Research Report

The accumulation of brain water-free sodium is associated with ischemic damage independent of the blood pressure in female rats

Manabu Sumiyoshi⁎, Keiko T. Kitazato, Kenji Yagi, Takeshi Miyamoto, Yoshitaka Kurashiki, Nobuhisa Matsushita, Tomoya Kinouchi, Kazuyuki Kuwayama, Junichiro Satomi, Shinji Nagahiro

Department of Neurosurgery, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

Abstract

Estrogen deficiency worsens ischemic stroke outcomes. In ovariectomized (OVX+) rats fed a high-salt diet (HSD), an increase in the body Na+/water ratio, which characterizes water-free Na+ accumulation, was associated with detrimental vascular effects independent of the blood pressure (BP). We hypothesized that an increase in brain water-free Na+ accumulation is associated with ischemic brain damage in OVX+/HSD rats. To test our hypothesis we divided female Wistar rats into 4 groups, OVX+ and OVX-/C0 rats fed HSD or a normal diet (ND), and subjected them to transient cerebral ischemia. The brain Na+/water ratio was increased even in OVX+/ND rats and augmented in OVX+/HSD rats. The increase in the brain Na+/water ratio was positively correlated with expansion of the cortical infarct volume without affecting the BP. Interestingly, OVX+ was associated with the decreased expression of ATP1α3, a subtype of the Na+ efflux pump. HSD increased the expression of brain Na+ influx-related molecules and the mineralocorticoid receptor (MR). The pretreatment of OVX+/HSD rats with the MR antagonist eplerenone reduced brain water-free Na+ accumulation, up-regulated ATP1α3, down-regulated MR, and reduced the cortical infarct volume. Our findings show that the increase in the brain Na+/water ratio elicited by estrogen deficiency or HSD is associated with ischemic brain damage BP-independently, suggesting the importance of regulating the accumulation of brain water-free Na+. The up-regulation of ATP1α3 and the down-regulation of MR may provide a promising therapeutic strategy to attenuate ischemic brain damage in postmenopausal women.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

⁎Corresponding author. Tel.: +81 88 633 7149; fax: +81 88 632 9464. E-mail address: mansumiyoshi@yahoo.co.jp (M. Sumiyoshi).
1. Introduction

The functional outcome after acute ischemic stroke is poorer in women, especially postmenopausal women, than men (Turtzo and McCullough, 2008). Epidemiological studies have shown that a high-salt intake is a risk factor for stroke (Li et al., 2012; Strazzullo et al., 2009), a leading cause of morbidity and mortality worldwide. Elsewhere we demonstrated that in ovariectomized (OVX) rats fed a high-salt diet (HSD), the increase in the body Na\(^+\)/water ratio was associated with the formation of cerebral aneurysms without a rise in the blood pressure (BP) (Matsushita et al., 2012). Excessive salt intake is thought to induce hypertension by Na\(^+\) and concomitant water retention. However, a large amount of Na\(^+\) can be accumulated without accompanying water retention in the form of water-free Na\(^+\) (Heer et al., 2000), which can be characterized by an increase in the Na\(^+\)/water ratio. The skin interstitium is a water-free Na\(^+\) reservoir that can buffer the impact of Na\(^+\) accumulation on the intravascular volume (Coffman, 2011).

Compared to age-matched men, women are resistant to the hypertensive effects of dietary salt (Titze et al., 2003). Although the detailed mechanisms underlying this sex difference remain obscure, they may involve disturbance of the function of the renin-angiotensin-aldosterone system (RAAS) (Rands et al., 2012) and oxidative stress (Sartori-Valinotti et al., 2008). Estrogen deficiency exacerbates experimental ischemic brain damage in rats (Shimada et al., 2011). As the expression of Na\(^+\)/K\(^+\)-ATPase is modified by estrogen (Palacios et al., 2004), the down-regulation of Na\(^+\)/K\(^+\)-ATPase may be partly involved in the pathophysiology of brain ischemia (Pimentel et al., 2013). Elsewhere we demonstrated that the incidence of cerebral aneurysms was reduced in rats treated with the angiotensin II type 1 receptor blocker olmesartan (Matsushita et al., 2012) or the mineralocorticoid receptor (MR) antagonist eplerenone (EPL) (Tada et al., 2009). Olmesartan decreased the body Na\(^+\)/water ratio and increased the expression of ATP1\(\alpha\)2, a subtype of the Na\(^+\) efflux pump, in the cerebral blood vessels of OVX/HSD rats (Matsushita et al., 2012) and EPL reduced the sodium intake of OVX/HSD rats without affecting the BP (Tada et al., 2009). These findings suggest that, apart from their hypotensive action, they may have vasoprotective effects regulating the accumulation of water-free Na\(^+\).

Based on our findings and those of others, we hypothesized that the accumulation of brain water-free Na\(^+\) in estrogen-deficient female rats is associated with ischemic brain damage. To test our hypothesis and to verify the mechanisms underlying the accumulation of brain water-free Na\(^+\) we
focused on Na\(^+\) influx-related molecules, the Na\(^+\) efflux pump, and MR.

Here we demonstrate for the first time that the increase in the accumulation of brain water-free Na\(^+\) is associated with ischemic brain damage in female rats without affecting the BP.

2. Results

2.1. HSD increases the BP of male—but not of female rats

First we examined the effect of HSD on BP, body Na\(^+\) and water retention, and the body Na\(^+\)/water ratio in male and female Wistar rats. As shown in Fig. 1A, HSD increased the systolic blood pressure (SBP) in male but not female rats (p < 0.01). Before the feeding of HSD, the SBP in the 4 experimental groups of female rats was not different. We measured the 24-h BP in female rats by telemetry to record daily fluctuations and found that the feeding of HSD for 2 weeks did not increase their BP (Supplementary Fig. 2).

While HSD elevated the body Na\(^+\) retention in both sexes (p < 0.01, Supplementary Fig. 3), it increased body water retention only in males (p < 0.05, Fig. 1B). Therefore, although HSD increased the body Na\(^+\)/water ratio in both sexes, this increase was more pronounced in females (p < 0.05, Fig. 1C). OVX did not affect body Na\(^+\) retention.

2.2. The accumulation of brain water-free Na\(^+\) is associated with ischemic brain damage in both sexes

As shown in Supplementary Fig. 4A, HSD increased the brain Na\(^+\) content in male rats (p < 0.05). In females, even OVX\(^+\) rats fed ND and OVX\(^-\) rats fed HSD, the brain Na\(^+\) content was significantly increased (p < 0.05). The combination of OVX and HSD further augmented the accumulation of brain Na\(^+\) (p < 0.05). As the brain water content was not changed in any of the groups (Supplementary Fig. 4B), the brain Na\(^+\)/water ratio paralleled the brain Na\(^+\) content (Fig. 2A). Importantly, the infarct volume in the cortex (Fig. 2B) but not the basal ganglia region (data not shown) was significantly greater in rats whose brain Na\(^+\) content and Na\(^+\)/water ratio were high before 90-min middle cerebral artery occlusion (MCAO) (p < 0.05 vs male ND rats, and vs OVX\(^-\)/ND, OVX\(^+\)/ND, and OVX\(^-\)/HSD rats).

2.3. The accumulation of brain water-free Na\(^+\) is associated with the up-regulation of Na\(^+\) influx-related molecules and the MR in rats fed HSD, and with down-regulation of the Na\(^+\) efflux pump in OVX\(^-\) rats

To further clarify the mechanisms underlying the increase in the accumulation of brain water-free Na\(^+\) we focused on the Na\(^+\) influx-related molecules NHE1 (Na\(^+\)-H\(^+\) exchanger 1), NKCC1 (Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter 1), and NCX (Na\(^+\)-Ca\(^2+\) exchanger), the Na\(^+\) efflux pump Na\(^+\)/K\(^+\)-ATPase subtypes (ATP1a1, ATP1a2, and ATP1a3), and MR. In the brain cortex, HSD led to a higher mRNA level of NHE1 (p < 0.05, Supplementary Fig. 5A), NKCC1 (p < 0.05, Supplementary Fig. 5B), and NCX (p < 0.05, Supplementary Fig. 5C), while the mRNA and protein levels of ATP1a3 were significantly reduced even in OVX\(^+\)/ND rats (p < 0.05, Supplementary Fig. 5D and Fig. 3A) and much lower in OVX\(^-\)/HSD rats (p < 0.05, Supplementary Fig. 5D and Fig. 3A). ATP1a1 or ATP1a2 was not changed in any group (data not shown). The mRNA and protein levels of MR were increased in rats fed HSD (p < 0.05, Supplementary Fig. 5E and Fig. 3B).

2.4. The MR antagonist EPL increases the expression of ATP1a3, reduces the accumulation of brain water-free Na\(^+\), and attenuates the cortical infarct size in OVX\(^-\)/HSD rats

EPL pretreatment significantly decreased the cortical infarct size (p < 0.01, Fig. 4A), the brain Na\(^+\) content (p < 0.01, Supplementary Fig. 6A), and the Na\(^+\)/water ratio (p < 0.01, Fig. 4B) but not the brain water content (Supplementary Fig. 6B) in OVX\(^-\)/HSD rats. EPL markedly increased the protein level of ATP1a3 in the brain cortex (p < 0.01, Fig. 4C) and decreased the level of MR (p < 0.01, Fig. 4D).

3. Discussion

We newly document that in female Wistar rats the pre-ischemia increase in the accumulation of brain water-free Na\(^+\) is associated with exacerbation of ischemic brain damage without affecting the BP. Fig. 5 summarizes the results.

First we found that HSD increased the BP in male—but not female rats. Rands et al. (2012) reported that male rats display salt-sensitive BP increases during Ang II infusion and the ingestion of HSD while female rats do not. Their findings support our observation of sex differences in salt sensitivity. The effect of HSD on BP seems to be correlated with body water retention. The higher body Na\(^+\)/water ratio in female rats may reflect the buffer effect of water-free Na\(^+\) accumulation against the impact of Na\(^+\) and the concomitant water retention on the intravascular volume. In both sexes the cortical infarct volume was associated with the increase in the brain Na\(^+\) content and the Na\(^+\)/water ratio but not with BP. This suggests that there are no sex differences in the importance of water-free Na\(^+\) accumulation with respect to ischemic brain damage. Our results are supported by clinical studies that found that while a high-salt intake was not associated with hypertension in females (OR 0.85, 95% CI 0.44–1.65), it was associated in males (OR 2.26, 95% CI 1.27–4.02) (Thrift et al., 2011) and by an epidemiological study of Miura et al. (2010) that demonstrated a positive relationship between the dietary salt intake and BP, especially in males.

Next we demonstrated that OVX per se increased the accumulation of brain water-free Na\(^+\) without increasing the body Na\(^+\)/water ratio and that HSD augmented this OVX-induced accumulation of brain water-free Na\(^+\). Most importantly, the increase in the accumulation of brain-water-free Na\(^+\) was positively correlated with the cortical infarct size in rats of both sexes.

Titze et al. (2004) reported that water-free Na\(^+\) accumulates and binds to proteoglycans and glycosaminoglycans in the extracellular matrix (ECM) under the skin. A large proportion of the ECM in the central nervous system contains...
proteoglycans such as chondroitin/dermatan sulfate that play a significant role in the functional diversity of neurons, in brain development, and in some brain diseases (Zamfir et al., 2012). This suggests interactions in the brain with respect to the accumulation of water-free Na⁺.

Matsushita et al. (2012) indicated that the reduced gene and protein expression of cerebral vascular ATP1α2 in OVX⁺/HSD rats is associated with an increased incidence of cerebral aneurysms, suggesting an association with the accumulation of water-free Na⁺ in the cerebral vascular wall. Our study

![Graph A](image1)

**Fig. 2** – Effects of a high-salt diet (HSD, 8% NaCl) on the brain Na⁺/water ratio (A) and the cortical infarct volume (B) in male and female Wistar rats. The cortical infarct volume was measured 24 h after 90-min middle cerebral artery occlusion and is shown as a percentage of the contralateral hemisphere. Each datum represents the mean ± SD (the number of each group is shown in parentheses). *p < 0.05 vs male ND by Student’s t-test or OVX⁻/ND by ANOVA followed by Scheffe’s test, †p < 0.05 vs OVX⁺/ND, and #p < 0.05 vs OVX⁻/HSD by ANOVA followed by Scheffe’s test. ND indicates a normal diet containing 0.3% NaCl; OVX, ovariectomy.

![Graph B](image2)

![Graph C](image3)

**Fig. 3** – Effects of HSD on the protein levels of ATP1α3 (A) and MR (B) in the brain cortex of OVX⁻ and intact (OVX⁺) rats. The protein levels were measured as described in Experimental Procedures. Each datum represents the mean ± SD (each group, n = 8). *p < 0.05 vs OVX⁻/ND, †p < 0.05 vs OVX⁺/ND, and #p < 0.05 vs OVX⁻/HSD by ANOVA followed by Scheffe’s test. MR indicates mineralocorticoid receptor.
confirmed that the down-regulation of ATP1α3 is associated with the accumulation of brain water-free Na⁺ and ischemic brain damage in OVX⁺ rats. In a preliminary study we treated OVX⁺/HSD rats with 17β-estradiol and confirmed the finding of Palacios et al. (2004) that estrogen increases Na⁺/K⁺ ATPase (data not shown). Our study confirmed that the down-regulation of ATP1α3 is associated with the accumulation of brain water-free Na⁺ and ischemic brain damage both in male rats fed HSD and in OVX⁺/HSD rats. EPL up-regulated the mRNA and protein levels of ATP1α3 in both male- (data not shown) and OVX⁺/HSD rats, suggesting an estrogen-independent effect. The detailed mechanisms underlying this up-regulation and its precise control in the brain remain unclear. According to Benfante et al. (2005), the expression of the human neuronal ATP1α3 gene is regulated by the transcription factors Sp1/3/4 (transcription factors 1/3/4 purified from sephacryl and phosphocellulose columns) and NF-Y (nuclear factor-Y). The activity of brain Na⁺/K⁺-ATPase was reduced under oxidative stress (Simao et al., 2011) and oxidative stress was increased by OVX (Behr et al., 2012). As pretreatment with EPL reduced oxidative stress in a mouse cerebral ischemia model (Iwanami et al., 2007), its antioxidative effects may partly contribute to the up-regulation of ATP1α3. The expression patterns of ATP1α3 in the brain are cell-type specific and cortical neurons harbor a large amount of ATP1α3 (Bottger et al., 2011). Verification of the detailed mechanisms underlying the EPL-induced ATP1α3 modulation may help to develop new therapeutic strategies for dealing with stroke.

MR activation is a contributing factor in the pathophysiology of a wide range of diseases. Elevated levels of aldosterone, a physiological MR activator, are related to various detrimental effects (Queisser and Schupp, 2012; Brown, 2008). However, as HSD lowers the plasma aldosterone level (Matsushita et al., 2012), we think that the contribution of aldosterone to the ischemic brain damage in our OVX⁺/HSD rats is minimal. On the other hand, the mRNA expression of MR was increased in the human hippocampus after brief cerebral ischemia and there were no sex differences in the expression of MR (Lai et al., 2009). In the striatum of mice subjected to MCAO, most MR-positive cells were astrocytes (Oyamada et al., 2008), confirming the activation of MR in the ischemic brain. In our study the gene and protein expression

---

**Fig. 4** – Effects of eplerenone (EPL) on the cortical infarct volume (A), brain Na⁺/water ratio (B), and the protein level of brain ATP1α3 (C) and MR (D) in ovariectomized rats fed a high-salt diet. Each datum represents the mean ± SD (the number of each group is shown in parentheses). *p < 0.01 vs the vehicle control (VC) by Student's t-test.

**Fig. 5** – Relationship between the brain Na⁺/water ratio and ischemic brain damage. The effect of a high-salt diet (HSD) on the blood pressure (BP) shows a sex difference. In female rats, an increase in the body Na⁺/water ratio is not always accompanied by body water retention and a BP increase. In both sexes, the brain Na⁺/water ratio is positively correlated with the ischemic brain damage. Ovariectomy (OVX) itself increased the brain Na⁺/water ratio.
of MR was up-regulated by HSD. The exacerbation of ischemic brain damage in OVX+/HSD rats may be attributable to the up-regulation of MR by HSD and the down-regulation of ATP1a3 by estrogen deficiency. Since MR antagonists had no effects on the infarct size in female stroke-prone hypertensive rats (Rigsby et al., 2007), the effects of EPL and the role of MR may depend on the experimental conditions (Dinh et al., 2012).

While the putative benefits from a low-salt diet have not been fully established, lower levels of excreted urinary Na⁺ were predictive of mortality rates from cardiovascular diseases (Matyas et al., 2011; Stolarz-Skrzypek et al., 2011). There seems to be no discrepancy between these findings and our hypothesis that the EPL-induced removal of accumulated water-free Na⁺ from target tissues may be crucial for their protection. As BP control helps to prevent stroke, many drugs have been developed to treat hypertension. Our study suggests that water-free Na⁺ accumulation in the brain may be another modifiable factor in addition to BP control. To detect the accumulation of brain water-free Na⁺, sodium magnetic resonance imaging (²³Na MRI) studies may yield the necessary information (Kopp et al., 2013; Wetterling et al., 2012).

Our study has some limitations. Our experimental conditions cannot be extrapolated to humans. At present we do not know the detailed mechanisms that lead to the accumulation of brain water-free Na⁺. Nonetheless, our findings highlight the importance of the accumulation of brain water-free Na⁺ in ischemic stroke and suggest that its management, besides a reduction in the salt intake, is a promising strategy especially in women, both normotensive and postmenopausal. Our findings warrant further studies to explore new translatable methods and to assess whether the suppression of brain water-free Na⁺ accumulation helps to limit ischemic brain damage in the clinical setting.

4. Experimental procedures

4.1. Animals

Wistar rats obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) were housed in temperature- and light-controlled animal quarters and provided with a normal diet (ND, 0.3% NaCl) ad libitum.

All experiments and protocols were approved by the Ethics Committee of the Institute of Health Biosciences, the University of Tokushima Graduate School, and were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Surgical procedures were performed under anesthesia with 2% isoflurane in 30% oxygen and 70% nitrous oxide; the body temperature was monitored and maintained at 37 °C using a warming plate (NISSIN, Tokyo, Japan). The rats were ovariec-tomized (OVX) at the age of 7 weeks, one week after their arrival to allow their acclimatization. The investigators involved in all surgical procedures, drug treatments, and endpoint assessments were blinded to the group to which each animal had been assigned.

4.1.1. Experimental study 1

We randomly divided 7-week-old female rats (n=80) into 4 equal groups. Group 1 did not undergo OVX and was fed ND (OVX+/ND), group 2 underwent bilateral OVX and was fed ND (OVX+/ND), group 3 was OVX⁻ and fed a high-salt diet (HSD, 8% NaCl) (OVX-/HSD), and group 4 was OVX⁻ and fed HSD (OVX-/HSD). HSD was provided for 2 weeks before 90-min middle cerebral artery occlusion (MCAO) performed at the age of 11 weeks.

BP at the age of 9- and 11 weeks was measured by tail-cuff plethysmography (Softron, Tokyo, Japan). Body water retention, Na⁺ retention, and the Na⁺/water ratio at the age of 11 weeks were recorded for all rats as described previously (Matsushita et al., 2012).

At the age of 11 weeks, 24-h BP was measured by telemetry (Data Science Inc., MN, USA) and recorded using the Dataquest Advanced Research Technologies Acquisition program (UNIQUE MEDICAL, Osaka, Japan) (each group, n=4). Another 8 rats in each group were euthanized without MCAO and their brain tissues were harvested to measure the brain water content, Na⁺ content, the Na⁺/water ratio, and the expression of Na⁺ transport-related molecules. The remaining 8 rats in each group were subjected to 90-min MCAO and the infract size 24 h post-reperfusion was recorded.

To examine sex differences we used a set of 9-week-old male rats (n=32) and divided them into 2 equal groups. They were fed ND or HSD for 2 weeks and the body water retention, Na⁺ retention, and the Na⁺/water ratio were determined. At the age of 11 weeks, 8 males from each group were subjected to 90-min MCAO and the other 8 rats in each group were euthanized without MCAO. The BP at the age of 9- and 11 weeks and body water retention, Na⁺ retention, and the Na⁺/water ratio at the age of 11 weeks were recorded for all 32 male rats.

4.1.2. Experimental study 2

Another set of 32 OVX+/HSD rats was randomly divided into 2 equal groups. At the age of 9- and 11 weeks they underwent the same measurements performed in experimental study 1. Group 1 served as the vehicle control (VC). Group 2 was treated for 2 weeks with 100 mg/kg/day eplerenone (EPL) diluted in 5% gum arabic and administered orally once a day. At the age of 11 weeks, 8 VC- and 8 EPL rats were subjected to 90-min MCAO; the other 8 rats in each group were euthanized without MCAO.

4.2. Measurement of the brain water, Na⁺, K⁺, and Cl⁻ content

All rats without MCAO were euthanized and their brains were removed immediately. Using a brain matrix (Bioresearch Center, Nagoya, Japan), the extracted brain tissues were cut into equal 2-mm-spaced slices and 6 serial coronal sections were prepared; they did not contain olfactory tissues or tissues from the cerebellum. The anterior 3rd coronal sections were divided into right- and left hemispheres. The right hemispheres were stored at –80 °C until quantitative real-time PCR assay and the left hemispheres were processed to measure the brain Na⁺, K⁺, and Cl⁻ content. The 1st, 2nd,
4th, 5th, and 6th coronal sections were processed to measure the brain water content. The brain tissue samples used for water- and Na⁺, and for K⁺ and Cl⁻ content measurements were weighed on an analytical balance (GH120, A&D, Japan) to obtain the wet weight (Ww); the precision was 0.01 mg.

The samples used for water measurements were then dried in a freeze dryer (EYELA FDU-1000, Rikakikai, Tokyo, Japan) for 24 h and weighed again to obtain the dry weight (Dw). The brain water content, expressed in milliliters per gram wet tissue weight (Ww g wt⁻¹), was calculated using the formula:

\[ \frac{W_w}{W_w - Dw} \times 1000 \text{ (mL/mL)} \]

The brain tissue samples for Na⁺, K⁺ or Cl⁻ measurements (the left hemisphere of the 3rd coronal sections) were weighed and dried as described above and then immersed in 0.5 mL of deionized H₂O₂; this was followed by 30-s homogenization (ISO Inc.), 1-min sonication on ice using a pulse ultrasonicator (Tomy Seiko Co.), and 15-min centrifugation at 14,000 rpm (Tomy Seiko). Supernatant aliquots were collected and the concentration of Na⁺, K⁺, and Cl⁻ was measured using an ion-selective electrode method (SRL Inc., Tokyo, Japan). The brain content of Na⁺, K⁺, and Cl⁻ per gram tissue weight was calculated using the formula:

\[ [\text{Na}^+], [\text{K}^+], \text{or } [\text{Cl}^-]_{\text{brain}} \text{ (mmol/g wt)} = \left[ \frac{[\text{Na}^+], [\text{K}^+], \text{or } [\text{Cl}^-]}{\text{Ww g wt}} \right] \times 5.0 \text{ (mL)} \times 1000 \text{ (mL/mL)} \]

### 4.3. RNA isolation and quantitative RT-PCR assay

The relative gene expression of Na⁺ transport-related molecules in the brain cortex was determined by quantitative realtime PCR (qRT-PCR) assay as described previously (Matsushita et al., 2012). LightCycler FastStart DNA master and SYBR green I (Roche) were used for NCX1, NHE1, NKCC1, ATP1α1, ATP1α2, ATP1α3, MR, and GAPDH. We used the following primers:

- for rat NCX1: forward (F), 5'-TTC CCT CTA CGG TAA TCA GCA-3', reverse (R), 5'-ATT TCT GCA ATG CCG CTC T-3';
- for NHE1: F, 5'-TCT CCC TCT GGA TGC TTC TG-3', R, 5'-CAC CAG CCC CCC CAC TAC-3';
- for NKCC1: F, 5'-CTG TAT TCC ATC ATC AGG CAG CCA-3', R, 5'-TGT TGT TGT AGT ACT TGA ACG CAG GAC-3';
- for ATP1α1: F, 5'-AAG CTC ATC ATC AGT AGC CCA CGA-3', R, 5'-CCA CAT GTG TTG GTG TCT TAC-3';
- for ATP1α2: F, 5'-AAG TGC ACC CAC TCA AGG TG-3', R, 5'-CCT GGT CTT CCT TTT GTC-3';
- for ATP1α3: F, 5'-AAG GAG CAG CCT CTG AGT-3', R, 5'-GTT CCT CCG GCA GGT AGT AA-3';
- for MR: F, 5'-CCTTCCGCGCGGTGATG-3', R, 5'-GAGCCATCATTCGCCACACA-3';
- for GAPDH: F, 5'-TAC ACT GAG GAC CAG GGT G-3', R, 5'-CCC TGT TGC TGT AGT CAT A-3'.

### 4.4. Western blot analysis

Total protein in the tissues was measured with the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein (50 μg) was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline solution Tween 20 (T-TBS) and then incubated with anti-rabbit ATP1α3 antibody (Ab) (Millipore, CA, USA), anti-mouse MR Ab (Abcam, Tokyo, Japan), or anti-mouse β-actin Ab (Sigma, Steinheim, Germany) in T-TBS or Can Get signal immunoreaction enhancer solution (Toyobo, Osaka, Japan). After incubation with horse-radish peroxidase-conjugated secondary antibodies, signals were detected by chemiluminescence using an ECL prime Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK). Images were visualized using Image Quant Las 4000 mini (GE Healthcare, Tokyo, Japan) and analyzed with image software from Image J and quantified as the relative increase over the controls after normalization with β-actin.

### 4.5. Focal cerebral ischemia induction

MCAO was induced with a 4–0 or 4.5–0 monofilament suture as described previously (Longa et al., 1989; Yagi et al., 2009). Blood flow to the MCA region (rCBF) was measured intraoperatively with a laser Doppler flow probe (UNIQUE MEDICAL, Osaka, Japan) through the temporal bone. The baseline rCBF just before suture insertion was recorded. Rats whose rCBF after MCAO was reduced by less than 30% of the baseline were classified as successful MCAO. The suture was withdrawn after 90 min to allow reperfusion; the ipsilateral blood flow was restored to approximately 80–100% of the baseline value. The extent of the rCBF decrease and increase, respectively, before and after reperfusion, was identical in all rats with successful MCAO.

In approximately 10% of rats from each group, MCAO was judged to be unsuccessful. Unlike rats that manifested successful MCAO, they did not consistently display circling behavior, decreased resistance to lateral push, forelimb flexion, and shoulder adduction. These rats were excluded from further experiments by a blinded observer.

#### 4.5.1. Measurement of the infarct volume

Sliced brain tissues were immersed in a 2% 2,3,5-triphenyltetrazolium chloride solution in phosphate-buffered saline (PBS). The extent of ischemic infarction was traced and the integrated volume was calculated using NIH 1.36b Image J software. Artifact from brain edema was reduced by the indirect measurement method based on the contralateral brain volume (Hosomi et al., 2005). The infarct volume was calculated as a percentage of the contralateral hemisphere.

### 4.6. Statistical analysis

Sequentially obtained data (mean ± SD) were analyzed with the Student’s t-test for 2-group comparisons and analysis of variance (ANOVA) followed by Scheffe’s test for multiple comparisons. Statistical analyses were on a Windows computer running statistical software (StatView 5). Differences of \( p < 0.05 \) were considered statistically significant.
Acknowledgments

We thank Pfizer Pharmaceutical Company (Tokyo, Japan) for providing eplerenone. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (C26462159) and a Grant-in-Aid for the Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation from the Japan Society for the Promotion of Science (S2407).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2015.04.051.

References

Behr, G.A., et al., 2012. Increased blood oxidative stress in experimental menopause rat model: the effects of vitamin A low-dose supplementation upon antioxidant status in bilateral ovariecetomized rats. Fundam. Clin. Pharmacol. 26, 235–249.

Benfante, R., et al., 2005. The expression of the human neuronal alpha3 Na+,K+-ATPase subunit gene is regulated by the activity of the Sp1 and NF-Y transcription factors. Biochem. J. 386, 63–72.

Bottjer, P., et al., 2011. Distribution of Na/K-ATPase alpha 3 isoform, a sodium–potassium P-type pump associated with rapid-onset of dystonia parkinsonism (RDP) in the adult mouse brain. J. Comp. Neurol. 519, 376–404.

Brown, N.J., 2008. Aldosterone and vascular inflammation. Hypertension 51, 161–167.

Coffman, T.M., 2011. Under pressure: the search for the essential mechanisms of hypertension. Nat. Med. 17, 1402–1409.

Dinh, Q.N., et al., 2012. Aldosterone and the mineralocorticoid receptor in the cerebral circulation and stroke. Exp. Transl. Stroke Med. 4, 21.

Heer, M., et al., 2000. High dietary sodium chloride consumption may not induce body fluid retention in humans. Am. J. Physiol. Ren. Physiol. 278, F585–F595.

Hosomi, N., et al., 2005. Tumor necrosis factor-alpha neutralization reduced cerebral edema through inhibition of matrix metalloproteinase production after transient focal cerebral ischemia. J. Cereb. Blood Flow Metab. 25, 959–967.

Iwanami, J., et al., 2007. Pretreatment with eplerenone reduces stroke volume in mouse middle cerebral artery occlusion model. Eur. J. Pharmacol. 566, 153–159.

Kopp, C., et al., 2013. 23Na magnetic resonance imaging-determined tissue sodium in healthy subjects and hypertensive patients. Hypertension 61, 635–640.

Lai, M., et al., 2009. Mineralocorticoid receptor mRNA expression is increased in human hippocampus following brief cerebral ischaemia. Neuropathol. Appl. Neurobiol. 35, 156–164.

Li, X.Y., et al., 2012. High salt intake and stroke: meta-analysis of the epidemiologic evidence. CNS Neurosci. Ther. 18, 691–701.

Longa, E.Z., et al., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.

Matsushita, N., et al., 2012. Increase in body Na+/water ratio is associated with cerebral aneurysm formation in oophorectomized rats. Hypertension 60, 1309–1315.

Matyas, E., et al., 2011. Benefit assessment of salt reduction in patients with hypertension: systematic overview. J. Hypertens. 29, 821–828.

Miura, K., et al., 2010. Dietary salt intake and blood pressure in a representative Japanese population: baseline analyses of NIPPPON DATA80. J. Epidemiol. 20 (Suppl. 3), S524–S530.

Oyamada, N., et al., 2008. The role of mineralocorticoid receptor expression in brain remodeling after cerebral ischemia. Endocrinology 149, 3764–3777.

Palacios, J., et al., 2004. Estradiol-induced expression of Na(+)-K(+)-ATPase catalytic isoforms in rat arteries: gender differences in activity mediated by nitric oxide donors. Am. J. Physiol. Heart Circ. Physiol. 286, H1793–H1800.

Pimentel, V.C., et al., 2013. Hypoxia-ischemia alters nucleotide and nucleoside catabolism and Na+,K+-ATPase activity in the cerebral cortex of newborn rats. Neurochem. Res. 38, 886–894.

Queisser, N., Schupp, N., 2012. Aldosterone, oxidative stress, and NF-kappaB activation in hypertension-related cardiovascular and renal diseases. Free Radic. Biol. Med. 53, 314–327.

Rands, V.F., et al., 2012. Sexual dimorphism in urinary angiotensinogen excretion during chronic angiotensin II-salt hypertension. Gend. Med. 9, 207–218.

Rigsby, C.S., et al., 2007. Intact female stroke-prone hypertensive rats lack responsiveness to mineralocorticoid receptor antagonists. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R1754–R1763.

Sartori-Valinotti, J.C., et al., 2008. Sex differences in the pressor response to angiotensin II when the endogenous renin-angiotensin system is blocked. Hypertension 51, 1170–1176.

Shimada, K., et al., 2011. Activation of estrogen receptor-alpha and of angiotensin-converting enzyme 2 suppresses ischemic brain damage in oophorectomized rats. Hypertension 57, 1161–1166.

Simao, F.C., et al., 2013. Resveratrol prevents oxidative stress and inhibition of Na(+)+K(+)−ATPase activity induced by transient global cerebral ischemia in rats. J. Nutr. Biochem. 22, 921–928.

Stolarz-Skrzypek, K., et al., 2011. Fatal and nonfatal outcomes, incidence of hypertension, and blood pressure changes in relation to urinary sodium excretion. J. Am. Med. Assoc. 305, 1777–1785.

Strazzullo, P., et al., 2009. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. Br. Med. J. 339, b4567.

Tada, Y., et al., 2009. Role of mineralocorticoid receptor on experimental cerebral aneurysms in rats. Hypertension 54, 552–557.

Thrift, A.G., et al., 2011. Gender-specific effects of caste and salt on hypertension in poverty: a population-based study. J. Hypertens. 29, 443–450.

Titze, J., et al., 2003. Osmotically inactive skin Na+ storage in rats. Am. J. Physiol. Ren. Physiol. 285, F1108–F1117.

Titze, J., et al., 2004. Glycosaminoglycan polymerization may enable osmotically inactive Na+ storage in the skin. Am. J. Physiol. Heart Circ. Physiol. 287, H203–H208.

Turtzo, L.C., McCullough, L.D., 2008. Sex differences in stroke. Cerebrovasc. Dis. 26, 462–474.

Wetterling, F., et al., 2012. Regional and temporal variations in tissue sodium concentration during the acute stroke phase. Magn. Reson. Med. 67, 740–749.

Yagi, K., et al., 2005. Edaravone, a free radical scavenger, inhibits MMP-9-related brain hemorrhage in rats treated with tissue plasminogen activator. Stroke 40, 626–631.

Zamfir, A.D., et al., 2012. Brain chondroitin/dermatan sulfate from cerebral tissue to fine structure: extraction, preparation, and fully automated chip-electrospray mass spectrometric analysis. Methods Mol. Biol. 836, 145–159.