Sympathoexcitation by Oxidative Stress in the Brain Mediates Arterial Pressure Elevation in Salt-Sensitive Hypertension

Megumi Fujita, Katsuyuki Ando, Ai Nagae, Toshiro Fujita

Abstract—Central sympathoexcitation is involved in the pathogenesis of salt-sensitive hypertension. We have suggested that oxidative stress in the brain modulates the sympathetic regulation of arterial pressure. Thus, we investigated whether oxidative stress could mediate central sympathoexcitation in salt-sensitive hypertension. Five- to 6-week-old male Dahl salt-sensitive rats and salt-resistant rats were fed with a normal (0.3%) or high- (8%) salt diet for 4 weeks. In urethane-anesthetized and artificially ventilated rats, arterial pressure, renal sympathetic nerve activity, and heart rate decreased in a dose-dependent fashion, when 20 or 40 μmol of tempol, a membrane-permeable superoxide dismutase mimetic, was infused into the lateral cerebral ventricle. The same degree of reduction was noted in salt-sensitive and salt-resistant rats without salt loading. Salt loading significantly increased central tempol-induced reductions in arterial pressure (−29.1±4.8% versus −10.6±3.3% at 40 μmol; P<0.01), sympathetic nerve activity (−18.7±2.0% versus −7.1±1.8%; P<0.01), and heart rate (−10.7±2.8% versus −2.0±0.7%; P<0.05) in salt-sensitive rats but not in salt-resistant rats. Intracerebroventricular diphénylèneiodonium, a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, also elicited significantly greater reduction in each parameter in salt-loaded salt-sensitive rats. Moreover, salt loading increased reduced nicotinamide-adenine dinucleotide phosphate–dependent superoxide production in the hypothalamus in salt-sensitive rats but not in salt-resistant rats. In addition, reduced nicotinamide-adenine dinucleotide phosphate oxidase subunits p22phox, p47phox, and gp91phox mRNA expression significantly increased in the hypothalamus of salt-loaded salt-sensitive rats. In conclusion, in salt-sensitive hypertension, increased oxidative stress in the brain, possibly via activation of reduced nicotinamide-adenine dinucleotide phosphate–dependent superoxide production, may elevate arterial pressure through central sympathoexcitation. (Hypertension. 2007;50:360-367.)

Key Words: salt-sensitive hypertension ■ oxidative stress ■ brain ■ hypertension ■ salt ■ sympathetic nervous system ■ Dahl rat

Substantial findings indicate that abnormal modulation of the sympathetic nervous system may be involved in salt-induced development of hypertension in humans1 and animals.2–5 In our previous study,2 salt loading impaired the arterial baroreceptor reflex associated with abnormal central properties in spontaneously hypertensive rats (SHRs); the sympathetic nerve activity (SNA) was less inhibited by sympathoinhibitory and pressor responses to ICV hyperosmotic saline that oxidative stress in the brain modulates the sympathetic regulation of arterial pressure. Thus, we investigated whether oxidative stress could mediate central sympathoexcitation in salt-sensitive hypertension. Five- to 6-week-old male Dahl salt-sensitive rats and salt-resistant rats were fed with a normal (0.3%) or high- (8%) salt diet for 4 weeks. In urethane-anesthetized and artificially ventilated rats, arterial pressure, renal sympathetic nerve activity, and heart rate decreased in a dose-dependent fashion, when 20 or 40 μmol of tempol, a membrane-permeable superoxide dismutase mimetic, was infused into the lateral cerebral ventricle. The same degree of reduction was noted in salt-sensitive and salt-resistant rats without salt loading. Salt loading significantly increased central tempol-induced reductions in arterial pressure (−29.1±4.8% versus −10.6±3.3% at 40 μmol; P<0.01), sympathetic nerve activity (−18.7±2.0% versus −7.1±1.8%; P<0.01), and heart rate (−10.7±2.8% versus −2.0±0.7%; P<0.05) in salt-sensitive rats but not in salt-resistant rats. Intracerebroventricular diphénylèneiodonium, a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, also elicited significantly greater reduction in each parameter in salt-loaded salt-sensitive rats. Moreover, salt loading increased reduced nicotinamide-adenine dinucleotide phosphate–dependent superoxide production in the hypothalamus in salt-sensitive rats but not in salt-resistant rats. In addition, reduced nicotinamide-adenine dinucleotide phosphate oxidase subunits p22phox, p47phox, and gp91phox mRNA expression significantly increased in the hypothalamus of salt-loaded salt-sensitive rats. In conclusion, in salt-sensitive hypertension, increased oxidative stress in the brain, possibly via activation of reduced nicotinamide-adenine dinucleotide phosphate oxidase, may elevate arterial pressure through central sympathoexcitation. (Hypertension. 2007;50:360-367.)

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was shown to be mediated by reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase–dependent production of superoxide in the brain. Thus, the sympathetic activation may be mediated by the increased ROS production in the brain of some types of hypertensive animals.

In recent animal studies, ROS overproduction has been demonstrated in salt-sensitive hypertension. Salt-sensitive essential hypertensive patients have also been reported to show significantly higher plasma isoprostane, a marker for oxidative stress, than non–salt-sensitive patients during salt loading. Recently we showed that high salt loading induced the augmentation of NADPH oxidase activity in the cardiac tissue of DSs. NADPH oxidase–induced ROS in the renal medulla has also been reported to contribute to salt-induced AP rise in DSs. Furthermore, salt loading increased superoxide production and NADPH oxidase activity in the brain of stroke-prone SHR.s

However, it has not yet been determined whether salt-induced ROS in the brain contributes to central sympathetic activation and AP elevation in salt-sensitive hypertension. The aim of the present study was, therefore, to elucidate whether the central sympathoexcitatory effect of oxidative stress mediates AP elevation in salt-sensitive hypertension. We examined the response of renal SNA (RSNA) and AP to central antioxidant agents and the level of NADPH oxidase–induced brain ROS in salt-sensitive animal model DSs and normotensive counterpart DRs.

**Methods**

**Animals**

The present study was conducted in 5- or 6-week-old male DSs and DRs, purchased from SLC (Shizuoka, Japan). They were randomly placed on a normal (0.3%) or high- (8%) salt diet for 4 weeks and were used for the experiments at 9 or 10 weeks of age. Some of the salt-loaded DSs were given hydralazine hydrochloride (4 mg/mL in drinking water for 4 weeks) to examine the effect of AP change. All of the animal procedures conformed to the guiding principles for animal experimentation as enunciated by the University of Tokyo Faculty of Medicine Ethics Committees on Animal Research.

**Preparation of Animals for the Experimental Procedure of ICV Injection**

Rats were anesthetized with an intraperitoneal injection of urethane (1 g/kg). An additional dose (0.1 to 0.2 g/kg) was given when required to maintain a level of anesthesia, as judged by baseline stability of AP, heart rate (HR), and respiration. Mean AP (MAP), HR, and RSNA were recorded as described previously. After tracheal intubation, the animal was paralyzed with d-tubocurarine chloride (initially: 0.2 mg/kg; thereafter: 0.2 mg/kg per hour) given by an infusion pump and artificially ventilated with oxygen-enriched room air. For the ICV injection, a 27-gauge cannula was implanted and fixed to the skull with dental cement (coordinates: 1.0-mm posterior and 1.4-mm lateral to the bregma and the tip at 4.0-mm ventral depth).

**ICV Administration of Tempol and Diphenyleneiodonium**

After recording the basal MAP, HR, and RSNA, artificial cerebrospinal fluid or tempol (20 or 40 μmol in 10 μL of artificial cerebrospinal fluid) was infused into the lateral ventricle in 2 minutes, and changes in the parameters were recorded. The dose of tempol was determined as described previously. In DSs, the effect of ICV diphenyleneiodonium (DPI; 0.5 μmol in 10 μL of artificial cerebrospinal fluid), a flavoprotein inhibitor that blocks NADPH oxidase, was also examined. Accuracy of the ICV injection was confirmed by injecting Evans blue dye after each experiment.

**Measurement of NADPH-Induced Superoxide Production in the Isolated Hypothalamus**

Production of superoxide anions was measured in the isolated hypothalamus, where several nuclei critically involved in cardiovascular regulation are known to be located. The hypothalamic section was isolated after perfusion with PBS. The whole brain was quickly removed from decapitated rats. The brain stem, cerebellum, forebrain, and midbrain were removed, and the remaining tissue was considered as the hypothalamus. Lucigenin (bis-N-methylacridinium, at a final concentration of 25 μmol/L) chemiluminescence was measured as relative light units emitted, recorded every 30 seconds for 10 minutes as described previously. The chemiluminescence value was divided by the weight of the hypothalamic sections (grams). NADPH (at a final concentration of 100 μmol/L), the substrate of NADPH oxidase, was added to evaluate NADPH oxidase activity. In some experiments, we confirmed that pretreatment with DPI (at a final concentration of 100 μmol/L) suppressed superoxide production, which indicated that the increase of ROS reflected NADPH oxidase activity as described previously. Before the measurement, incubation was performed at 37°C for 10 minutes. DPI was added before the incubation, and lucigenin and NADPH were added just before the measurement.

**Real-Time Quantitative RT-PCR for NADPH Oxidase Subunits**

NADPH oxidase subunits p22phox, p47phox, and gp91phox mRNA expression in the isolated hypothalamus were evaluated by real-time RT-PCR. RNA extraction, reverse transcription, and real-time PCR were performed as described previously. Assay-on-demand primers and probe sets (Applied Biosystems) were used for the rat β-actin, p22phox, p47phox, and gp91phox.

**Ganglionic Blockade With Hexamethonium Hydrochloride**

In conscious DSs, we examined the response of MAP to ganglionic blockade. The femoral artery and vein were cannulated under ether anesthesia. The experiment was begun after waiting recovery from anesthesia for ≥3 hours. After the baseline MAP determination, 30 mg/kg of hexamethonium was administered into the femoral vein of the conscious animals. The maximal decrease in MAP was considered as an index of sympathetic activity.

**Statistical Analysis**

In the ICV administration experiments, the baseline value was defined as the mean value over a 1-minute stabilization period before the administration of drugs, and the peak value was defined as the mean value for 10 seconds around the maximum response. Magnitudes of changes were expressed as percentages of change from the baseline. All of the values were presented as means ± SEM. Comparisons among groups were made with the ANOVA followed by a post hoc test (Tukey-Kramer methods). The ANOVA was performed with Statview computer software. P < 0.05 was considered to indicate statistical significance.

**Results**

**Baseline MAP and HR**

As shown in the Table, baseline MAP was significantly higher in DSs than in DRs without salt loading (P < 0.01). Salt loading significantly elevated the baseline MAP in DSs...
Baseline MAP and HR Before ICV Administration of Tempol

<table>
<thead>
<tr>
<th>Group of Rats</th>
<th>Normal-Salt</th>
<th>High-Salt</th>
<th>Normal-Salt</th>
<th>High-Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>119±4*</td>
<td>146±6†</td>
<td>103±3*†</td>
<td>100±1†</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>373±8</td>
<td>381±8</td>
<td>346±13</td>
<td>342±11</td>
</tr>
</tbody>
</table>

Values are means±SEM. N indicates number of rats in each group. With a normal-salt diet, MAP was slightly higher in DSs. Salt loading significantly increased MAP in DSs but not in DRs.

*P<0.01 vs high-salt DSs.
†P<0.01 vs normal-salt DSs.

Effects of the ICV Administration of Tempol

MAP, integrated RSNA, and HR started decreasing ≈1 to 2 minutes after ICV tempol administration and reached their lowest level within 5 minutes (Figure 1), after which they gradually returned to the respective control levels within ≈15 minutes. However, in some cases of salt-loaded DSs, the reduction continued longer than 15 minutes. In all of the rats, both MAP and SNA decreased in a dose-dependent fashion (Figure 2A and 2B). The reduction in MAP was significantly greater in salt-loaded DSs than in non–salt-loaded DSs (−29.1±4.8% versus −10.6±3.3% at 40 μmol tempol; P<0.01; Figure 2A), but it was not increased with salt loading in DRs (−9.6±1.7% versus −14.1±3.2%). Thus, the dose-response curve of MAP showed a significantly greater downward shift in salt-loaded DSs compared with the other 3 groups of rats (Figure 2A). Similarly, salt loading increased the reduction in SNA in DSs (−18.7±2.0% versus −7.1±1.8%; P<0.01; Figure 2B) but not in DRs (−6.7±2.5% versus −8.0±0.7%). There was also a significantly greater downward shift in the dose-response curve of SNA in salt-loaded DSs (Figure 2B). The reduction in HR was also stimulated with salt loading in DSs (−10.7±2.8% versus −2.0±0.7%; P<0.05; Figure 2C) but not in DRs (−3.0±1.9% versus −6.0±3.1%). Baseline MAP normalization by hydralazine hydrochloride without changes in HR (MAP: 110±5 mm Hg; HR: 398±13 bpm; N=7) did not affect the significantly augmented responses in salt-loaded DSs (MAP: −22.1±2.5%; SNA: −17.8±1.4%; HR: −10.5±1.9% at 40 μmol of tempol).

Effects of the ICV Administration of Diphenyleneiodonium

ICV DPI, an NADPH oxidase inhibitor, also decreased MAP, RSNA, and HR in salt-loaded DSs but not in non–salt-loaded DSs. ICV DPI-induced change in each parameter was significantly different between salt-loaded DSs and non–salt-loaded DSs (P<0.01; Figure 3).

Measurement of NADPH-Dependent Superoxide Production in the Isolated Hypothalamus

NADPH-dependent superoxide production in the hypothalamus was significantly higher in salt-loaded DSs than in the other groups of rats (P<0.01; Figure 4).

NADPH Oxidase Subunits p22phox, p47phox, and gp91phox mRNA Expression in the Hypothalamus

NADPH oxidase subunits p22phox, p47phox, and gp91phox mRNA expression significantly increased in salt-loaded DSs

(P<0.01) but not in DRs. The HR did not differ among the 4 groups.

Figure 1. Typical responses of MAP and RSNA to ICV administration of tempol (40 μmol) in normal-salt (left) and high-salt (right) DSs. MAP is expressed as an absolute value, and RSNA is expressed as percentage value from the baseline. Both MAP and RSNA decreased in response to ICV tempol. The reductions in MAP and RSNA are greater in salt-loaded DSs than in non-salt-loaded DSs.
compared with the other groups of rats ($P<0.01$; Figure 5). The gp91$^{phox}$ mRNA expression was significantly higher in non–salt-loaded DSs than in non–salt-loaded DRs ($P<0.05$), but the other subunits were not.

**Ganglionic Blockade With Hexamethonium Hydrochloride**

Under the conscious state, MAP (171±11 mm Hg) was significantly higher in salt-loaded DSs than in non–salt-loaded DSs.
The maximum hypotensive response to hexamethonium was significantly greater in salt-loaded DSs (118±2.5 mm Hg; P<0.01) than in non–salt-loaded DSs (59.6±2.5 mm Hg; P<0.05), suggesting that sympathetic activity significantly increased in salt-loaded DSs compared with non–salt-loaded DSs.

Discussion

In the present study, central administration of the antioxidant tempol elicited reductions in RSNA, AP, and HR. These reductions were enhanced with salt loading in DSs, along with augmented sympathoexcitation. In addition, ICV DPI, an NADPH oxidase inhibitor, also decreased RSNA, AP, and HR in salt-loaded DSs, whereas it did not in non–salt-loaded DSs. Moreover, hypothalamic NADPH oxidase activity and mRNA expression in salt-loaded DSs were significantly greater than in non–salt-loaded DSs. In DRs, however, salt loading did not enhance either the responses to ICV tempol or NADPH oxidase activation. Thus, this is the first report providing evidence that, in DSs, salt-induced hypertension may result from central sympathetic activation because of NADPH oxidase–induced ROS production in the brain. Actually, in our previous study, in mice with deficiency of adrenomedullin, an intrinsic antioxidant, salt loading enhanced ROS production and sympathetic activation in the brain.6 In a manner similar to the present results, dose-dependent renal sympathoinhibitory, hypotensive, and bradycardiac responses to ICV tempol have also been demonstrated in Sprague-Dawley rats with central Ang II.19 The augmented response shown in salt-loaded DSs was not a result from but a cause of the elevated baseline AP, because AP normalization by hydralazine did not affect the responses to ICV tempol in salt-loaded DSs.

In salt-loaded DSs, the NADPH-dependent superoxide production in the isolated hypothalamic sections from DSs and DRs. The chemiluminescence value was significantly higher in salt-loaded DSs than in the other groups of rats (P<0.01). Values are means±SEM. **P<0.01. RLU indicates relative light units.
mRNA expression. Moreover, salt loading had upregulated the inhibitory response of RSNA, AP, and HR to ICV DPI, an NADPH oxidase inhibitor, in the hypothalamus of DSs. In findings similar to the present data, brain oxidative stress production via NADPH oxidase had been reported in the salt-loaded stroke-prone SHR18 and a central Ang II-induced hypertensive rat.11,12 Primary cultures from the hypothalamus indicated that extracellular application of tempol and gp91ds-tat, a selective NADPH oxidase inhibitor, attenuated the Ang II-induced increase in the neuronal firing rate.24 Moreover, in sympathetic neurons in DOCA salt-hypertensive rats, superoxide anions were elevated via the activation of NADPH oxidase.25 Thus, NADPH oxidase may play an important role in ROS production in the brain, and our data are compatible with the other reports. In addition, gp91phox mRNA expression was elevated in the hypothalamus of DSs compared with that of DRs, even without salt loading, although NADPH oxidase activity was not different. The slight increment of NADPH oxidase subunit expression might suggest that DS has a predisposition to ROS overproduction by any stimulator, such as salt loading.

ICV administration can act in the hypothalamic area, which is supposed to contain sodium and osmosensors, and several nuclei critically involved in the cardiovascular regulation, such as the subfornical organ, the organum vasculosum of the stria terminalis, and the paraventricular nucleus.20,26 Although the results obtained from the hypothalamus experiments ex vivo suggest that the hypothalamus is one of the candidates where ROS overproduction induces sympathoexcitation to cause hypertension, we cannot deny the importance of the other autonomic areas in the brain. For example, rostral ventrolateral medulla is also an important central sympathetic regulatory area,26,27 where some investigators injected tempol and showed its hypotensive effect in stroke-prone SHRs7 and its inhibitory effect on the acute pressor effect induced by bilateral microinjection of Ang II.12 A number of studies, including ours, have indicated the pivotal role of central superoxide in cardiovascular regulation by the central administration of superoxide scavengers.7–12,19 It remains to be elucidated, however, which central nuclei are the key sites responsible for mediating the cardiovascular response to antioxidants such as tempol and DPI. Further study, including a microinjection or in vitro experiment, is required to determine the specific central site at which brain ROS may cause sympathetic activation in salt-sensitive hypertension.

Several factors in the brain had been demonstrated to play important roles in the sympathetic activation in salt-induced AP elevation. The central renin-angiotensin system might stimulate SNA and mediate salt-induced hypertension.1,18,27 There are reports suggesting that ROS in the brain might...
mediate the Ang II-induced pressor effect,\(^8\)\(^–\)\(^12\),\(^18\),\(^19\),\(^26\) 11-\(\beta\)-hydroxysteroid dehydrogenase type 2\(^28\) and the epithelial sodium channel, etc., could be involved in sympathoexcitation in salt-sensitive hypertension.\(^26\) However, the involvement and interaction of these factors have not been established. The mechanism of salt-induced sympathoexcitation through oxidative stress in the brain may be complicated, and further study is needed to clarify a possible candidate mediator to cause hypertension through sympathoexcitation via central ROS excess.

In the present study, salt loading was demonstrated to upregulate the inhibitory response of RSNA, AP, and HR to ICV DPI, an NADPH oxidase inhibitor, as well as tempol, a superoxide dismutase mimic, associated with NADPH oxidase activity and mRNA expression in the hypothalamus of DSs. These findings suggest that, in salt-sensitive hypertension, increased oxidative stress in the brain, possibly via activation of NADPH oxidase, might mediate AP elevation through central sympathetic activation.

**Perspectives**

We found that, in the salt-sensitive hypertensive rat, salt-induced ROS overproduction in the brain led to sympathoexcitation and AP elevation. Sympathetic activation\(^29\) and ROS\(^13\)\(^–\)\(^18\) have been reported to lead not only to hypertension but also to organ damage. Therefore, sympathetic inhibition with an antioxidant can be a useful target for treatment of hypertension and its complications. Similar to salt-sensitive hypertension, oxidative stress\(^30\) and sympathoexcitation\(^11\) may be important pathogenic mechanisms of the metabolic disorder, which is also a powerful predictor of cardiovascular disease. A number of studies, including ours, have demonstrated that salt-sensitive hypertension exhibits a metabolic disorder.\(^32\),\(^33\) Thus, increased ROS production in the brain may be a common feature in salt-sensitive hypertension and the metabolic disorder. High dietary fat has, in fact, been reported to induce NADPH oxidase–associated oxidative stress and inflammation in the brain.\(^34\) Thus, the present results might suggest a reason why the metabolic disorder accelerates the risk of salt-sensitive hypertension\(^35\) and why the opposite is true.\(^32\),\(^33\) Based on our new insights, a novel stress and inflammation in the brain.\(^34\) Thus, the present study is needed to clarify a possible candidate mediator to cause hypertension through sympathoexcitation via central ROS excess.

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**Disclosures**

None.


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