Acquisition of Brain Na Sensitivity Contributes to Salt-Induced Sympathoexcitation and Cardiac Dysfunction in Mice With Pressure Overload

Koji Ito, Yoshitaka Hirooka, Kenji Sunagawa

Abstract—In animal models of salt-sensitive hypertension, high salt augments sympathetic outflow via central mechanisms. It is not known, however, whether pressure overload affects salt sensitivity, thereby modifying central sympathetic outflow and cardiac function. We induced left ventricular hypertrophy with aortic banding in mice. Four weeks after aortic banding (AB-4), the left ventricle wall thickness was increased without changing the percentage fractional shortening. AB-4 mice were then fed either a high-salt (8%) diet or regular-salt diet for additional 4 weeks. Cardiac dysfunction, wall thickness, and 24-hour urinary catecholamine excretion were increased with high-salt diet compared with regular-salt diet. We then examined brain Na sensitivity. Intracerebroventricular infusion of high-Na (0.2 mol/L) artificial cerebrospinal fluid into AB-4 mice and mice Sham-4 increased urinary catecholamine excretion, arterial pressure, and heart rate more in AB-4 mice than in Sham-4 mice. Intracerebroventricular infusion of an epithelial Na channel blocker (benzamil) into mice with high-salt diet significantly decreased urinary catecholamine excretion and improved cardiac function. Infusion of either an angiotensin II type 1 receptor blocker or a Rho-kinase inhibitor also attenuated the salt-induced sympathetic hyperactivation and cardiac dysfunction in mice with high-salt diet. The levels of angiotensin II type 1 receptor and phosphorylated moesin, a substrate of Rho-kinase, were significantly greater in AB-4 mice than in Sham-4 mice. These results suggest that mice with pressure overload acquire brain Na sensitivity because of the activation of epithelial Na channel via Rho-kinase and angiotensin II, and this mechanism contributes to salt-induced sympathetic hyperactivation, further pressure overload, and cardiac dysfunction. (Circ Res. 2009;104:1004-1011.)

Key Words: hypertension ■ heart failure ■ hypertrophy ■ sympathetic nervous system ■ brain ■ sodium chloride
ENaCs in the enhanced sympathetic activity and cardiac dysfunction induced by high salt intake in mice with pressure overload and the relationship of brain ENaCs to the Rho/Rho-kinase pathway and the renin-angiotensin system (RAS) in the brain, because ENaCs in kidney are reported to be activated by the Rho/Rho-kinase pathway and RAS.

**Materials and Methods**

**Animals**
The study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male Institute of Cancer Research (ICR) mice (10 weeks old; SLC, Fukuoka, Japan) were used.

**Mouse Pressure Overload Model Preparation**
The suprarenal abdominal aorta was banded in ICR mice (AB mice) to create the pressure overload model or sham operation (Sham mice) as a control. We divided these mice into the groups represented in Figure 1. For details, see the online data supplement, available at http://circres.ahajournals.org.

**Evaluation of Cardiac Function**
Cardiac function was evaluated by echocardiography. Serial M-mode echocardiography was performed under light sodium pentobarbital anesthesia with spontaneous respiration. Cardiac function was also evaluated by the left ventricular end-diastolic pressure (LVEDP). A conductance catheter (1.4 Fr; Miller Instruments) was inserted into the right carotid artery and advanced across the aortic valve into the left ventricle. See the online data supplement for details.

**Measurement of Arterial Pressure and Heart Rate**
Under sodium pentobarbital anesthesia and mechanical ventilation, a catheter was inserted into the right carotid artery and arterial pressure and heart rate were measured. In another protocol, we also measured arterial pressure and heart rate in awake AB mice fed a high-salt diet (AB-H) and AB mice fed with a regular salt diet (AB-R) using a radiotelemetry system implanted in the left carotid artery. See the online data supplement for details.

**Evaluation of Sympathetic Activity**
Sympathetic activity was evaluated by measuring 24-hour urinary norepinephrine (U-NE) and urinary epinephrine (U-E) excretion using high-performance liquid chromatography.

**Evaluation of Na Sensitivity**
We evaluated U-NE, U-E, arterial pressure, and heart rate responses to a high-salt diet or high-Na aCSF (0.2 mol/L, 1 μL/min for 10 minutes) ICV infusion in each group. In addition, we measured Na concentrations in the brain tissue (circumventricular tissues including the hypothalamus) of mice in each group. Furthermore, to examine the response of other central stimuli, we performed ICV infusion of angiotensin II (0.5 nmol/L, 1 μL/min for 5 minutes) and carbachol (0.1 mmol/L, 1 μL/min for 5 minutes). See the online data supplement for details.

**Measurement of Organ Weight**
After completion of the experiments, mice were killed with an overdose of sodium pentobarbital, and the heart and lungs were removed and weighed.

**Measurement of Serum Parameters**
We measured the serum concentrations of sodium, creatinine, and aldosterone in each group. See the online data supplement for details.

**Evaluation of the Effects of Na Channel Blockade in the Brain**
To assess the effects of Na channel blockade in the brain, benzamil, a specific ENaC blocker, was infused ICV (1 mg/ml, 0.11 μL/h for 28 days). The U-NE and U-E excretion, arterial pressure, heart rate, and organ weight were measured, and echocardiography was performed.
formed as described earlier. See the online data supplement for details.

Evaluation of the Effects of Rho-Kinase and Angiotensin Type 1 Receptor Blockade in the Brain

To assess the effects of Rho-kinase or angiotensin II type 1 receptor (AT1R) blockade in the brain, Y-27632 (a specific Rho-kinase inhibitor) or telmisartan (an AT1R blocker) was infused ICV (Y-27632: 5 mmol/L, 0.11 L/h for 28 days; telmisartan: 4 and 20 mmol/L, 0.11 L/h for 28 days). The U-NE and U-E excretion, arterial pressure, heart rate, and organ weight were measured, and echocardiography was performed as described earlier. See the online data supplement for details.

Evaluation of AT1R Expression and Rho-Kinase Activity

To assess AT1R expression levels and Rho-kinase activity, we performed a Western blot analysis for AT1R (1:1000, Santa Cruz Biotechnology, Santa Cruz, Calif) and phosphorylated-moesin, a substrate of Rho-kinase (p-moesin, 1:1000, Santa Cruz Biotechnology) in the circumventricular tissues, including the hypothalamus and brain stem tissues, of Sham-4 mice and AB-4 mice. See the online data supplement for details.

Statistical Analysis

All values are expressed as means±SE. ANOVA was used to compare U-NE and U-E excretion, organ weight, left ventricular end-diastolic diameter (LVDD), left ventricular wall thickness (LVWT), percentage fractional shortening (%FS), and arterial pressure measured by telemetry between groups. An unpaired t test was used to compare changes in arterial pressure and heart rate after high-Na ICV infusion, as well as protein levels, between Sham mice and AB mice. Differences were considered to be significant when \( P<0.05 \).

Results

Characteristics of the Pressure Overload Model

Relative heart weight (heart weight/body weight) was not increased in AB-4 mice compared with Sham-4 mice (Figure 2A). AB-R mice, however, had a significantly higher relative heart weight than Sham-R mice and a significantly lower relative heart weight than AB-H mice. Relative lung weight (lung weight/body weight) did not differ between groups (Figure 2A). Body weight of AB mice was significantly lower than that of Sham mice (body weight: Sham-4, 44.7±1.4 g; AB-4, 45.3±1.1 g; Sham-R, 47.8±0.5 g; AB-R, 42.5±0.6 g; AB-H 40.6±0.9 g, n=6 for each); however, the absolute heart weight in AB-H was significantly greater than that in AB-R or Sham-R mice (heart weight: AB-H 0.26±0.01 g; Sham-R 0.24±0.01 g; AB-R 0.24±0.01 g; n=6 for each).

Echocardiography revealed the following characteristics: LVWT was greater in AB-4 mice than in Sham-4 mice, but %FS did not differ between the groups (Figure 2B). After an additional 4 weeks, cardiac function declined in AB-R mice compared with Sham-R mice and declined significantly more in AB-H mice compared with AB-R mice. LVDD was also higher in AB-H mice than in Sham-R or AB-R mice (Table I in the online data supplement).

Arterial Pressure Monitoring

Measurement Under Anesthesia

Mean arterial pressure and heart rate were significantly higher in AB-4 mice compared with Sham-4 mice. In AB-R and AB-H mice, arterial pressure was reduced to levels similar to that in the Sham-R mice. Heart rate was significantly higher in AB-4, AB-R, and AB-H mice than in Sham-4
or Sham-R mice. There were no significant differences in arterial pressure and heart rate between the groups of Sham mice (Online Table II).

**Measurement in Awake Mice Using Radio-Telemetry System**

In AB-4 mice (AB day 28), mean arterial pressure and heart rate were significantly higher than that in the mice before aortic banding. Furthermore, high salt intake (AB-H mice) dramatically increased mean arterial pressure to 171 ± 5 mm Hg by day 35 after aortic banding (1 week after the starting high salt intake). The general conditions deteriorated in all AB-H mice, however, likely because of severe lung congestion (lung/body weight ratio, 7.0 ± 0.1). In AB-R mice, mean arterial pressure increased mildly, and the highest mean arterial pressure value was 145 ± 5 mm Hg on day 38 after aortic banding and thereafter gradually decreased to 124 ± 7 mm Hg on day 56 after aortic banding (n = 3 for each; see the online data supplement for details).

**Salt Sensitivity in Sham Mice and AB Mice**

High salt intake did not increase U-NE or U-E excretion in Sham mice (Figure 3A). In AB mice, however, high salt intake significantly increased U-NE and U-E excretion. Furthermore, U-NE excretion in AB mice began to increase within 5 days (AB-Hd5 mice) of beginning the high-salt diet (Figure 3A), although cardiac function was preserved (%FS 43 ± 1%; n = 5). ICV infusion of regular-Na aCSF did not significantly increase U-NE or U-E excretion in Sham mice or AB mice (Figure 3B). ICV infusion of high-Na aCSF significantly increased U-NE and U-E excretion in both Sham mice and AB mice. The increase in U-NE excretion in AB mice, however, tended to be greater than that in Sham mice (P < 0.1), and the increase in U-E excretion was significantly greater in AB mice than in Sham mice (Figure 3B).

In the acute experiments, high-Na aCSF ICV infusion increased arterial pressure and heart rate in both Sham-4 mice and AB-4 mice, but the degree of these changes was significantly greater in AB-4 mice (Figure 3C). The pressor response to angiotensin II ICV infusion was greater in AB-4 mice than Sham-4 mice (ΔMAP: 8.6 ± 1.2 mm Hg in Sham-4, 22.3 ± 3.4 mm Hg in AB-4 mice; n = 4 for each), however, the pressor response to carbachol ICV infusion did not differ between groups (ΔMAP: 9.5 ± 1.8 mm Hg in Sham-4, 13.7 ± 1.5 mm Hg in AB-4 mice; n = 4 for each).

Na concentration in the brain tissues (circumventricular tissues including hypothalamus) was higher in AB-H mice than in the other groups (AB-H, 116 ± 2 ppm; AB-R, 102 ± 4 ppm; Sham-R, 104 ± 2 ppm; Sham-H, 104 ± 2 ppm; n = 5 for each; P < 0.05).

**Effects of High-Na aCSF ICV Infusion on Cardiac Function**

In AB mice, high-Na aCSF ICV infusion significantly increased LVDD (3.4 ± 0.4 mm) and decreased %FS (32 ± 1%) compared with regular-Na aCSF ICV infusion (LVDD, 3.2 ± 0.5 mm; %FS, 41 ± 1%; n = 5 for each; P < 0.05). Arterial pressure did not differ between AB mice with high-Na aCSF and regular-Na aCSF (94 ± 3 mm Hg in high-Na aCSF,
101±5 mmHg in regular-Na aCSF; n=4 for each). In Sham mice, high-Na aCSF ICV infusion had no significant effects on cardiac function compared with regular-Na aCSF ICV infusion (LVDD, 3.1±0.2 mm in high-Na aCSF versus 3.1±0.3 mm in regular-Na aCSF; %FS, 46±2% in high-Na aCSF versus 48±3% in regular-Na aCSF; n=5 for each).

Effects of ENaC Blocker ICV Infusion on Cardiac Function
In comparison with AB-H mice, ICV infusion of the ENaC blocker benzamil (AB-HB mice) significantly decreased U-NE and U-E excretion (Figure 4A). Cardiac function (LVDD and %FS) significantly improved in AB-HB mice compared with AB-H mice (Figure 4B). Relative heart weight decreased in AB-HB mice compared with AB-H mice (Figure 4C). Arterial pressure was significantly higher and heart rate was lower in AB-HB mice than in AB-H mice (Online Table II). ICV infusion of benzamil did not affect these measures in AB-R mice, and ICV infusion of vehicle in AB-H mice also did not significantly decrease U-NE and U-E excretion (data not shown).

Rho-Kinase Activity and AT_{1}R Expression in the Brain
The amount of AT_{1}R and the expression of p-moesin, a substrate of Rho-kinase, in the brain stem and circumventricular tissue were significantly higher in AB-4 mice than in Sham-4 mice (Figure 5).

Effects of ICV Infusion of Rho-Kinase Inhibitor and AT_{1}R Blocker on Cardiac Function
In comparison with AB-H mice, ICV infusion of the Rho-kinase inhibitor Y-27632 (AB-HY mice) or AT_{1}R blocker telmisartan (AB-HT mice) induced a significant decrease in U-NE and U-E excretion (Figure 6A). In AB-HT mice, U-NE and U-E decreased in a dose-related manner. Cardiac function was also significantly improved in AB-HY mice or AB-HT mice compared with AB-H mice (Figure 6B). Relative heart weight was decreased in AB-HY mice or AB-HT mice compared with AB-H mice (Figure 6C). Heart rate was significantly decreased in AB-HY mice or AB-HT mice compared with AB-H mice (Online Table II). Infusion of vehicle (aCSF or DMSO) did not have these effects.
Serum Parameters
Serum Na concentration did not differ between groups (Sham-4, 151 ± 2 mEq/L; AB-4, 151 ± 1 mEq/L; AB-R, 150 ± 1 mEq/L; AB-H, 152 ± 1 mEq/L). Serum creatinine concentration, as a marker of renal function, also did not differ between groups (Sham-4, 0.11 ± 0.01 mg/dL; AB-4, 0.09 ± 0.01 mg/dL; AB-R, 0.12 ± 0.01 mg/dL; AB-H, 0.11 ± 0.01 mg/dL). Serum aldosterone levels were not different between Sham-4 and AB-4 mice and were significantly lower in AB-H mice than in AB-4 mice, AB-R mice, and Sham-4 mice (Sham-4, 120 ± 11 pg/dL; AB-4, 145 ± 28 pg/dL; AB-R, 163 ± 17 pg/dL; AB-H, 54 ± 6 pg/dL; n = 6 to 7; P < 0.05).

Discussion
The major findings of the present study were that mice with pressure overload produced by aortic banding acquired brain Na sensitivity via the activation of brain ENaCs through stimulation of the Rho/Rho-kinase pathway and RAS. Because of the acquired brain Na sensitivity, high salt intake led to sympathetic activation, which led to the deterioration of cardiac function. These findings are novel and suggest new targets for studies of the prevention and treatment of cardiac deterioration in patients with pressure overload, such as hypertensive heart disease.

The most important finding of the present study was that the mice with pressure overload acquired brain Na sensitivity and a high-salt diet increased the sympathetic outflow before cardiac dysfunction was detected. In AB-4 mice, only LVWT tended to increase compared to the Sham-4 mice, but there was no effect on cardiac function. Both a high-salt and regular-salt diet for an additional 4 weeks, however, induced cardiac dysfunction in AB mice compared with Sham mice. Furthermore, AB mice on the high-salt diet exhibited significantly more severe cardiac dysfunction and greater activation of the sympathetic system than AB mice on the regular-salt diet. This high-salt induced enhanced sympathetic drive was obvious before cardiac function was impaired. In Sham mice, a high salt intake did not increase U-NE and U-E excretion and had no effect on cardiac function. These results strongly suggest that the mice with pressure overload acquired the salt sensitivity before cardiac function began to deteriorate and that a high salt intake augmented cardiac dysfunction by inducing sympathetic activation.

To clarify the contribution of central mechanisms to the acquisition of salt sensitivity in mice with pressure overload, we examined the effects of high-Na in the CSF on sympathetic activity and arterial pressure after ICV infusion of high-Na or regular-Na aCSF. Compared with ICV infusion of regular-Na aCSF, high-Na aCSF induced significant increases in U-NE and U-E excretion in both groups of mice. The increased U-NE excretion in AB mice, however, tended to be greater than that in Sham mice (P = 0.1), and the increase in U-E excretion was significantly greater in AB mice than in Sham mice. Furthermore, ICV infusion of high-Na aCSF induced significantly greater increases in arterial pressure and heart rate in AB-4 mice than in Sham-4 mice. To assess the specificity of the pressure response to a high-Na ICV infusion, we examined the response to other central stimuli, such as angiotensin II and carbachol. The response to angiotensin II was greater in AB-4 mice than Sham-4 mice. In contrast, the response to carbachol was not different between groups. The effect of the angiotensin II ICV infusion was supported by the findings that the extent of brain AT1R was greater in AB-4 mice than Sham-4 mice, and the effect of carbachol ICV infusion indicated the specific activation of the brain RAS and Na sensing system. Together with the findings from the systemic salt loading, our findings suggest that the acquisition of Na sensitivity in the brain of...
mice with pressure overload results from two different mechanisms: (1) the enhancement of Na uptake into the brain and (2) the increase in responsiveness to Na within the brain.

Another important finding of the present study was the high-Na aCSF-induced activation of the sympathetic system, which further deteriorates cardiac function in mice with pressure overload. There are some reports that enhanced sympathetic drive plays an important role in the progression of heart failure. In the present study, in comparison with ICV infusion of regular-Na aCSF, high-Na aCSF induced a significant decline in cardiac function. To evaluate the possibility that the increase in the afterload induced by increased arterial pressure affected cardiac function, we measured arterial pressure 2 weeks after ICV infusion of high-Na aCSF and confirmed that arterial pressure did not significantly increase compared with regular-Na ICV infusion. These results suggest that high-Na aCSF-induced sympathetic hyperactivation may lead to cardiac dysfunction in mice with pressure overload and the deterioration of cardiac function may not be attributable to the increase in the afterload induced by the arterial pressure elevation. However, high-salt loading caused further decreases in cardiac function in AB mice, indicating that high-salt loading may induce further decrease in cardiac function both by sympathetic activation and an increase in arterial pressure in AB mice.

Arterial pressure in AB-4 mice was significantly higher than that in Sham-4 mice; and arterial pressure in AB-H 1-week mice, which were loaded with a high-salt diet for 1 week, was further increased compared with that in AB-4 mice. Arterial pressure in AB-R mice and AB-H mice decreased to levels similar or lower than that in Sham mice within 8 weeks. This may relate to cardiac dysfunction. In fact, the LVEDP in AB-H mice was significantly greater than that in AB-R or Sham-R mice and the LV %FS in AB-H mice was significantly smaller than that in AB-R or Sham-R mice. To validate the arterial pressure measurements, we measured arterial pressure and heart rate using a radio-telemetry system with mice in the awake state. At day 28 after aortic banding (AB-4 mice), arterial pressure was significantly higher than that before aortic banding. Thereafter, in AB-H mice, arterial pressure was significantly further increased at day 35 (1 week after the starting high-salt diet), but the general health of the mice deteriorated, likely because of severe lung congestion, which was supported by the high lung/body weight ratio. In AB-R mice, arterial pressure peaked at around day 40 and then gradually decreased. Implantation of the telemetry catheter in the carotid artery might further augment the pressure overload and induce severe lung congestion in AB-H mice. Therefore, we examined the arterial pressure under anesthesia in acute experiments. The findings indicate that aortic banding causes a pressure overload for LV and high-salt loading superimposed on aortic banding further augments the pressure overload.

To explore the mechanisms of the acquisition of brain Na sensitivity, we examined the effects of an ENaC blocker, benzamil. Brain ENaCs are involved in the high salt–induced increase in central sympathetic outflow in salt-sensitive hypertensive rats. In the present study, brain ENaC blockade by benzamil attenuated the high salt–induced activation of the sympathetic nervous system and the deterioration of cardiac function. Furthermore, we examined the brain Na concentrations in each group. We were unable to measure Na concentrations in the CSF in the present study, because in mice it is difficult to obtain the volume of CSF required to measure Na concentration. Therefore, we measured the Na concentrations in the brain tissues and confirmed that AB-H mice had higher Na concentrations than the other groups. These findings support our hypothesis that the pressure overload activates brain ENaCs and augments Na transport from plasma to the CSF, resulting in sympathoexcitation. However, we did not examine the effects of brain ENaCs on Na transport directly and ENaCs have both epithelial and neural components. Therefore, it is possible that the benzamil may affect ENaCs on neural components and cause sympathoinhibitory effects. The role of ENaCs on neural components in sympathetic modulation remains unclear. A similar dose of benzamil was used as specific ENaC blocker in previous studies, and the estimated benzamil concentration in the CSF in the present study was considered to be specific for ENaCs (<100 nmol/L). Therefore, the dose of benzamil used in the present study was adequate for use as a specific ENaC blocker. Further studies are required to measure ENaC activity directly. Although some studies have demonstrated that salt intake induces sympathoexcitation via central mechanisms and the effects of brain ENaCs on cardiac function, these previous studies used genetic models of salt-sensitive hypertension or heart failure induced by myocardial infarction, whereas we used the pressure overload produced by aortic banding model in mice without a genetic background of salt sensitivity.

Finally, we focused on Rho-kinase and angiotensin II as the mechanisms involved in brain ENaC activation in the mice with pressure overload, because ENaCs in kidney are reported to be activated by Rho-kinase and angiotensin II. In addition, we recently reported that Rho-kinase and angiotensin II in the brain contribute to cardiovascular regulation via the sympathetic nervous system. In the present study, we confirmed that compared to Sham-4 mice, the brains of AB-4 mice had higher levels of AT1R and higher Rho-kinase activity, and blockade of either AT1R or Rho-kinase attenuates high salt–induced sympathetic activation and cardiac dysfunction. These findings suggest that enhanced brain Na sensitivity results from the activation of brain ENaCs via the Rho/Rho-kinase pathway and RAS in mice with pressure overload. However, ENaCs may be upstream of RAS in brain. In the present study, we did not address this issue. Further studies are needed to clarify the relationship between RAS and ENaCs in brain. It is possible that renal blood flow is reduced in mice with suprarenal abdominal aortic banding, resulting in renal dysfunction concomitant with activation of the systemic RAS. It is unlikely that this occurred in the present study because we confirmed that serum creatinine and aldosterone levels were not significantly different between groups and the mean arterial pressure in the AB-4 mice measured from the right femoral artery was above 90 mm Hg, suggesting that the aortic banding procedure did not significantly reduce renal blood flow and impair renal function. Previous studies...
demonstrated that excess stimulation of cardiopulmonary and arterial baroreceptors impair baroreflex function, and RAS or Rho-kinase in the brain might contribute to the impaired baroreflex function. In the present study, we demonstrated that arterial pressure measured from the carotid artery and LVEDP were significantly greater in AB-4 mice than in Sham-4 mice. The excess stimulation of cardiopulmonary and arterial baroreceptor may contribute to the activation of the Rho/Rho-kinase pathway and RAS in the brains of the mice with pressure overload, even before high-salt loading.

In conclusion, the present findings strongly suggest that mice with pressure overload acquire brain Na sensitivity because of the activation of brain ENaCs via the Rho/Rho-kinase pathway and RAS. The acquired brain Na sensitivity contributes to high salt–induced sympathetic activation, leading to deteriorating cardiac function in mice with pressure overload.

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Disclosures
None.

References
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Expanded Materials and Methods

Animals
The study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male Institute of Cancer Research (ICR) mice (10 weeks old; SLC, Fukuoka, Japan) were used.

Mouse LVH Model Preparation
The suprarenal abdominal aorta\(^1\) was banded in mice (AB mice) under sodium pentobarbital (25–40 mg/kg i.p.) anesthesia. The abdominal aorta was constricted at the suprarenal level with 5-0 silk sutures guided by a blunted 27-gauge needle, which was withdrawn as quickly as possible. Sham-operated (Sham) mice served as controls. Four weeks later, AB and Sham mice were each divided into 2 groups: 1) mice fed a high-salt (8% NaCl) diet for 4 weeks (AB-H mice and Sham-H mice) and 2) mice fed a regular-salt (0.3% NaCl) diet for 4 weeks (AB-R mice and Sham-R mice; Figure 1; protocol-1).

Evaluation of Cardiac Function
Cardiac function was evaluated by echocardiography.\(^2,3\) Serial M-mode echocardiography was performed on mice under light sodium pentobarbital anesthesia with spontaneous respiration. An echocardiography system (SSD5000; Aloka, Tokyo, Japan) with a dynamically focused 7.5-MHz linear array transducer was used. M-mode tracings were recorded from the short-axis view at the
level of the papillary muscle. Left ventricle (LV) end-diastolic diameter (LVDD), LV end-systolic diameter (LVSD), and LV wall thickness (LVWT) were measured. LVWT was calculated as the average of the thickness of the interventricular septum and the posterior LV wall. Percent fractional shortening (%FS) was calculated as follows: %FS = (LVDD) − (LVSD) / (LVDD) × 100. Cardiac function was also evaluated by LV End-Diastolic Pressure (LVEDP). LVEDP was measured with a conductance catheter (1.4 Fr; Miller Instruments®) inserted into the right carotid artery and advanced across the aortic valve into the left ventricle.

**Measurement of Arterial Pressure and Heart Rate**

Under sodium pentobarbital anesthesia (25–40 mg/kg i.p.), mice were intubated using a 20-gauge soft catheter and ventilated with a tidal volume of 1.0-1.5 mL at 120 cycles/min with the fraction of inspired oxygen equal to 0.21. A catheter was then inserted into the right carotid artery to measure arterial pressure and heart rate. In another protocol, arterial pressure and heart rate were measured in awake AB-H and AB-R mice using a radio-telemetry system (Data Sciences International). Under sodium pentobarbital anesthesia (25–40 mg/kg i.p.), the telemetry catheter was implanted into the left carotid artery and the transducer unit was inserted into a subcutaneous pouch along the abdomen. Each mouse was housed in an individual cage after operation and unrestricted and free move in their cage. The case was placed over the receiver panel connected to the computer for data acquisition. Arterial pressure and heart rate were recorded continuously for 5 minutes and averaged.

**Evaluation of Sympathetic Activity**

Sympathetic activity was evaluated by measuring 24-h urinary norepinephrine (U-NE) and urinary epinephrine (U-E) excretion using high-performance liquid chromatography.
Evaluation of Na Sensitivity

U-NE and U-E after high salt intake was compared between Sham mice and AB mice. Four weeks after AB (AB-4 mice) or sham operation (Sham-4 mice), mice were fed a high-salt (8% NaCl) diet. Five days after starting the high-salt diet, 24-h U-NE and U-E excretion were measured, and echocardiography was performed to confirm that cardiac function was preserved. In addition, U-NE and U-E excretion in response to high-Na (0.2 mol/L) aCSF ICV infusion (0.25 L/h for 14 days, using an osmotic minipump) were measured in Sham mice and AB mice.

Under anesthesia with sodium pentobarbital (25–40 mg/kg i.p.), mice were placed on a stereotaxic frame. The skin overlying the midline of the skull was incised, and a small hole with the following coordinates was bored using a dental drill: 0.3 mm posterior and 1 mm lateral relative to the bregma, and 3 mm ventral to the skull surface. An Alzet® brain infusion kit 3 (DURECT Corporation, CA) connected to an osmotic minipump (Alzet model 1004; DURECT) was fixed to the skull surface with tissue adhesive. The pump was inserted subcutaneously on the back. Mice with ICV infusion of regular-Na (0.145 mol/L) aCSF served as the controls (R-Na ICV-mice; Figure 1; protocol-2). Before and 2 weeks after starting the ICV infusion, sympathetic activity, cardiac function, arterial pressure, and heart rate were measured by the methods described above.

The effects of high-Na (0.2 mol/L) aCSF ICV infusion on arterial pressure and heart rate were also evaluated in Sham-4 mice and AB-4 mice in acute experiments. Arterial pressure and heart rate were measured via a catheter in the right carotid artery under anesthesia. High-Na (0.2 mol/L) aCSF was infused ICV with a microsyringe pump (infusion rate: 1 L/min for 10 min) and changes in arterial pressure and heart rate were measured. Furthermore, the effects of other central stimuli, such as angiotensin II or carbachol ICV infusion on arterial pressure were
examined. Angiotensin II (Sigma) (0.5 nmol/L, 1 L/min for 5 min) or carbachol (Sigma) (0.1 mmol/L, 1 L/min for 5 min) was infused. The dose of each chemical was also determined according to the previous reports.\textsuperscript{7,8}

**Measurement of Brain Na Concentration**

Under anesthesia with an overdose of sodium pentobarbital, the mice were perfused with dH\textsubscript{2}O. After adequate perfusion to remove blood, the brain circumventricular tissues and hypothalamus were dissected out. The tissues (0.10 ± 0.01 g) were homogenized in 200 L of dH\textsubscript{2}O, centrifuged, and the supernatant was collected. The Na concentration in each sample was measured.

**Measurement of Organ Weight**

After completion of the experiments, mice were killed with an overdose of sodium pentobarbital, and the heart and lungs were removed and weighed.

**Measurement of Serum Parameters**

Within minutes after the mice were injected with an overdose of sodium pentobarbital, a blood sample was collected from the right ventricle and rapidly centrifuged (6000 rpm for 10 min). The obtained serum sample was then stored at \textdegree\textsubscript{C} before measuring serum components. We evaluated the aldosterone concentration by radioimmunoassay, Na concentration by electrode methods, and creatinine by enzymatic methods.

**Evaluation of the Effects of Na-Channel Blockade in the Brain**

Benzamil (Sigma), a specific epithelial Na-channel (ENaC) blocker\textsuperscript{9} (1 mg/mL, dissolved in
aCSF), was infused ICV in AB-H mice (AB-HB mice) and AB-R mice (AB-RB mice) using an osmotic minipump (0.11 µL/h for 4 weeks). Four weeks later, 24-h U-NE and U-E excretion, arterial pressure, heart rate, and organ weight were measured, and echocardiography was performed as described earlier. Mice with ICV infusion of only aCSF (vehicle) served as controls (aCSF mice; Figure 1; protocol-1).

Evaluation of the Effects of Rho-Kinase and Angiotensin Type 1 Receptors (AT1R)

Blockade in the Brain

A specific Rho-kinase inhibitor, Y-27632\textsuperscript{10} (Calbiochem, 5 mmol/L, dissolved in aCSF), or an AT1R blocker, telmisartan (Sigma, 4 mmol/L, 20 mmol/L, dissolved in demethyl sulfoxide [DMSO]) was infused ICV in ABH mice (AB-HY or AB-HT mice, respectively) using an osmotic minipump (0.11 µL/h for 4 weeks). Four weeks later, 24-h U-NE/U-E excretion, arterial pressure, heart rate, and organ weight were measured; echocardiography was performed in AB-HY mice and AB-HT mice as described earlier (Figure 1; protocol-1).

Evaluation of AT1R Expression and Rho-Kinase Activity

The animals were killed with an overdose of sodium pentobarbital, and circumventricular tissues including the hypothalamus and brainstem tissues were obtained. The tissues were homogenized in a lysing buffer containing 40 mmol/L HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 1% Triton\textsuperscript{®} X-100, 10% glycerol, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4} (sodium orthovanadate), and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged and the supernatant collected. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). A 15-µg aliquot of protein from each sample was separated on a polyacrylamide gel with 10% sodium dodecyl sulfate. The proteins were subsequently
transferred onto polyvinylidene difluoride membranes (Immobilon®-P membranes; Millipore, Billerica, MA). Membranes were incubated with rabbit immunoglobulin G (IgG) monoclonal antibody to angiotensin type-1 receptor (AT1Rs, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), with rabbit IgG polyclonal antibody to β-tubulin (1:1000; Santa Cruz Biotechnology) and with goat IgG polyclonal antibody to phosphorylated-moesin, a substrate of Rho-kinase (p-moesin, 1:1000, Santa Cruz Biotechnology). Membranes were then incubated with horseradish peroxidase-conjugated horse anti-rabbit or anti-goat IgG antibody (1:10,000). β-Tubulin (1:5000; Santa Cruz Biotechnology) was used as an internal control for the brain tissues. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL™ Western blotting detection kit; Amersham Pharmacia Biotech, Uppsala, Sweden), and the film was analyzed using the public domain software NIH Image (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Statistical Analysis**

All values are expressed as mean ± SE. Analysis of variance was used to compare U-NE and U-E excretion, organ weight, LVDD, LVWT, %FS, and arterial pressure by telemetry system between groups. An unpaired t-test was used to compare changes in arterial pressure and heart rate after high-Na ICV infusion, and protein levels between Sham mice and AB mice. Differences were considered to be significant when $P < 0.05$. 
References


8. Wang N, Jiang CL, Wang CY, Yao QY. Role of brain angiotensin AT1 receptor in the


### Online Table I  
**LVEDP for Each Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham- 4</td>
<td>7.0 ± 0.8</td>
</tr>
<tr>
<td>Sham-R</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>AB-4</td>
<td>13.5 ± 1.6 *</td>
</tr>
<tr>
<td>AB-R</td>
<td>13.3 ± 1.1</td>
</tr>
<tr>
<td>AB-H</td>
<td>18.6 ± 0.8 #</td>
</tr>
</tbody>
</table>

n=4 for each, *P<0.05 versus Sham-4, #P<0.05 versus Sham-R
### Online Table II  MAP and HR for Each Group

<table>
<thead>
<tr>
<th>Group (Number)</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-4 (8)</td>
<td>87 ± 2</td>
<td>388 ± 9</td>
</tr>
<tr>
<td>Sham-R (5)</td>
<td>88 ± 2</td>
<td>390 ± 5</td>
</tr>
<tr>
<td>Sham-H (5)</td>
<td>95 ± 3</td>
<td>401 ± 6</td>
</tr>
<tr>
<td>AB-4 (8)</td>
<td>107 ± 4 *</td>
<td>429 ± 28 *</td>
</tr>
<tr>
<td>AB-R (5)</td>
<td>94 ± 3</td>
<td>460 ± 14 #</td>
</tr>
<tr>
<td>AB-H (5)</td>
<td>80 ± 5</td>
<td>497 ± 3 $#,#,+$</td>
</tr>
<tr>
<td>AB-H 1w (5)</td>
<td>123 ± 7</td>
<td>490 ± 15 *</td>
</tr>
<tr>
<td>AB-HB (4)</td>
<td>95 ± 5</td>
<td>435 ± 17 $^5$</td>
</tr>
<tr>
<td>AB-HY (5)</td>
<td>94 ± 3</td>
<td>372 ± 15 $^5$</td>
</tr>
<tr>
<td>AB-HT (4)</td>
<td>88 ± 1</td>
<td>402 ± 6 $^5$</td>
</tr>
<tr>
<td>AB-4 (FA) (6)</td>
<td>92 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 versus Sham-4, #P<0.05 versus Sham-R, $^+P<0.05$ versus AB-R

$^5P<0.05$ versus AB-H

FA measured from femoral artery.
Online Figure I: Graphs showing mean arterial pressure (MAP) (upper panel) and heart rate (HR) (lower panel) measured by telemetry before and after aortic banding (arrow). Circles indicate the data from mice fed a high salt diet and squares indicate the data from mice fed a regular salt diet. Please see details in the Results section of the text.