Original Article

High Salt Intake Enhances Blood Pressure Increase during Development of Hypertension *via* Oxidative Stress in Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats

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High salt intake increases blood pressure (BP) in spontaneously hypertensive rats (SHR), and central neural mechanisms are suggested to be involved. Increased generation of reactive oxygen species (ROS) in the rostral ventrolateral medulla (RVLM) contributes to the neural mechanism of hypertension in SHR. We sought to examine whether high salt intake increases hypertension in SHR and whether the increased ROS in the RVLM contributes to this mechanism. Male SHR and Wistar-Kyoto rats (WKY) (6 weeks old) were fed a high-salt diet (8%: HS-S; HS-W) or a regular-salt diet (0.5%: RS-S; RS-W) for 6 weeks. Systolic BP was significantly higher in HS-S than in RS-S at 12 weeks of age (244±5 *vs.* 187±7 mmHg, $n=8$; $p<0.05$). Urinary norepinephrine excretion was significantly higher in HS-S than in RS-S. Thiobarbituric acid–reactive substances levels in the RVLM were significantly higher in HS-S than in RS-S (9.9±0.5 *vs.* 8.1±0.6 μmol/g wet wt, $n=5$; $p<0.05$). Microinjection of tempol or valsartan into the RVLM induced significantly greater BP reduction in HS-S than in RS-S. The increase in angiotensin II type 1 receptor (AT_1R) expression and the increase in reduced nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase activity in the RVLM were significantly greater in HS-S than in RS-S. These findings indicate that high salt intake exacerbates BP elevation and sympathetic nervous system activity during the development of hypertension in SHR. These responses are mediated by increased ROS generation that is probably due to upregulation of AT_1R/NAD(P)H oxidase in the RVLM. (*Hypertens Res* 2008; 31: 2075–2083)

***Key Words*: salt, hypertension, sympathetic nervous system, brain, oxidative stress**

Introduction

High salt intake is an important environmental factor in the exacerbation of hypertension (*1–3*). Although the kidneys have a key role in salt-induced hypertension (*4*), increasing evidence suggests that central nervous system mechanisms are also involved in salt-induced hypertension, including data from spontaneous hypertensive rats (SHR) obtained through activation of the sympathetic nervous system (*5*, *6*). In salt-sensitive rats, brain areas such as the anteroventral third ventricular region, paraventricular nucleus of the hypothalamus, anterior hypothalamus, and the rostral ventrolateral medulla (RVLM) are considered to be responsible for activating the

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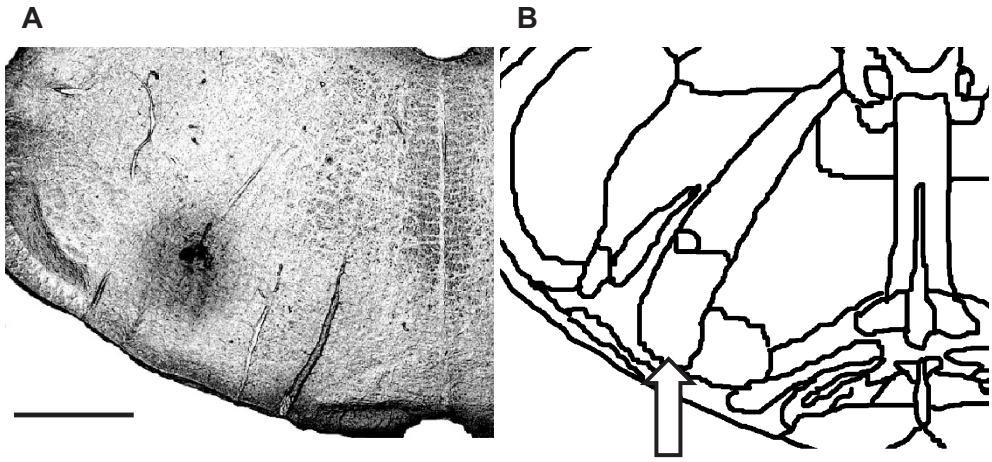

Fig. 1. *Verification of injection site. Methylene blue was observed locally in the RVLM. Scale bar, 1 mm (A). Schematic of a section that includes the RVLM. Arrows indicate the RVLM (B).*

sympathetic nervous system (*7–10*). Of these areas, the RVLM is the cardiovascular center that determines basal sympathetic tone and receives inputs from various autonomic areas within the central nervous system, including the hypothalamus (*11*, *12*). However, it is not known whether activation of the RVLM is involved in the development of hypertension in SHR with high salt intake. Furthermore, the mechanisms by which salt intake-induced activation of sympathetic nerve activity involves the RVLM are not known.

We recently demonstrated that increased reactive oxygen species (ROS) in the RVLM contribute to activation of the sympathetic nervous system, thereby increasing blood pressure (BP) in stroke-prone SHR (*13–15*). The major source of ROS is reduced nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase, which is activated by angiotensin II type 1 receptor (AT_1R) stimulation (*16–19*). The brain renin-angiotensin system is activated in salt-sensitive hypertensive rats, including SHR (*20*, *21*). Therefore, in the present study, we investigated whether high salt intake exacerbates BP elevation during the progression of hypertension *via* further activation of the sympathetic nervous system. If so, we sought to determine whether an increase in ROS generation within the RVLM is involved in this mechanism. Further, we determined whether the source of the increased ROS is NAD(P)H oxidase activated by AT_1R stimulation in the RVLM.

Methods

Animals and General Procedures

Male SHR/Izm rats (6 weeks old; SLC Japan, Hamamatsu, Japan) were fed a standard rodent diet containing 0.5% NaCl (RS diet group: RS-S) or 8% NaCl (HS diet group: HS-S). Male Wistar-Kyoto rats (WKY/Izm; SLC Japan) were fed a standard rodent diet containing 0.5% NaCl (RS diet group: RS-W) or 8% NaCl (HS diet group: HS-W) during a 6-week experimental period. Food and tap water were available ad libitum throughout the study. BP and heart rate (HR) were measured using the tail-cuff method. Urinary norepinephrine concentration of SHR and WKY was measured at 6 and 12 weeks of age, and urinary norepinephrine excretion for 24 h was calculated as an indicator of sympathetic nerve activity, as described previously (*14*). To obtain the RVLM tissues, the rats at 12 weeks of age were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) 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 ± 1°C. The RVLM was defined according to a rat brain atlas, as described previously (*14*). 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.

Measurement of Thiobarbituric Acid–Reactive Substances

The RVLM tissues were homogenized in 1.15% KCl (pH 7.4) and 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 min, followed by heating to 100°C for 60 min. After cooling, the mixture was extracted with distilled water and *n*-butanolpyridine (15:1) and centrifuged at $1{,}600\times g$ for 10 min. The absorbance of the organic phase was measured at 532 nm. The amount of thiobarbituric acid–reactive substances (TBARS) was determined by absorbance, as described previously (*14*).

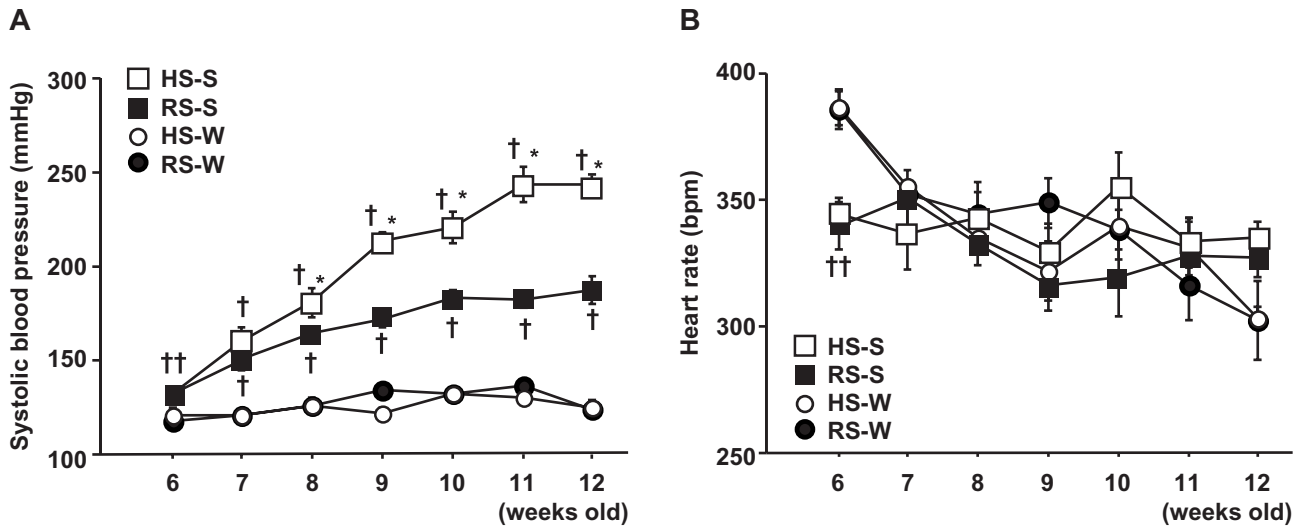

Fig. 2. *Time course of systolic BP (A) and HR (B). RS-W, regular-salt diet Wistar-Kyoto rats (WKY); HS-W, high-salt diet WKY; RS-S, regular-salt diet spontaneously hypertensive rats (SHR); HS-S, high-salt diet SHR. HS-S and RS-S: n = 8; HS-W and RS-W: n = 5. *p < 0.05 HS-S vs. RS-S; †p < 0.05 vs. RS-W.*

Table 1. Blood Pressure and Heart Rate at 12 Weeks of Age

Values	HS-S	RS-S	HS-W	RS-W
N	8	8	5	5
Systolic BP	244±5†,*	187±7†	125±3	124±5
Diastolic BP	198±9†,*	136±7†	94±4	90±3
HR	335±8†	327±7†	303±4	302±16

Data represent baseline systolic BP, diastolic BP, and HR. *$p<0.05$ HS-S *vs*. RS-S, †$p<0.05$ *vs*. RS-W. HS-S, high-salt diet spontaneously hypertensive rats (SHR); RS-S, regular-salt diet SHR; HS-W, high-salt diet Wistar-Kyoto rats (WKY); RS-W, regular-salt diet WKY; BP, blood pressure; HR, heart rate.

Microinjection of Tempol and Valsartan into the RVLM and Intravenous Infusion of Hexamethonium Chloride

Rats were initially anesthetized with sodium pentobarbital (50 mg/kg i.p. followed by 20 mg/kg/h i.v.). A catheter was inserted into the femoral artery to record arterial BP and HR, and another catheter was inserted into the femoral vein to allow for intravenous drug injections. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. The rats were placed in a stereotaxic frame with the incisor bar and the dorsal surface of the medulla was surgically exposed to allow for positioning of the microinjection pipettes into the RVLM (with the pipette angled rostrally 18°, 1.8 mm lateral, 3.5 mm below the calamus scriptorius), as described previously (*14*, *22*, *23*). Drugs were microinjected into the brain in a 50-nL volume of PBS. The drugs injected included tempol (10, 100, 1,000 pmol; Sigma, St. Louis, USA), which is a stable, metal-independent, membrane-permeable SOD mimetic (*24–26*), and valsartan (Val, 100 pmol; Novartis Pharma AG, Basel, Switzerland), an angiotensin II receptor blocker. Drug doses were based on previous reports (*8*, *14*). Prior to the microinjection of tempol and Val into the RVLM, glutamate was injected to verify that the pipette was placed into a functional pressor site. For bilateral injections, the injections were made on one side, and then the pipette was moved to the contralateral side; the two injections were made ~1 min apart. To determine whether basal sympathetic nerve activity is activated in HS-S, hexamethonium chloride was administered intravenously (40 mg/kg), as described previously (*27*). To verify the injection site histologically, 50 nL of methylene blue was injected into the site at the end of the microinjection experiments. The rats were deeply anesthetized with an excessive dose of sodium pentobarbital, and perfused with 4% paraformaldehyde in PBS. The brain was removed and the coronal sections (50 μm) were observed (Fig. 1).

Western Blot Analysis for AT_1R in the RVLM

Western blot analysis was performed for AT_1R (1:1,000; Santa Cruz Biotechnology, Santa Cruz, USA) and GAPDH (1:1,000; Santa Cruz Biotechnology) in the RVLM of HS-S, RS-S, and in RS-W as a control.

NAD(P)H-Dependent Superoxide Production

NAD(P)H-dependent superoxide production in the RVLM was measured by lucigenin luminescence (*28–30*). A 10% (w/v) RVLM tissue homogenate was homogenized in 50

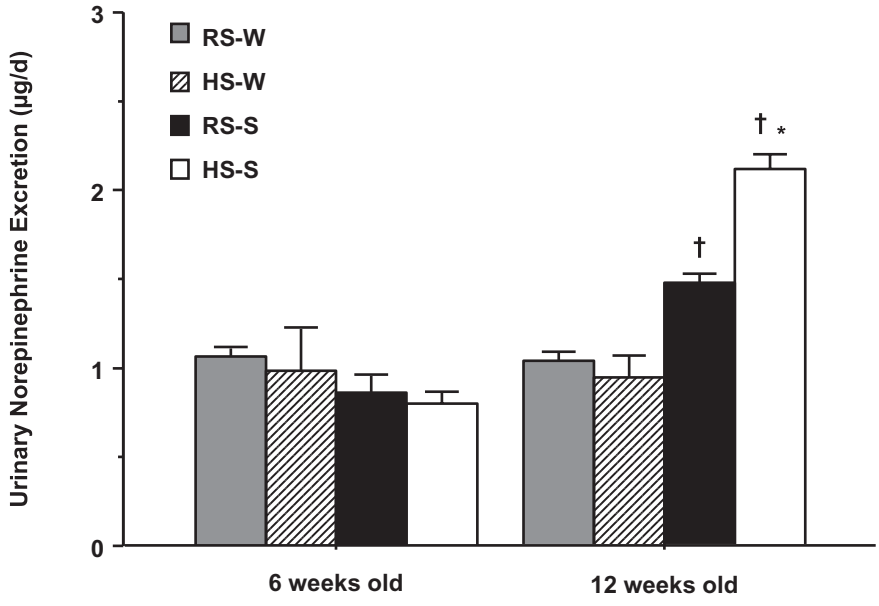

Fig. 3. *Grouped data of urinary norepinephrine excretion for 24 h in the SHR (at 12 weeks old, RS-S: 1.5±0.1 μg/d; HS-S: 2.1±0.1 μg/d; n=5; *p<0.05 HS-S vs. RS-S, †p<0.05 vs. RS-W).*

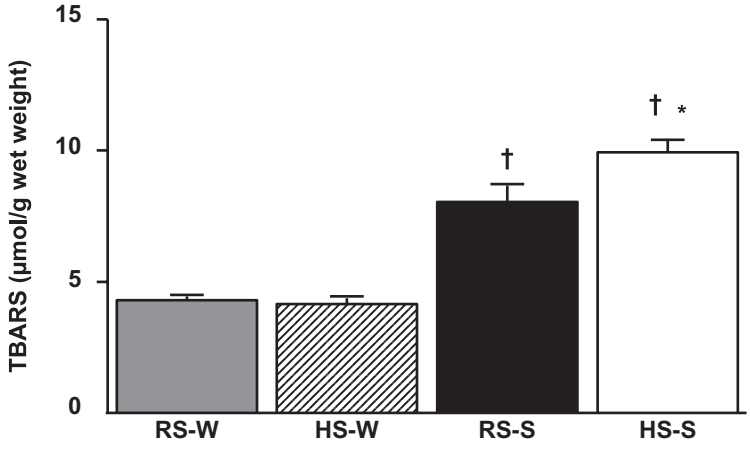

Fig. 4. *Lipid peroxidation as indicated by TBARS levels in RVLM tissues from each group of SHR and WKY (n=5; *p<0.05 HS-S vs. RS-S, †p<0.05 vs. RS-W).*

mmol/L phosphate buffer and centrifuged at 1,000 × *g* for 10 min to remove unbroken cells and debris. An aliquot was kept for protein determination, and supernatants (100 μL) were assayed immediately for superoxide production. The luminescence assay was performed in a balanced salt solution buffer containing 5 μmol/L lucigenin (Sigma) with a luminescence reader (Berthold Technology, Bad Wildbad, Germany). The reaction was started by adding 100 μmol/L β-NAD(P)H (Sigma) as the substrate. No activity was measured in the absence of β-NAD(P)H. The lucigenin count was recorded every 60 s for 10 min; results were expressed as relative light units (RLU)/mg/s.

Statistical Analysis

All values are expressed as mean±SEM. Two-way ANOVA was used to compare the time course of systolic BP and HR, and the expression of AT_1R in the RVLM between any two groups. Comparisons between any two mean values were performed using Fisher's PLSD correction for multiple comparisons. Differences were considered to be statistically significant at a *p* value of less than 0.05.

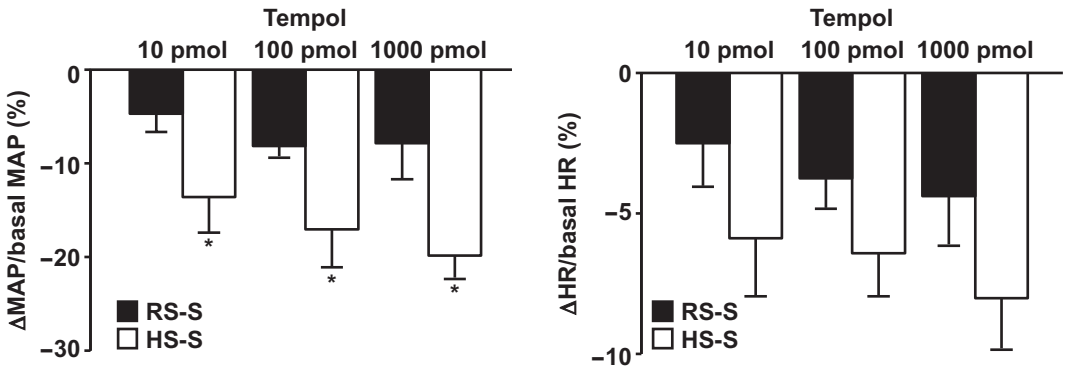

Fig. 5. *Grouped data of MAP and HR responses evoked by microinjection of tempol into the RVLM (n = 7; *p < 0.05 vs. RS-S).*

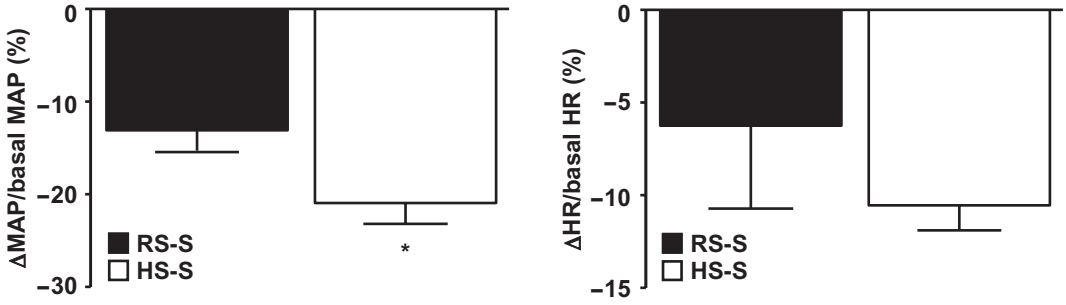

Fig. 6. *Grouped data of MAP and HR responses evoked by microinjection of valsartan into the RVLM (n = 5; *p < 0.05 vs. RS-S).*

Results

BP, HR, and Urinary Norepinephrine Excretion

Systolic BP of both SHR groups was significantly higher than that of RS-W throughout the study. The HS-S group exhibited a significantly higher systolic BP than the RS-S group, starting at 8 weeks of age ($n=8$, $p<0.05$; Fig. 2A). No significant differences in HR were detected between HS-SHR and RS-SHR (Fig. 2B). Systolic BP and HR values for all four groups at 12 weeks of age are shown in Table 1. Urinary norepinephrine excretion was significantly higher in HS-S than in RS-S and that of both SHR was significantly higher than RS-W at 12 weeks of age (SHR groups: $n=5$, WKY groups: $n=3$, $p<0.05$; Fig. 3). Urinary norepinephrine excretion at 12 weeks of age was not significantly different between the WKY groups.

TBARS Levels in the RVLM Tissues

RVLM TBARS levels were significantly higher in HS-S than in RS-S ($n=5$, $p<0.05$; Fig. 4). RVLM TBARS levels in both SHR groups were significantly higher than those in both WKY groups (HS-S *vs*. RS-W, $n=5$, $p<0.05$; RS-S *vs*. RS-W, $n=5$, $p<0.05$). RVLM TBARS levels were not significantly different between WKY groups.

Microinjection of Tempol into the RVLM

Basal mean arterial pressure (MAP) and HR were significantly higher in HS-S than in RS-S (201±3 *vs*. 155±3 mmHg, 312±10 *vs*. 275±8 bpm, $n=7$ for each, $p<0.05$). Microinjection of tempol into the RVLM in HS-S induced a larger decrease in MAP than in RS-S at each dose (Fig. 5). Because the MAP and HR baseline values were different between HS-S and RS-S before microinjection, the changes in MAP/basal MAP and HR/basal HR were expressed as the changes in MAP and HR. These responses in HR evoked by tempol microinjection were not significantly different between the SHR groups.

Microinjection of Val into the RVLM

Basal MAP were significantly higher in HS-S than in RS-S (204±5 *vs*. 149±3 mmHg, $n=5$ for each, $p<0.05$). Basal HR were significantly higher in HS-S than in RS-S (314±12 *vs*. 276±11 bpm, $n=5$ for each, $p<0.05$). Microinjection of Val

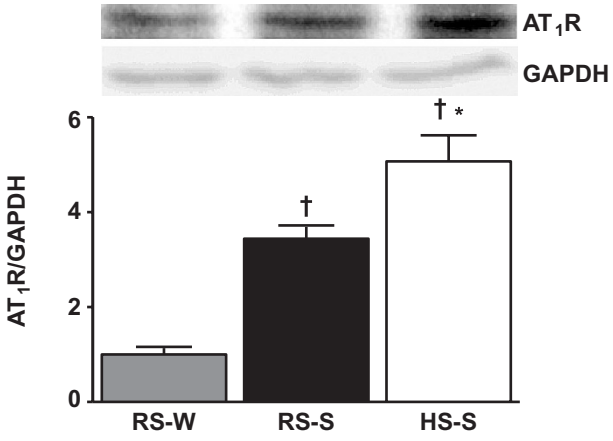

Fig. 7. *The expression of AT_1 receptors in the RVLM. Western blots of AT_1R protein and densitometric analysis of AT_1R protein from the RVLM in the three groups of rats. The data were the mean ratio of AT_1R to GAPDH protein; these were expressed as the relative ratio to that of RS-W ($n=6$; *$p<0.05$ HS-S vs. RS-S group, †$p<0.05$ vs. RS-W group).*

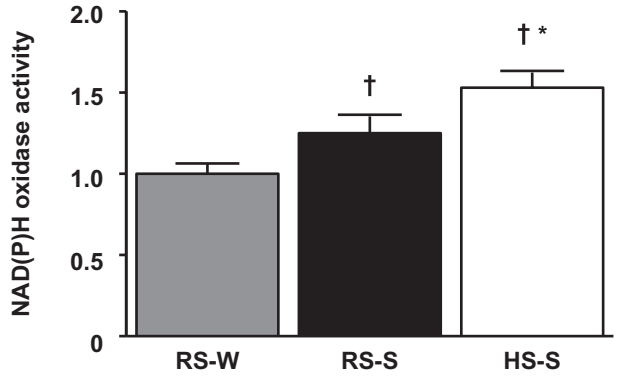

Fig. 8. *NAD(P)H oxidase activity evaluated by lucigenin chemiluminescence in the RVLM. Quantification of NAD(P)H-dependent superoxide production was expressed as the relative ratio to control (RS-W rats), which was assigned a value of 1 ($n=9$; *$p<0.05$ HS-S vs. RS-S, †$p<0.05$ vs. RS-W).*

into the RVLM in HS-S decreased MAP to a significantly greater extent than in RS-S (Fig. 6). The responses in HR evoked by the microinjection of Val were not significant between HS-S and RS-S.

Effects of Systemic Administration of Hexamethonium Chloride

Basal MAP were significantly higher in HS-S than in RS-S (186±8 *vs*. 135±5 mmHg, $n=7$ for each, $p<0.05$). The effects of the intravenous injection of hexamethonium chloride on MAP were significantly greater in HS-S than in RS-S (ΔMAP/basal MAP: −57.1±0.4 *vs*. −46.7±0.4%, $n=7$, $p<0.05$). Intravenous injection of hexamethonium chloride decreased MAP in both HS-S and RS-S to equivalent levels (79±4 *vs*. 73±3 mmHg, $n=7$, n.s.).

AT_1 Receptor Expression in the RVLM

The AT_1R expression levels in the RVLM were significantly higher in HS-S than in RS-S ($n=6$, $p<0.05$; Fig. 7). The AT_1R expression levels in both SHR groups were significantly higher than in RS-W (HS-S *vs*. RS-W, $n=6$, $p<0.05$; RS-S *vs*. RS-W, $n=6$, $p<0.05$). AT_1R expression levels were expressed as the ratio to GAPDH expression in the RVLM. Those values were expressed as the relative ratio to RS-W, which was assigned a value of 1.

NAD(P)H Oxidase Activity

NAD(P)H-dependent superoxide production was also significantly higher in the HS-S RVLM group than in the RS-S RVLM group (1.5±0.1 *vs*. 1.3±0.1, $n=9$, $p<0.05$; Fig. 8). NAD(P)H-dependent superoxide production was significantly higher in both HS-S and RS-S groups than in the RS-W one (HS-S *vs*. RS-W, $n=9$, $p<0.05$; RS-S *vs*. RS-W, $n=9$, $p<0.05$). Quantification of NAD(P)H-dependent superoxide production was expressed as the relative ratio to control (RS-W), which was assigned a value of 1.

Discussion

The present study reports novel observations on the effect of high salt intake on sympathetic mechanisms regulating BP in SHR. Specifically, our results suggest that angiotensin II induces increases in the level of ROS in the RVLM, thereby enhancing sympathetic nervous system activity and hypertension in SHR. This conclusion is based on the findings that 1) high salt intake induced enhanced BP elevation during the development of hypertension in SHR; 2) there was a greater generation of ROS in the RVLM of SHR; and 3) tempol-induced inhibition of ROS in the RVLM elicited a greater reduction of BP in SHR with a high salt intake than in those with a regular salt intake. Higher AT_1R expression levels were observed in the RVLM of HS-S than in that of the RS-S, and there was a greater reduction in BP induced by microinjection of an AT_1R blocker into the RVLM in the HS-S than in RS-S.

Higher BP is considered to be due to activation of the sympathetic nervous system, because HS-S had a greater 24-h urinary norepinephrine excretion than did RS-S. Furthermore, intravenous infusion of hexamethonium chloride induced a greater decrease in BP in HS-S than in RS-S, indicating that activation of the sympathetic nervous system contributes to a high BP in HS-S. In contrast, BP did not increase in HS-W. In fact, TBARS levels did not differ between HS-W and RS-W.

The increases in cerebrospinal fluid (CSF) [Na^+] led to activation of the brain angiotensin system in SHR but not in WKY on the high-salt diet (*31–33*). Considering the importance of brain angiotensin system as a source of ROS generation, this mechanism might be related to our observations.

We used SHR during the development of hypertension in the present study because SHR are salt-sensitive and the central nervous system mechanisms for salt sensitivity have been explored (*9*, *34*). High salt intake augmented the development of hypertension in SHR beginning at the age of 6 to 12 weeks in the present study. A higher BP was observed with HS-S intake than with RS-S. Dahl salt-sensitive rats were used in a recent study (*35*); consistent with our observations, increased oxidative stress in the brain, possibly *via* the activation of NAD(P)H oxidase, may elevate BP through central sympathoexcitation in Dahl salt-sensitive rats, although the role of the RVLM in sympathoexcitation was not explored in that study (*35*). In SHR, AT_1R in the RVLM contributes to the development of hypertension (*8*, *20*). Our findings support this model and further indicate that activation of the angiotensin II/AT_1R pathway exacerbates BP elevation during the development of hypertension in SHR with high salt intake. It is reported that the brain angiotensin system is upregulated in SHR or Dahl-salt sensitive rats with high salt intake due to increased Na^+ transport into the CSF. Nonetheless, the precise mechanism(s) involved are not clear from the results of the present study (*32*, *33*, *36*). It has been demonstrated that distribution of AT_1R is particularly dense in brain areas where autonomic regulation of BP is important, including the RVLM (*37*, *38*). In fact, the expression levels of AT_1R have been shown to be significantly higher in the RVLM of SHR as opposed to Wistar rats (*37*, *38*). Administration of angiotensin II into the RVLM increases BP and sympathetic nerve activity, and microinjections of an AT_1R antagonist to the RVLM in SHR induced a greater BP reduction than in WKY (*20*, *37*). Taken together, these results suggest that AT_1R in the RVLM was upregulated and further BP elevation occurred in HS-S in the present study.

We focused on the role of oxidative stress in the RVLM in the present study because the RVLM is a cardiovascular center that determines basal sympathetic tone and modulates sympathetic nervous system activity based on inputs from other autonomic areas within the brain (*11*). There is considerable evidence suggesting that high salt intake increases sympathetic nervous system activity *via* the central nervous system in hypertensive models, such as SHR, Dahl salt-sensitive hypertensive rats, and deoxycorticosterone acetate–salt rats (*7*, *9*). The anteroventral third ventricular region, the paraventricular nucleus of the hypothalamus, and the anterior hypothalamus are involved in salt-sensitive hypertension (*7*, *9*). The RVLM receives inputs from these areas and integrates basal sympathetic nervous system activity; therefore, the role of the RVLM in salt-sensitive hypertension and the mechanism(s) involved are important issues to investigate further. In the present study, we demonstrated that increased ROS generation in the RVLM promotes hypertension in SHR, probably due to activation of the AT_1R/NAD(P)H oxidase system (*39*). Interestingly, there is an angiotensinergic projection from the paraventricular nucleus of the hypothalamus to the RVLM (*40*, *41*). Further studies are needed to clarify the pathways by which high salt intake affects BP and the nervous system.

High salt intake increased ROS generation in the RVLM of SHR. In fact, it increased TBARS levels in the RVLM of SHR, and microinjection of tempol, a radical scavenger, decreased BP to a greater extent in HS-S than in RS-S. Increased ROS generation and upregulation of AT_1R in the RVLM contribute to hypertension in SHR, even with a regular salt intake (*14*, *20*). In the present study, we observed significantly greater ROS generation and AT_1R upregulation in the RVLM of HS-S than in RS-S, suggesting that increased ROS generation contributes to augmented BP elevation in HS-S (*14*, *15*, *19*). In addition, we also observed greater urinary norepinephrine excretion in RS-S than in WKY groups. These results are consistent with those of previous studies in which the effects of ROS generation in the RVLM on BP were compared between stroke-prone SHR and WKY (*13–15*, *42*). Activation of AT_1R increases superoxide generation *via* NAD(P)H oxidase; NAD(P)H oxidase activity was greater in HS-S than in RS-S. It is now well accepted that angiotensin II can stimulate NAD(P)H oxidase–dependent ROS generation (*43*, *44*). Microinjection of Val into the RVLM induced a greater depressor response in HS-S than RS-S, although we did not examine the TBARS or NAD(P)H oxidase levels after Val administration because of the technical difficulty involved. Taken together, our results suggest that activation of the angiotensin system in the RVLM contributes to the increases in ROS generation, thereby enhancing BP elevation in HS-S. In the present study, we examined the dose-response effects of microinjection of tempol into the RVLM on BP. The doses of tempol utilized do not induce BP reduction when administered systemically, indicating that the effect of tempol does not result from BP reduction.

The results of the present study do not conclusively define the precise mechanism(s) by which high salt intake increases ROS generation in the RVLM, thereby increasing BP *via* activation of the sympathetic nervous system during development of hypertension in SHR. It is clear, however, that the central nervous system is a primary determinant of salt-sensitive hypertension (*10*, *32*). A high salt intake increases Na^+ transport through aldosterone–mineralocorticoid receptor–epithelial sodium channels (*31*). These alterations stimulate the release of an endogenous ouabain-like compound, which binds to angiotensin II/AT_1R to exert sympathoexcitatory effects throughout increasing superoxide generation (*21*, *40*, *45*, *46*). The enhanced superoxide generation increases the intracellular Ca^{2+} influx, which augments neuronal excitability (*45–47*). Further studies are needed to clarify how high salt intake activates the RVLM neurons in SHR.

In conclusion, the results of the present study indicate that

HS intake exacerbates BP elevation during the development of hypertension in SHR by activation of the sympathetic nervous system *via* an increase in ROS generation, probably due to activation of the AT_1R/NAD(P)H oxidase pathways.

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