly, we demonstrated that inhibition of Rac1-derived ROS in the RVLM decreased blood pressure, heart rate, and urinary norepinephrine excretion in SHRSP (Figure 3).²⁷ A similar response occurs after inhibition of Rac1-derived ROS in the nucleus tractus solitarius (NTS).²⁸

In addition to the cytosolic production of ROS, mitochondria are the primary source of ROS production in many cells. Ang II increases mitochondrial ROS production in the RVLM, leading to sympathoexcitation.²⁹ Furthermore, NAD(P)H oxidase-derived ROS might trigger Ca^{2+} accumulation, which leads to mitochondrial ROS production.²⁹ This suggestion is based on the finding that gene transfer of dominant negative Rac1 attenuated the Ang II-induced increase in reduced Mito-Tracker red fluorescence.²⁹ In contrast, impairment of mitochondrial electron transport chain complexes in the RVLM might be involved in the neural abnormality underlying hypertension in SHR.³⁰ This issue was recently discussed by Zimmerman and Zucker.³¹ Although we did not detect impairment of brain mitochondrial respiratory complexes in SHRSP, we propose that mitochondria-derived ROS mediate sympathoexcitation via NAD(P)H oxidase activation.²⁹

Another possibility for ROS generation is uncoupling nitric oxide synthase (NOS). In the absence of L-arginine or with tetrahydrobiopterin, NO production from inducible NOS (iNOS) causes uncoupling from the oxidation of NADPH, resulting in superoxide generation.⁹ iNOS overexpression in the RVLM causes hypertension and sympathoexcitation that is mediated by an increase in oxidative stress.³² This might be relevant to our observation that iNOS expression levels in the RVLM are greater in SHRSP than in WKY rats.³³ In addition, microinjection of iNOS antagonists into the RVLM reduces blood pressure only in SHR, but not in WKY rats.³³

ROS-Mediated Activation of Transcriptional Factors

It has been suggested that an Ang II-mediated influx of Ca^{2+}

in neurons depends on increased superoxide generation by a Rac1-dependent NAD(P)H oxidase.³⁴ Ang II also regulates neuronal activity via inhibition of the delayed rectifier potassium current.³⁵ Ang II-mediated upregulation of L-type Ca^{2+} currents in neurons isolated from the NTS is inhibited by scavenging ROS, indicating a role for NAD(P)H oxidase-derived superoxide in the activation of Ca^{2+} channels in the NTS.²⁴

NAD(P)H oxidase-derived superoxide mediates an Ang II-induced pressor effect via the activation of p38 mitogen-activated protein kinase (MAPK) in the RVLM.³⁶ Recently, we suggested that AT1 receptor-activated caspase-3 acting through the Ras/p38 MAPK/extracellular signal-related protein kinase pathway in the RVLM is involved in sympathoexcitation in SHRSP.³⁷ These pathways may be downstream effectors of ROS in the RVLM, which in turn plays a crucial role in the pathogenesis of hypertension. Interestingly, the pro-apoptotic proteins Bax and Bad were enhanced and the anti-apoptotic protein Bcl-2 was decreased in the RVLM of SHRSP, and inhibition of caspase-3 normalized these changes in pro- and anti-apoptotic protein levels.³⁷ These alterations in the RVLM of SHRSP were stimulated by Ang II via activation of the AT1 receptors, which are upregulated in this strain and other hypertensive models.³⁸ It would be reasonable to consider that different mechanisms may be responsible for sympathoexcitation in different brain sites (influx of Ca^{2+} for RVLM, apoptosis for NTS), and activation of the apoptotic pathway is involved in sympathoexcitation in the RVLM.³⁷ The exact physiologic implication of these observations requires further evaluation.

Effects of Angiotensin Receptor Blockers on Brain Oxidative Stress

The existence of an independent renin-angiotensin system in the brain is well established. Activation of the brain renin-angiotensin system substantially contributes to the development and maintenance of hypertension through activation of the sympathetic nervous system, vasopressin release, and drinking behavior.^{39,40} There is considerable evidence that

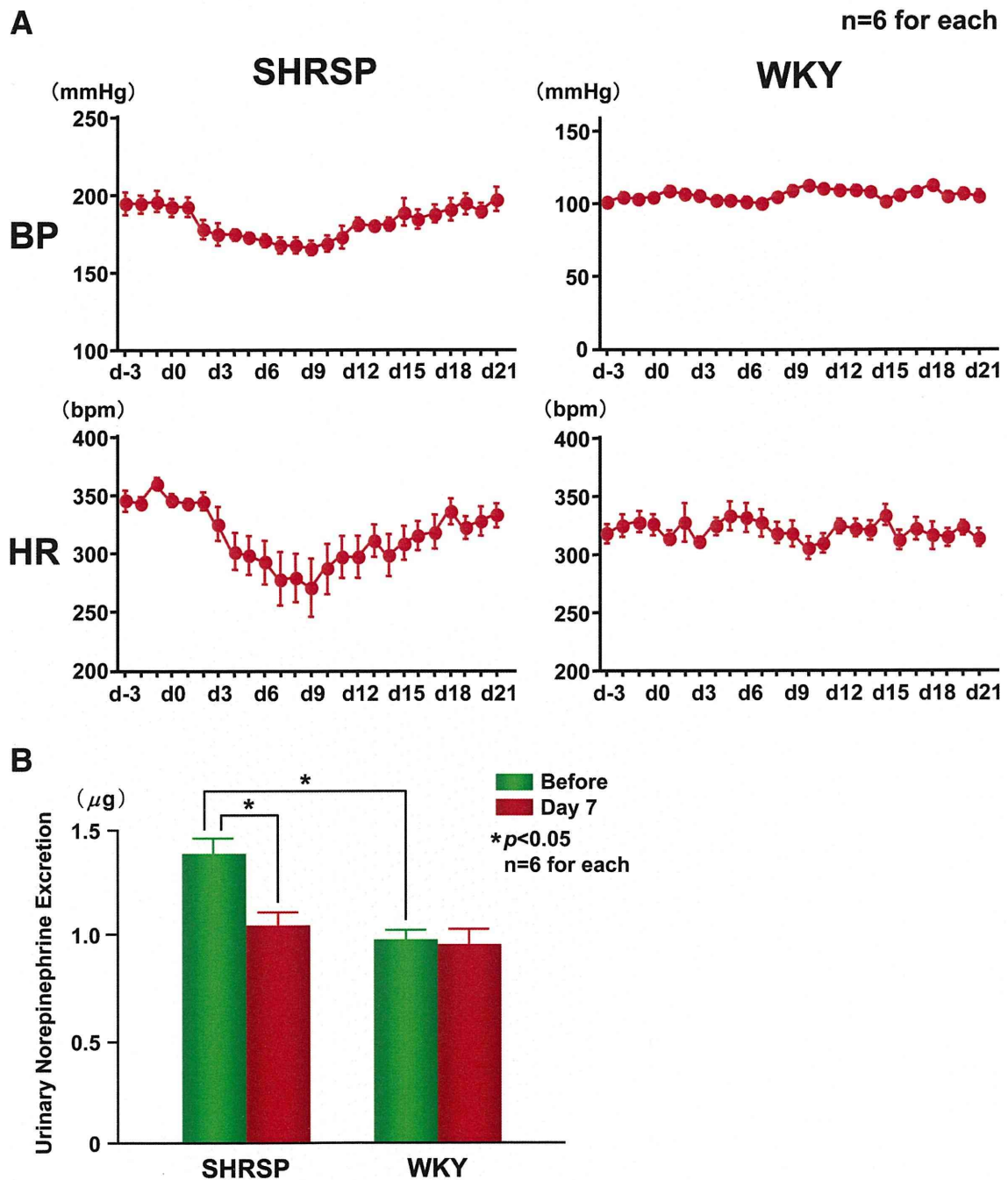
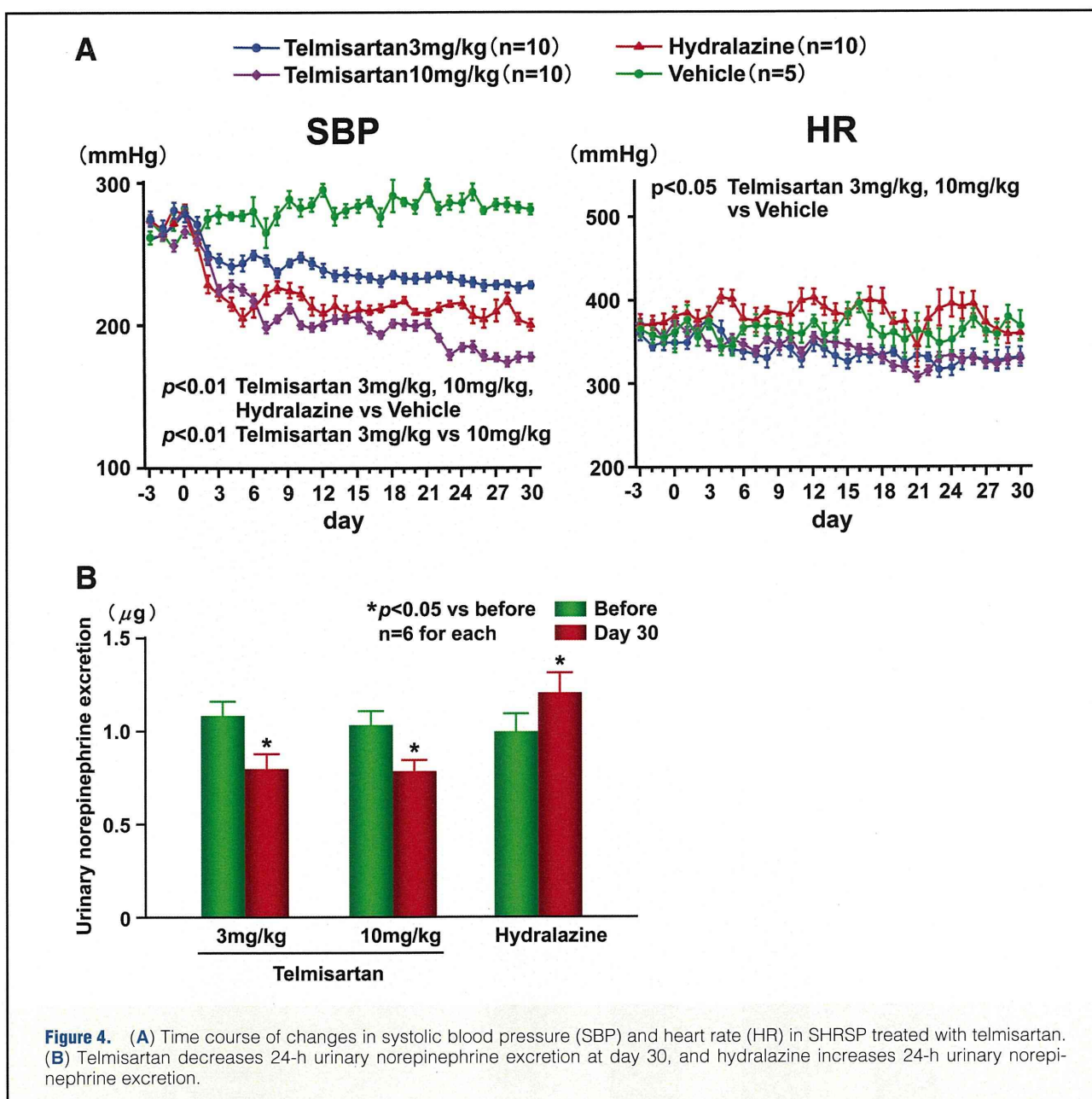


Figure 3. Inhibition of Rac1-dependent superoxide production in the rostral ventrolateral medulla (RVLM) decreases blood pressure, heart rate, and urinary norepinephrine excretion in stroke-prone spontaneously hypertensive rats (SHRSP). **(A)** Time course of mean blood pressure (BP) and heart rate (HR) before and after gene transfer of dominant negative Rac1 (DNRac1). **(B)** Twenty-four-hour urinary norepinephrine excretion before and after gene transfer of DNRac1 into the RVLM. d, day; WKY, Wistar-Kyoto rats.

peripherally administered angiotensin receptor blockers (ARBs) penetrate the blood–brain barrier, although there are some differences among ARBs.^{41,42} AT1 receptors are abundant in the circumventricular organs, such as the subfornical organ and the organum vasculosum lamina terminalis, and the area postrema, which lack a blood–brain barrier.^{39–42} Therefore, peripherally administered ARBs can also bind to

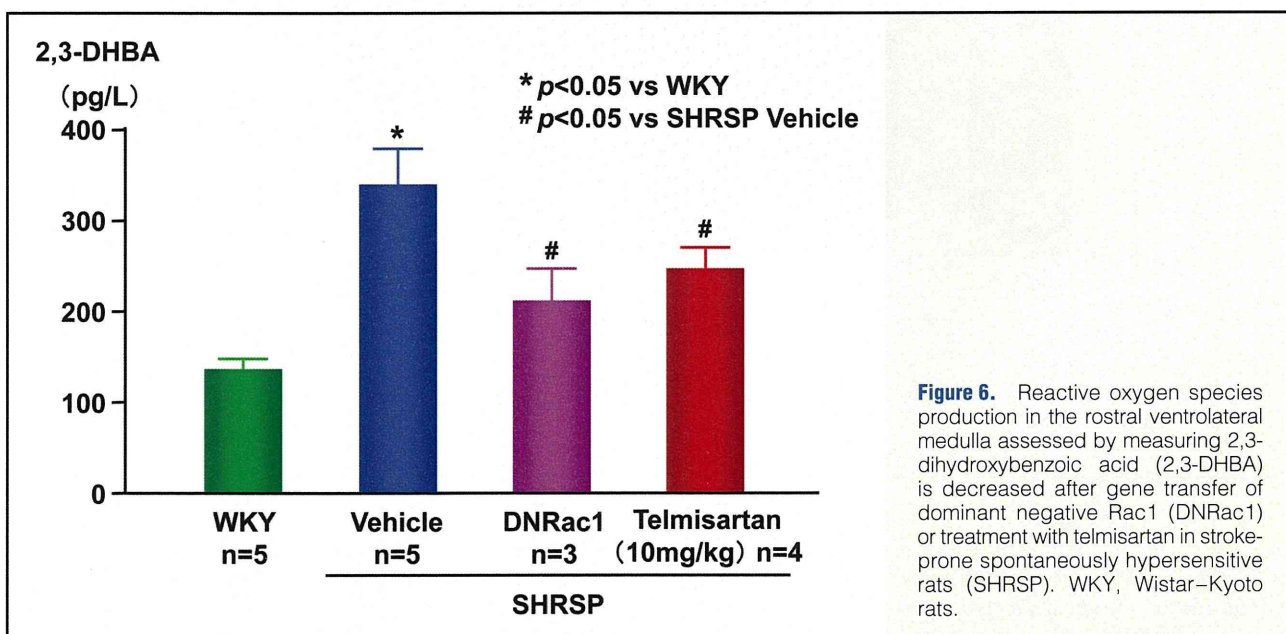
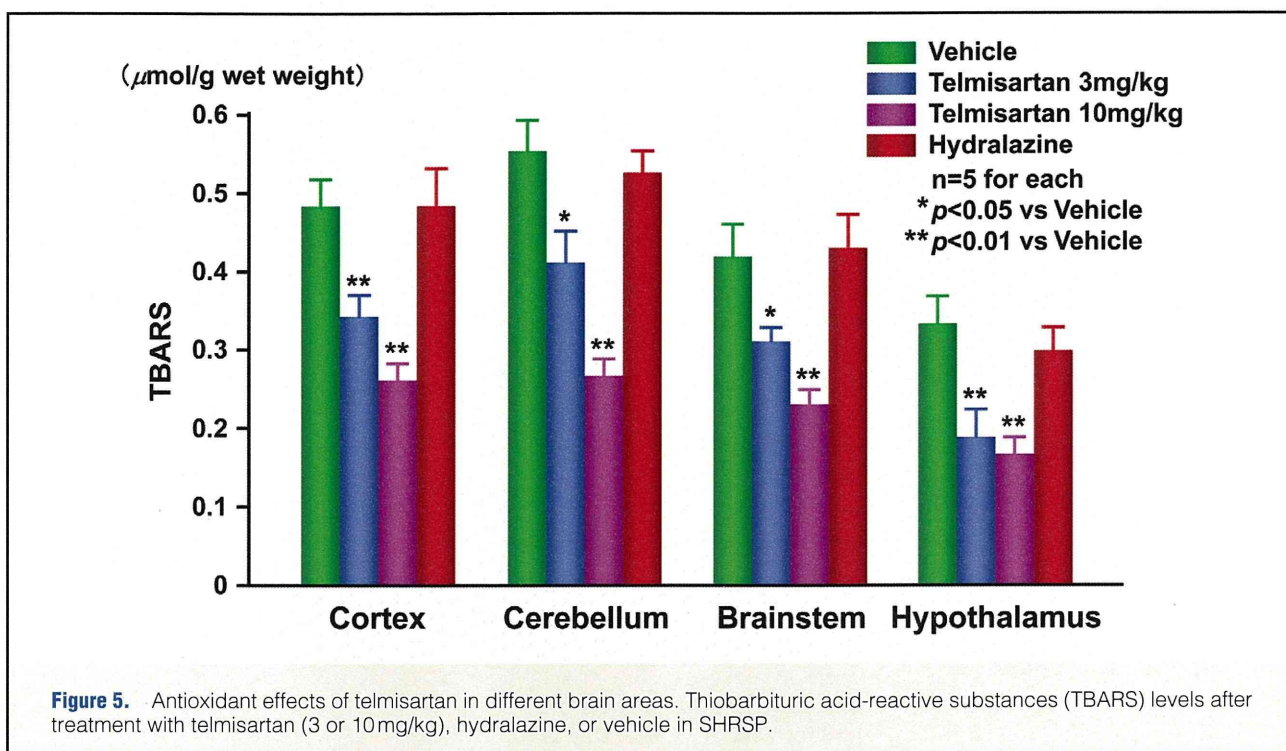
those areas, thereby inhibiting the central actions of Ang II. Oral treatment with the ARB telmisartan appears to inhibit the central responses to Ang II in awake rats.⁴³ Although other ARBs also inhibit the central actions of Ang II within the brain beyond the blood–brain barrier,^{41,42,44} these effects might differ depending on the pharmacokinetics and properties of each drug (ie, lipophilicity etc).⁴³ We evaluated the



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

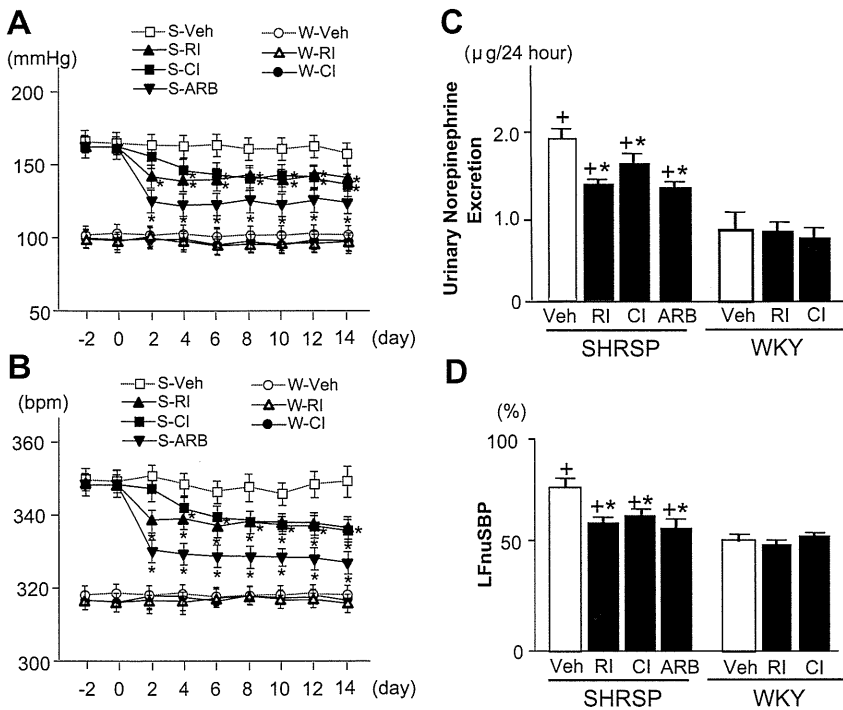


Figure 1. Time course of MBP (A, mm Hg) and HR (B, bpm) in S-RI (n=5), S-CI (n=5), S-ARB (n=5), S-Veh (n=5), W-RI (n=5), W-CI (n=5), and W-Veh (n=5). **P*<0.05 for Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB vs vehicle (Veh) values in each strain. C and D, 24-hour uNE (µg) (C) and LFnusBP (%)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

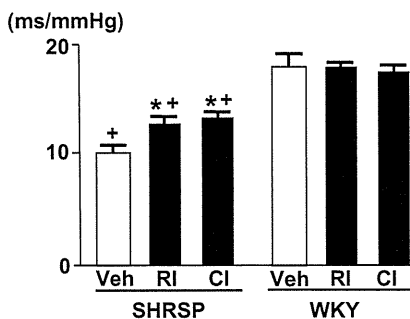


Figure 2. BRS (ms/mm Hg) in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), or caspase-3 inhibitor (CI) (n=5 for each). **P*<0.05 vs Veh in each strain. +*P*<0.05 vs W-Veh. Data are shown as mean±SEM.

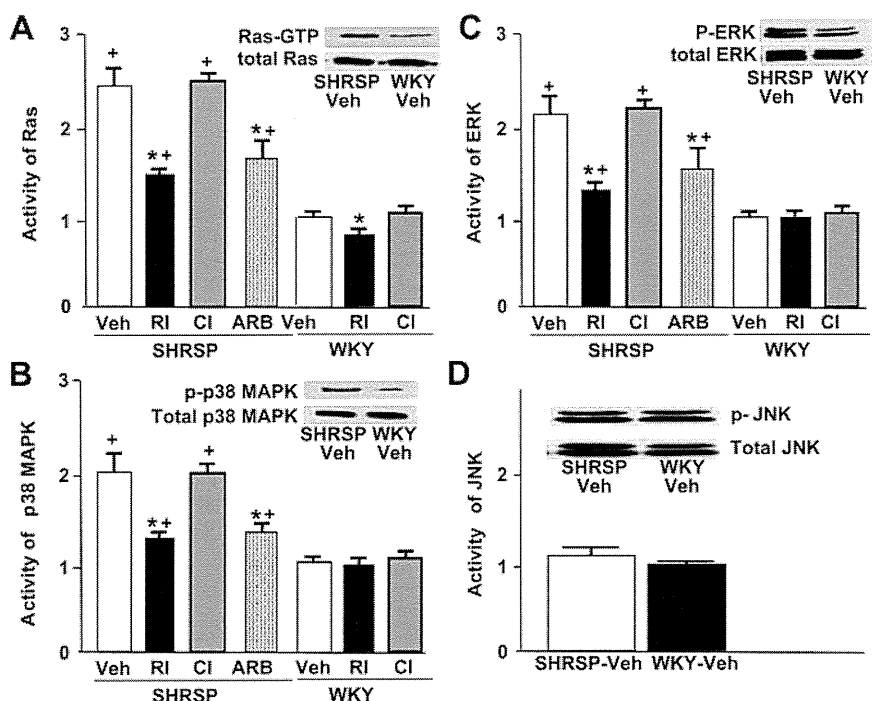


Figure 3. Activity of Ras (A), p38 MAPK (B), ERK (C), and JNK (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5/group). * $P < 0.05$ vs Veh in each strain. + $P < 0.05$ vs Veh-treated WKY. Activity is expressed relative to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

stated 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

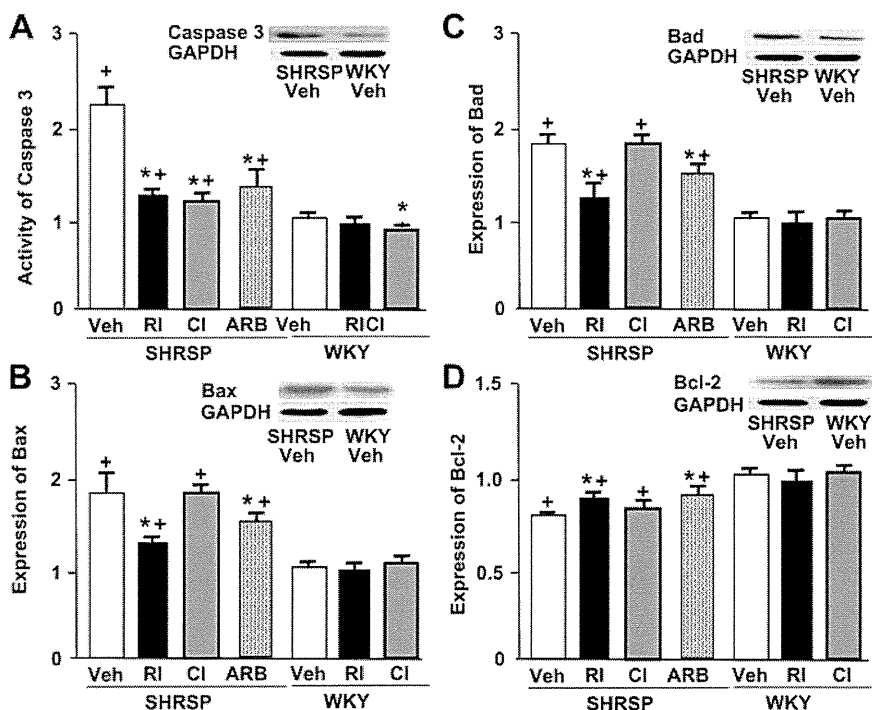


Figure 4. Activity of caspase-3 (A) and expression of Bax (B), Bad (C), and Bcl-2 (D) in the RVLM on day 14 in SHRSP and WKY treated with vehicle (Veh), Ras inhibitor (RI), caspase-3 inhibitor (CI), or ARB (n=5 for each). * $P < 0.05$ vs Veh in each strain. + $P < 0.05$ vs Veh-treated WKY. Activity and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

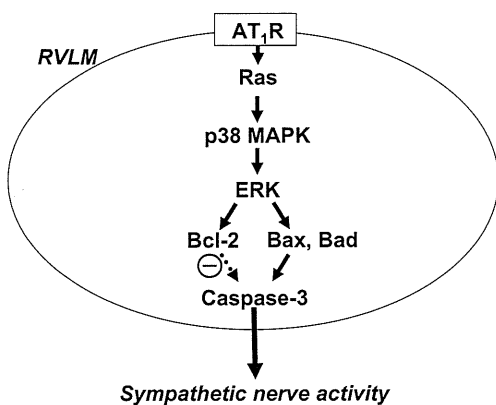


Figure 5. Illustration showing the major findings suggested by the results of the present study.

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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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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