Sympatho-Inhibitory Action of Endogenous Adrenomedullin Through Inhibition of Oxidative Stress in the Brain

Megumi Fujita, Tomoyuki Kuwaki, Katsuyuki Ando, Toshiro Fujita

Abstract—Central sympathetic activation is one of the possible mechanisms underlying hypertension, in which reactive oxygen species may play a role. Thus, we examined whether adrenomedullin, an antioxidant peptide, is involved in the central regulation of arterial pressure through sympatho-modulatory action. Adrenomedullin knockout mice were fed with high-salt diet for 4 weeks to stimulate adrenomedullin production. In the wild-type littermates, brain adrenomedullin content was significantly increased with salt loading, but not in the knockout mice. Intracerebroventricular hyperosmotic saline increased arterial pressure and sympathetic nerve activity in a dose-dependent fashion. With the normal salt diet, the hyperosmotic saline-induced response did not significantly differ between the knockout and wild-type mice; with the high-salt diet, however, the response was significantly greater in the knockout mice than in wild-type littermates (arterial pressure: 35.3±5.7% versus 20.1±2.1%, P<0.05; sympathetic nerve activity: 30.3±4.8% versus 15.9±1.5%, P<0.05; respectively). Moreover, pretreatment with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol), a membrane-permeable superoxide dismutase mimetic, inhibited the augmented response to central hyperosmotic saline in salt-loaded knockout mice. Consistently, the hyperosmotic saline-induced production of reactive oxygen species, measured by the lucigenin chemiluminescence method, was significantly greater in the isolated hypothalamus of salt-loaded knockout mice than in that of salt-loaded wild-type ones. In conclusion, endogenous adrenomedullin in the brain may inhibit sympathetic activation through its antioxidant action. (Hypertension. 2005;45:1165-1172.)

Key Words: central nervous system • free radicals • hypertension, arterial • mice • sodium, dietary • sympathetic nervous system

There is a growing body of evidences suggesting that abnormal modulation of the sympathetic nervous system is involved in the development of hypertension in human and animals. For example, the sympathetic nerve activity (SNA) was augmented in salt-loaded spontaneously hypertensive rats (SHRs) and Dahl salt-sensitive (S) rats, but not in their normotensive counterparts. In salt-loaded SHRs, norepinephrine turnover was enhanced in hypothalamus as well as kidney and heart, and the impaired arterial baroreceptor reflex was associated with abnormal central property because SNA was less inhibited by stimulation of the aortic depressor nerve compared with Wistar-Kyoto rats. Pressor response to intracerebroventricular (ICV) infusion of hyperosmotic saline was enhanced in salt-loaded Dahl S rats. Thus, the sympathetic activation may be mediated by the abnormal central mechanisms in some types of hypertension.

In addition, reactive oxygen species (ROS) have been proposed to play an important role in the development of hypertension. Elevated ROS production has been shown in various animal models with hypertension. Oral treatment with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol), a superoxide dismutase (SOD) mimetic, decreased arterial pressure (AP) and ROS in SHRs. Recently, intravenous injection of tempol elicited apparent decreases in SNA and AP in deoxycorticosterone acetate salt rats and SHRs. Moreover, ICV angiotensin II (Ang II)-induced changes in AP and heart rate (HR), which could be mediated by the sympathetic nervous system, were abolished by previous treatment with adenoviral vector–mediated expression of SOD. Also, ICV injection of an adenoviral vector encoding SOD significantly decreased the central sympathetic activation after myocardial infarction in mice, associated with the decreased Fos-positive neurons in the paraventricular nucleus (PVN) and supraventricular nuclei in the hypothalamus. The PVN is well-established as a key integrated center for regulating SNA via its inputs from the nucleus tractus solitarii and efferent projections to rostral ventrolateral medulla (RVLM) and spinal cord. In addition, microinjection of SOD into the RVLM decreased renal SNA and AP to a greater extent in pigs under chronic oxidative stress as compared with pigs without oxidative stress. In stroke-prone SHRs, ROS level was increased in the RVLM and

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bilateral microinjection of tempol into the RVLM decreased AP. Thus, the sympathetic activation may be mediated by the increased ROS production in the brain of hypertensive animals.

There are many endogenous antioxidant substances, one of which is adrenomedullin (AM), a hypotensive peptide originally isolated from pheochromocytoma cells. AM may be one of the negative feedback regulators to compensate overproduction of ROS, because AM not only reduced ROS production but also was increased by ROS. Furthermore, it should be noted that AM and its receptors are distributed not only in the cardiovascular organs but also in the brain, especially in the PVN and the supraoptic nucleus, which are critically involved in the central cardiovascular regulation. Several experimental studies with ICV administration of AM have demonstrated that AM in the brain possessed such properties as inhibition of salt appetite or drinking, natriuresis, and regulation of AP.

In the present study, therefore, we investigated whether brain AM modulated SNA in relation to AP control through ROS overproduction, using AM knockout mice. We used heterozygous AM knockout mice (AM+/−) because homozygous knockout mice were lethal. Thus, animals were fed high-salt diet, which stimulated AM production, to strengthen the difference of brain AM level between AM+/− and wild-type littermates (AM+/+); we have confirmed that AM+/− showed the blunted elevation of AM to the stimulus such as Ang II and salt loading. In these mice, we examined the effects of ICV injection of hyperosmotic saline on AP and SNA, because it has been used as a central sympathostimulating procedure in the experiments of hypertensive animals. To examine whether the antioxidant action of AM is involved in the central cardiovascular regulation; moreover, we studied the effects of tempol on the responses to ICV hyperosmotic saline and concurrently measured ROS production in the isolated hypothalamus in response to hyperosmotic saline.

Methods

Animals
Mice used in this study were genetically engineered AM knockout mice that were backcrossed into C57BL/6 for >10 generations. We used 4- to 8-month-old male AM+/− and AM+/+. They were fed with normal (0.6%) or high-salt (8%) diet for 4 weeks. Although proadrenomedullin N-terminal 20 peptide, which is produced by posttranslational splicing of a precursor of AM proadrenomedullin, is potential to inhibit SNA, AM gene alone was disrupted sparing normal production of proadrenomedullin N-terminal 20 peptide in our AM+/−. All mice were housed in a room maintained at 23°C to 25°C with lights on at 7:00 AM and off at 7:00 PM. Mice were given food and water available ad libitum. All animal procedures conformed to the guiding principles for animal experimentation as enunciated by the Ethics Committees on Animal Research of the University of Tokyo, Faculty of Medicine, and of Chiba University Graduate School of Medicine.

Preparation of Animals
Mice were anesthetized with intraperitoneal injection of urethane (1.2 g/kg). An additional dose (0.1 to 0.2 g/kg) was given when needed. Mean AP (MAP), HR, and SNA were recorded as described, except that we recorded splanchnic SNA instead of renal SNA. After tracheal intubation, the animal was paralyzed with pancuronium bromide given by infusion pump (0.1 mg/mL in a volume of 0.06 to 0.24 mL/h) and artificially ventilated with oxygen-enriched room air (Hugo Sachs Elektronik D-79232). For ICV injection, a guide cannula (C315GS-S/2.5; Plastics One) was implanted 1 mm lateral to the bregma and fixed onto the skull with dental cement.

For the experiment using unanesthetized mice, catheters were inserted into the right femoral artery and vein and the guide cannula was implanted to the lateral ventricle under isoflurane (2% to 4%) anesthesia. Experiments began after recovery from anesthesia for at least 3 hours.

ICV Administration of Hyperosmotic Saline
A 33-gauge needle connected to a Hamilton microsyringe was inserted into the guide cannula so that its tip protruded 1 mm into the lateral ventricle. After recording the basal MAP, HR, and SNA, artificial cerebrospinal fluid (ACSF) (containing 0.13 mol/L of NaCl) or hyperosmotic saline (0.3 to 1.5 mol/L) was infused into the lateral ventricle at 0.3 μL/min for 10 minutes and changes in the parameters were recorded. The administration speed was within the speed at which cerebrospinal fluid is produced in mice (0.33 μL/min) and ACSF did not affect MAP, HR, and SNA in the present study. Accuracy of ICV injection was confirmed by injecting Evans Blue dye after the each experiment. In the present study, responses of SNA and AP to ICV hyperosmotic saline in AM−/− were consistent with the results of previous experiments using cats and rats.

To exclude the influence of urethane anesthesia, effects of ICV administration of hyperosmotic saline on MAP and HR were examined in the unanesthetized freely moving mice. After recovery from isoflurane anesthesia, ACSF or hyperosmotic saline was infused into the lateral ventricle.

ICV Tempol Administration
Tempol, or 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-N-oxyl (3CP) that is structurally similar to tempol but has a minimal superoxide scavenging activity, was manually infused by 1 minute into the lateral ventricle 10 minutes before ICV administration of hyperosmotic saline. The effect of 3CP (6 μmol dissolved in 3 μL of ACSF [maximum concentration in our experimental condition]), and then that of tempol (6 or 10 μmol in 2 μL of ACSF), was examined.

Measurement of Superoxide Production in the Isolated Hypothalamus
Production of superoxide anion by the hyperosmotic saline was measured in the isolated hypothalamus, where putative sodium and osmo sensors were located and distribution of AM was confirmed. Whole brain was quickly removed from decapitated animals and brain stem, cerebellum, forebrain, and midbrain were removed. The remaining tissue was considered as hypothalamus. Its slice was weighed and placed in 0.25 mL of modified Krebs-Hepes buffer (in mmol/L; NaCl 99.0, KCl 4.69, CaCl2 1.87, MgSO4 1.20, K2HPO4 1.03, NaHCO3 25.0, Na-Hepes 20.0, glucose 11.1; pH 7.4), and maintained at 37°C for 10 minutes. After stabilization, 0.25 mL of physiological saline (final concentration of NaCl 0.13 mol/L, 3 mol/L LiNO3, final concentration of LiNO3, 1.5 mol/L), or 3 mol/L hyperosmotic saline (final concentration of NaCl, 1.55 mol/L) was added and background chemiluminescence was measured for 30 sec. After that, lucigenin (bis-N-methylacridinium, final concentration: 25 μmol/L) was added into the scintillation vial and chemiluminescence was recorded with the use of luminescence reader (Lumat LB9507; Berthold) for 15 minutes. The chemiluminescence values were standardized with the weight of hypothalamic slices (grams). Each hypothalamic slice was used for only one condition.

Measurement of Brain AM
AM concentration was measured with radioimmunoassay in the whole brain of mice with the 4 groups.
Effects of ICV Administration of Hyperosmotic Saline in Anesthetized Mice

MAP and integrated SNA increased within 5 minutes after ICV hyperosmotic saline was started to be infused, and subsequently they gradually returned to the respective control levels ~4 to 5 minutes after the infusion was finished in normal and high-salt diet AM$^{+/+}$ and normal salt diet AM$^{-/-}$ (Figure 2A and 2B). In some cases of salt-loaded AM$^{+/+}$, the elevation of MAP and SNA continued up to 10 minutes after the end of infusion. In all the groups, both MAP and SNA increased in a dose-dependent fashion (Figure 2C). With the normal salt diet, the elevation of MAP was not significantly different between AM$^{+/+}$ and AM$^{-/-}$ (22.0±2.7% versus 19.1±3.1% at 1.5 mol/L NaCl; Figure 2A and 2C). With the high-salt diet, however, the elevation of MAP was significantly greater in AM$^{+/+}$ than in AM$^{-/-}$ (35.3±5.7% versus 20.1±2.1%, P<0.05; Figure 2B and 2C). Thus, dose–response curve of MAP was significantly upward shifted in salt-loaded AM$^{+/+}$ compared with the other 3 groups of mice (Figure 2C). Similarly, the elevation of SNA was not different between AM$^{+/+}$ and AM$^{-/-}$ fed on normal salt diet (18.7±3.8% versus 16.5±2.3%; Figure 2A and 2C), whereas with the high-salt diet, it was significantly greater in AM$^{+/+}$ than in AM$^{-/-}$ (30.3±4.8% versus 15.9±1.5%, P<0.05; Figure 2B and 2C). Dose–response curve of SNA was also significantly upward shifted in salt-loaded AM$^{+/+}$ (Figure 2C). The responses of HR varied with each animal even in the same groups and did not show any significant difference among the 4 groups.

ICV Administration of Hyperosmotic Saline in Unanesthetized Mice

As was the case in anesthetized mice, the response of MAP to ICV hyperosmotic saline was significantly greater in salt-loaded AM$^{-/-}$ than the other groups of mice (Figure 3A through 3C).

ICV Tempol Administration

In all the groups of mice, pretreatment with 3CP (6 μmol) did not affect the elevation of MAP and SNA in response to ICV hyperosmotic saline. However, pretreatment with the same dose of tempol almost completely inhibited the response of MAP and SNA to ICV hyperosmotic saline in AM$^{+/+}$ (Figure 4A and 4B). In the case of AM$^{-/-}$, the pretreatment with 6 μmol of tempol

### Results

**Basal MAP and HR and Brain AM**

As shown in the Table, baseline MAP and HR did not differ among the 4 groups. Brain AM content was significantly elevated with the high-salt diet (P<0.05) in AM$^{+/+}$, whereas salt loading did not increase brain AM in AM$^{-/-}$. As a result, brain AM content was significantly lower in salt-loaded AM$^{+/+}$ than in salt-loaded AM$^{-/-}$ (P<0.05; Figure 1).

**Table: Baseline MAP and HR Before the Administration of Hyperosmotic Saline in Anesthetized and Unanesthetized Mice**

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Anesthetized Mice</th>
<th>Unanesthetized Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>MAP, mm Hg</td>
</tr>
<tr>
<td>Normal salt AM$^{+/+}$</td>
<td>7</td>
<td>73±3</td>
</tr>
<tr>
<td>High-salt AM$^{-/-}$</td>
<td>7</td>
<td>73±4</td>
</tr>
<tr>
<td>Normal salt AM$^{-/-}$</td>
<td>6</td>
<td>73±2</td>
</tr>
<tr>
<td>High-salt AM$^{+/+}$</td>
<td>6</td>
<td>73±4</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

No. indicates number of mice in each group.

AM$^{+/+}$ indicates wild-type mice; AM$^{-/-}$, adrenomedullin (AM) knockout mice; normal salt, fed with normal salt diet; high-salt, fed with high-salt diet; HR, heart rate; MAP, mean arterial pressure.
partially inhibited the response to hyperosmotic saline, but the pretreatment with 10 μmol of tempol completely inhibited it (Figure 4A and 4B).

Measurement of Superoxide Production in the Isolated Hypothalamus
In the hypothalamic slice of normal salt diet AM+/-, the lucigenin chemiluminescence immersed in 1.55 mol/L NaCl was significantly higher than that in physiological saline or 1.5 mol/L LiNO₃ (Figure 5A). Then, the superoxide generation from the hypothalamic slice immersed in 1.55 mol/L NaCl was compared among the 4 groups. The production of superoxide was significantly higher in the hypothalamus of salt-loaded AM+/- than in that of salt-loaded AM++ (P<0.05) (Figure 5B).

Discussion
The results clearly showed that the lack of high-salt diet-induced increment of brain AM in AM+/- had the augmented...
response of SNA and AP to the ICV infusion of hyperosmotic saline. Moreover, the administration of tempol, a membrane-permeable SOD mimetic, could inhibit it. Evidence suggests that endogenous AM in the brain modulates sympathetic regulation of AP through its antioxidant effect. This is the first report providing evidence that endogenous AM plays a role in the central nervous system using AM deficient mice, although possible contribution of AM to the central cardiovascular regulation has already been suggested by several experimental studies with the administration of exogenous AM.28–31

Brain AM was not increased in salt-loaded AM+/−, which showed the exaggerated hypertension and sympathoexcitation in response to ICV hyperosmotic saline, whereas AM+/+ had the apparent increase in brain AM with salt loading. It suggests that endogenous AM in the brain might inhibit the AP elevation and sympathetic activation induced by ICV hyperosmotic saline. ICV administration of exogenous AM has been reported to cause either pressor28,29 or depressor30,31 effect in rats. Apparent inconsistency may be explained by difference in distribution of exogenous administered AM. Actually, in rats, microinjection of AM into PVN30,31 caused depressor response, whereas microinjection into area postrema elicited pressor response.43 Thus, our data using AM+/− are compatible with those of microinjection of AM into PVN,30,31 and endogenous AM in the brain might play an important role in PVN. However, it is still controversial what nuclei in the brain are involved in the sympatho-inhibitory action of AM.

Hyperosmotic saline-induced sympathetic activation might be related to the increased release of vasopressin, because, besides the direct vasoconstrictive action of vasopressin, there is an intimate relationship between vasopressin and SNA; vasopressin increases AP through stimulating SNA.44 ICV administration of AM inhibited the release of vasopressin45 and the microinjection of AM into the PVN, where vasopressin-containing neurons are located, elicited hypotension.30,31 In our preliminary experiment, however, pretreatment with intravenous vasopressin V1 receptor antagonist did not affect the elevation of AP and SNA induced by ICV hyperosmotic saline (data not shown), indicating lesser involvement of vasopressin. Alternatively, several investigators have demonstrated that the local renin-angiotensin system in the brain could play an important role in the sympathetic

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Figure 3. A and B, Typical responses of MAP to ICV administration of hyperosmotic saline (0.3 mol/L) in the conscious freely moving mice fed with normal (A) and high-salt (B) diet. C, Percent changes in MAP in the 4 groups of mice. Values are mean±SEM. *P<0.05, **P<0.01.
activation induced by ICV hyperosmotic saline. In the present study, however, we did not measure Ang II in the brain nor investigated the effect of Ang II type 1 receptor antagonist on the response to ICV hyperosmotic saline.

Increased responses of AP and SNA to hyperosmotic saline in salt-loaded AM should not be attributed to the influence of anesthesia, because the similar response was observed between anesthetized and unanesthetized mice. Nor is it caused by the difference in the vascular reactivity to vasoactive substances in AM and AM, because the effect of intravenous phenylephrine and sodium nitroprusside did not differ among the 4 groups (data not shown). In the present study, apparently, there is a significant difference in the response of SNA, which was directly measured, to ICV hyperosmotic saline between salt-loaded AM and AM.

Pretreatment with ICV tempol could inhibit the increase in SNA and AP with ICV hyperosmotic saline, whereas pretreatment with 3CP did not affect it. Thus, it suggests that ROS might be involved in the pressor effect by ICV hyperosmotic saline. It is compatible with recent studies supporting that ROS plays a role in regulation of SNA and AP in PVN and RVLM. In the present study, accordingly, hyperosmotic saline-induced production of superoxide anion was significantly greater in the hypothalamic slice of salt-loaded AM than that of salt-loaded AM with the apparent elevation in brain AM. It is consistent with the results of our previous study showing that Ang II and salt loading increased ROS production in AM. Similar findings were observed in cuff-injured arteries and hypoxic lung in AM. Moreover, a higher dose of tempol was required to inhibit the augmented response to ICV hyperosmotic saline in salt-loaded AM. It led us to a plausible hypothesis that redox mechanisms in the brain may play a role in regulating sympathetic outflow in salt-loaded AM. In the present study, salt loading could upregulate AM expression in the brain of AM but not in that of AM. Consistent with the present results, chronic salt loading increased plasma AM concentrations and upregulated expression of AM in adrenal glands and kidney in rats. Moreover, salt loading increases ROS production in hypertensive rats. Because oxidative stress could increase AM production in cultured vascular smooth muscle cells and endothelial cells, the increase in brain AM content might be attributable to the increased ROS production. In turn, locally
generated AM in the brain might counteract the hypertensive saline-induced stimulation of the central SNA, possibly through inhibiting ROS production; brain AM might be an endogenous sympato-inhibitory substance.

**Perspectives**

AM could suppress the sympathoexcitatory activation induced by ROS-producing stimuli such as ICV hypertensive saline, resulting in reduction of AP increase. The enhanced ROS production and central sympathetic activation appear to be related to the development of hypertension. Thus, it is speculated that AM, as a neural antioxidant, might be an interesting goal for the development of new therapeutic strategies in cardiovascular medicine.

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**References**


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