Increase in Body Na+/Water Ratio Is Associated With Cerebral Aneurysm Formation in Oophorectomized Rats

Nobuhisa Matsushita, Keiko T. Kitazato, Yoshiteru Tada, Manabu Sumiyoshi, Kenji Shimada, Kenji Yagi, Yasuhisa Kanematsu, Junichiro Satomi, Shinji Nagahiro

Abstract—The incidence of cerebral aneurysms is higher in women than in men, especially postmenopause. Although hypertension is thought to be associated with a high incidence of stroke, not all patients with unruptured cerebral aneurysms are hypertensive. The possibility of water-free Na+ storage associated with hypertension has been raised. However, whether the increase in the body Na+/water ratio that characterizes water-free Na+ accumulation is associated with the formation of cerebral aneurysms remains obscure. To examine this relationship, Sprague-Dawley female rats subjected to carotid artery ligation were divided into 3 groups: a high-salt diet group (HSD) without and another with bilateral oophorectomy (HSD/OVX) and a third group that underwent additional renal artery ligation (HSD/OVX/RL). Compared with rats receiving a normal diet (shams), water retention was increased in HSD rats but not in HSD/OVX rats. Interestingly, compared with HSD rats, the incidence of cerebral aneurysms and the body Na+/water ratio were significantly higher in HSD/OVX and HSD/OVX/RL rats, independent of hypertension. In their aneurysmal wall, ATP1c2, a subtype of Na+/K+-ATPase, was downregulated, whereas inflammatory-related molecules were upregulated. Treatment with low-dose olmesartan that did not affect the blood pressure in hypertensive HSD/OVX/RL rats reduced the rate of cerebral aneurysm formation, body Na+ retention, and the Na+/water ratio and upregulated ATP1c2. These results suggest that the increase in the Na+/water ratio and a reduction in ATP1c2 may be associated with cerebral aneurysm formation. We provide the new insight that the management of water-free Na+ is important to prevent their development. (Hypertension. 2012;60:1309-1315.)

Key Words: cerebral aneurysm ■ sodium retention ■ hypertension ■ oophorectomy ■ renin-angiotensin system ■ Na+/K+-ATPase

Cerebral aneurysms are a major cause of subarachnoid hemorrhage. Despite the catastrophic consequences of aneurysmal rupture, not all mechanisms underlying the formation, progression, and rupture of cerebral aneurysms are fully understood. Hypertension is thought to be associated with excessive salt intake, and stroke1 and epidemiological studies have shown that the incidence of aneurysmal subarachnoid hemorrhage is higher in postmenopausal than in premenopausal women and higher than in men and that aneurysmal walls are exposed to high shear stress.2 On the basis of these findings, we established an aneurysm rat model in which the animals were subjected to oophorectomy (OVX).3 Using these rats, we demonstrated that, in the aneurysmal wall, the renin-angiotensin system (RAS) was upregulated and estrogen receptor (ER)-α was downregulated and that several pharmacological treatments including hormone replacement and antihypertensive drug therapy effectively reduced the incidence of cerebral aneurysms via their antioxidative and anti-inflammatory effects.4-8 Furthermore, our group reported that the saline intake and the incidence and growth of aneurysms were reduced in rats treated with the mineralocorticoid receptor antagonist eplerenone and that their blood pressure was not affected. In contrast, compared with rats with experimental hypertension, rats treated with the mineralocorticoid receptor agonist deoxycorticosterone acetate and saline manifested high saline intake and low blood pressure despite the same incidence of cerebral aneurysms. Titze et al9 reported that in deoxycorticosterone acetate and saline rats the total body Na+ content was increased by 40% to 50% within 5 weeks and that an increase in Na+ accumulation by ≈20% resulted in water-volume retention. The remaining 75% to 80% of Na+ was retained in abundance over water. This suggests that deoxycorticosterone acetate treatment results in an internal Na+ escape achieved by osmotically inactive storage as evidenced by the concomitant increase in the tissue Na+/water and the Na+K+/water ratios. On the basis of our findings and those reported by others, we hypothesized that the water-free accumulation of Na+, characterized by an increase in the Na+/water ratio, is associated with the incidence of cerebral aneurysms without inducing hypertension.
The physiological and pathophysiological stimulation of Na⁺ transporters or the Na⁺ pump because of high-salt loading is thought to be related to the retention of Na⁺ in the heart, brain, and blood vessels and to contribute to the vascular remodeling process. Some studies suggested that the increase in the Na⁺ influx molecules Na⁺-K⁺-2Cl⁻ exchanger, Na⁺-Ca²⁺ exchanger, and Na⁺-H⁺ exchanger contributes to this remodeling process and to cardiac hypertrophy. In contrast, the Na⁺ efflux pump Na⁺/K⁺-ATPase plays a key role in cellular osmotic regulation via the maintenance of the transmembrane gradients of Na⁺ and K⁺ and contributes to the regulation of vascular contractility, which has been reported to be impaired in smokers. To examine the relationship between body Na⁺/water ratio during high-salt intake and the formation of cerebral aneurysms in rats, we focused on the molecules related to cellular Na⁺ regulation.

Here, we provide new evidence that in OVX rats fed a high-salt diet, the increase in the body Na⁺/water ratio is associated with cerebral aneurysm formation, irrespective of hypertension. We also demonstrate an association between the down-regulation of vascular Na⁺/K⁺-ATPase and increase in the body Na⁺/water ratio. Our findings suggest that the dysfunction of the vascular Na⁺ efflux pump in rats manifesting a high Na⁺/water ratio is associated with the promotion of aneurysm formation.

Materials and Methods

Induction of Experimental Cerebral Aneurysms

We modified the protocol of Jamous et al. to induce cerebral aneurysms in rats.

Experimental Study 1

We first subjected 13-week-old female Sprague-Dawley rats (n=128) to right common carotid artery ligation to induce hemodynamic stress on the left anterior cerebral artery-olfactory artery bifurcation. The rats were fed a high-salt diet (8.0% sodium chloride) and divided into 3 groups (each group, n=28) without and with bilateral oophorectomy (HSD and HSD/OVX rats) and rats subjected to additive bilateral posterior renal artery ligation (HSD/OVX/RL rats) to induce hypertension. These groups were compared with sham rats (n=28) that received a normal diet (0.3% sodium chloride).

Experimental Study 2

Another set of HSD/OVX/RL rats (n=56) was randomized into 2 equal groups: one group served as the vehicle control and the other was treated with olmesartan, an angiotensin II type 1 receptor blocker (0.3 mg/kg per day, p.o.). We chose the angiotensin receptor (ATR) blocker dose based on our earlier findings that olmesartan at 0.3 mg/kg was neuroprotective without affecting the blood pressure.

On the day of assessment, blood pressure measurements were taken in the morning and evening, and there was no remarkable difference between the morning and evening values. All rats were housed individually in metabolic cages (Shinano, Tokyo, Japan), and their intake of food and water (provided in 500-mL bottles) and their urine volume were recorded. Their body water, Na⁺ and K⁺ retention, and the Na⁺/water ratio were calculated from their food and water intake, the urinary Na⁺ and K⁺ concentration, and the urine volume using the formulas:

- Water retention (L/kg) = food intake (g/kg) × concentration of salt (%)/58.5 (g/mol) − urine volume (L/kg) × urinary Na⁺ concentration (mol/L)
- K⁺ retention (mol/kg) = food intake (g/kg) × concentration of K⁺/39.1 (g/mol) − urine volume (L/kg) × urinary K⁺ concentration (mol/L)

For a detailed description, see the online-only Data Supplement.

Results

Excessive Salt Intake Facilitates Cerebral Aneurysm Formation Without Affecting the Blood Pressure, Especially in OVX Rats

Cerebral aneurysms frequently arise at the anterior cerebral artery-olfactory artery bifurcation. As shown in Figure 1A, in 6 of 20 HSD rats, we observed the formation of cerebral aneurysms at that site. Significantly, more HSD/OVX (16 of 20) than HSD rats developed these aneurysms (P<0.01), although their blood pressure was not affected (Figure 1B). Although the incidence (Figure 1A) and size (Figure S1 in the online-only Data Supplement) of the aneurysms were not markedly different between HSD/OVX and HSD/OVX/RL rats, some HSD/OVX/RL rats suffered aneurysmal rupture.

In preliminary studies, the incidence of cerebral aneurysms was moderately increased in rats subjected to renal hypertension alone (RL rats), the combination of RL and OVX (RL/OVX rats), or RL plus HSD (RL/HSD rats); it was similar to the incidence in HSD rats despite differences in the blood pressure (Figure S2A and S2B). These observations suggest that, in the presence of excessive salt intake, the formation of cerebral aneurysms is affected more by OVX than by hypertension.

Cerebral Aneurysm Formation Is Strongly Associated With the Increase in the Body Na⁺/Water Ratio

To further examine the relationship between cerebral aneurysm formation and excessive salt intake, we assessed plasma and urinary biomarkers related to the regulation of Na⁺. In HSD but not in HSD/OVX and HSD/OVX/RL rats, body water retention was significantly increased compared with sham rats fed a normal diet (Figure 2A and Table S1 in the online-only Data Supplement). Body Na⁺ retention was higher in rats fed a high-salt diet than in sham rats fed a normal diet (Figure 2B). These findings suggest that body Na⁺ retention is not necessarily correlated with body fluid retention. More importantly, the increase in the Na⁺/water ratio was significantly higher in HSD/OVX than in HSD rats (Figure 2C). The body Na⁺/water ratio was similar in HSD and HSD/RL rats (data not shown) and lower than in HSD/OVX rats, suggesting that it is affected by OVX rather than by hypertension. In contrast, body K⁺ retention was not significantly different among all groups of rats (Figure S3A). The K⁺/water ratio was significantly lower in rats fed a high-salt diet than in sham rats (Figure S3B). Interestingly, the Na⁺/K⁺/water ratio was different between sham and HSD rats, although it was significantly increased in HSD/OVX and HSD/OVX/RL rats (Figure S3C). Because osmotically inactive Na⁺ retention is characterized by
In increase in Na⁺/Water Ratio on Cerebral Aneurysms

A simultaneous change in the Na⁺+K⁺/water ratio,⁹ the increase in the Na⁺/water ratio in HSD/OVX and HSD/OVX/RL rats may include partly osmotically inactive Na⁺. Plasma renin activity and the level of angiotensin II were significantly lower in rats fed a high-salt diet than in sham rats; there was no difference in the aldosterone level (Figure S4).

Increase in the Na⁺/Water Ratio Is Associated With the Activation of RAS and the Downregulation of the Na⁺ Efflux Pump in the Aneurysmal Wall

In the aneurysmal wall, there is an association with RAS activation and inflammatory changes.⁶ Therefore, we first examined molecules involved in these processes. In HSD rats, ATR1b was increased (Figure 3A); ATR1a was not affected (data not shown). In HSD/OVX rats, the mRNA level of ATR2 (Figure 3A) and angiotensin converting enzyme 2, but not of angiotensin-converting enzyme, was decreased (Figure 3B). Furthermore, in HSD/OVX/RL rats, we observed the augmented downregulation of angiotensin-converting enzyme 2 and the upregulation of angiotensin-converting enzyme. In HSD/OVX but not in HSD rats, the level of ERα mRNA was significantly decreased; it was even lower in HSD/OVX/RL rats (Figure 3C); ERβ and GPR30 were not affected (data not shown). The level of tumor necrosis factor-α mRNA was significantly increased in HSD/OVX and HSD/OVX/RL but not in HSD rats (Figure 3D). Immunohistochemically, the vascular expression of angiotensin II and tumor necrosis factor-α was increased in HSD/OVX and HSD/OVX/RL but not in HSD rats (Figure S5). These findings suggest that RAS activation and inflammation in the aneurysmal wall were associated with an increased incidence of cerebral aneurysms and the increase in the Na⁺/water ratio.

We then addressed the role of Na⁺ transport-related molecules in the vascular wall to examine their association with the increase in the Na⁺/water ratio. There was no difference among our rats with respect to the mRNA level of Na⁺-Ca²⁺ exchanger, Na⁺-H⁺ exchanger, and Na⁺-K⁺-2Cl⁻ exchanger related to Na⁺ influx into the vascular wall (data not shown). In contrast, the mRNA level of ATP1α2, a subtype of the sodium efflux pump Na⁺/K⁺-ATPase, was significantly lower in HSD/OVX than in HSD rats and the sham rats (Figure 4A). There was no significant difference in the other subtypes of Na⁺/K⁺-ATPase (Figure S6). The protein expression of ATP1α2 was decreased in HSD/OVX compared with sham rats (Figure 4B and 4C). In sham rats, ATP1α2-positive cells were found among CD31-positive endothelial and

Figure 1. Experimental cerebral aneurysms and systolic blood pressure (experimental study 1). A, Incidence of cerebral aneurysms. Rats fed a normal diet (sham), rats fed a high-salt diet (HSD) without and with bilateral oophorectomy (HSD/OVX), and rats subjected to additive bilateral posterior renal artery ligation (HSD/OVX/RL) to induce hypertension were used. Vascular corrosion casts were inspected at the bifurcation of the anterior cerebral artery-olfactory artery (ACA-OA). †P<0.05 vs HSD rats by Fisher exact test (each group, n=20). B, Average systolic blood pressure (3 measurements using the tail cuff method) recorded once a month. *P<0.05 vs sham; †P<0.05 vs HSD rats; ††P<0.05 vs HSD/OVX rats by ANOVA followed by Scheffe test (n=20, mean±SD).

Figure 2. A, Water retention, (B) Na⁺ retention, and (C) Na⁺/water ratio. They were calculated as described in Materials and Methods. *P<0.05 vs sham and †P<0.05 vs high-salt diet (HSD) rats by ANOVA followed by Scheffe test (n=28, mean±SD). HSD indicates high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation.
α-actin–positive smooth muscle cells (Figure S7). These results suggest a relationship between the downregulation of ATP1α2 and the accumulation of Na+ in the wall of cerebral aneurysms.

Olmesartan-Induced Decrease in the Na+/Water Ratio May Contribute, in Part, to the Inhibition of Aneurysm Formation

Lastly, we tested whether, at low doses, the angiotensin II type I receptor blocker antagonist olmesartan inhibits the formation of cerebral aneurysms and whether this is associated with the reduction in the Na+/water ratio, irrespective of the antihypertensive effects of the drug. Fukuda et al13 documented that, in humans, olmesartan increased natriuresis and reduced the glomerular filtration rate, irrespective of its antihypertensive effects. As shown in Figure 5A, in HSD/OVX/RL rats treated with olmesartan at 0.3 mg/kg per day, the formation of cerebral aneurysms was significantly decreased without affecting the blood pressure (Figure S8) and ATP1α2 was upregulated (Figure 5B). Immunohistochemically, the reduced expression of ATP1α2 in HSD/OVX/RL rats was reversed by olmesartan (Figure 5C). Notably, in olmesartan-treated compared with vehicle control rats, the Na+/water ratio was significantly decreased (Figure 5D). Water retention was not affected, whereas body Na+ retention and the Na+/K+ ratio were decreased; K+ retention was not affected (Figure S9A through S9D). In addition to blocking ATR1, the olmesartan-induced decrease in the Na+/water ratio, together with the upregulation of ATP1α2, may contribute importantly to the inhibition of cerebral aneurysm formation independent of the blood pressure.

Discussion

Studies on the long-term balance of Na+ in humans ingesting a high-salt diet suggested its storage in water-free form.14 Kopp et al,15 who performed 23Na magnetic resonance imaging studies of tissue sodium recently demonstrated Na+ storage in patients with hyperaldosteronism. According to Coffman,16 in these individuals, Na+ may accumulate in the subdermal interstitium, and tissues at hypertonic concentrations and its storage may be mediated by its interaction with glycosaminoglycans and proteoglycans. Glycosaminoglycans sulfation and polymerization have been reported to be linked with additional macrophage-dependent mechanisms for interstitial electrolyte clearance through the lymph capillary network.17 Our investigation is the first to demonstrate that the increased rate of aneurysm formation in OVX rats fed a high-salt diet was associated with an increase in the Na+/water ratio, irrespective of the blood pressure. The increased incidence of cerebral aneurysm formation was also associated with RAS activation, inflammatory changes, and the downregulation of the Na+ efflux pump ATP1α2 in the aneurysmal wall. Interestingly, in HSD/OVX/RL rats treated with olmesartan at a dose that did not affect the blood pressure, cerebral aneurysm formation and the Na+/water ratio were suppressed and ATP1α2 was upregulated. At present, we do not know whether this is a cause-and-effect phenomenon, although
our study yields the new insight that the Na\(^+/\)water ratio is an independent risk factor for the formation of cerebral aneurysms.

Because the retention of Na\(^+\) is thought to be accompanied by a commensurate retention of water to maintain iso-osmolarity, a proportional expansion of the intravascular volume can be expected. Titze,\(^8\) who suggested that Na\(^+\) handling during increased dietary salt intake is more complex, highlighting the dynamic role of the dermal interstitium as a reservoir that can buffer the impact of Na\(^+\) accumulation on the intravascular volume and blood pressure, proposed that accumulated water-free Na\(^+\) is associated with hypertension. However, the relationship between vascular injury and water-free Na\(^+\) remains to be addressed. Although differences in renal Na\(^+\)/water handling do not prove the presence of Na\(^+\) storage in the body, especially in the vascular wall, our finding that the increased Na\(^+\)/water ratio is associated with the formation of cerebral aneurysms is supported by the concept that water-free Na\(^+\) accumulation may exert deleterious effects.

Titze et al\(^9\) reported that Sprague-Dawley rats are not a salt-sensitive strain because they manifest a high ratio of osmotically inactive/active Na\(^+\) retention compared with Dahl salt-sensitive rats. This may partly explain why the blood pressure was not affected in our HSD/OVX rats.

Perry and Beevers\(^10\) found that a high-salt diet can increase the risk of stroke in humans independent of its effect on the blood pressure. Although there are studies reporting that excessive salt intake raises the risk for cardiovascular events, the putative benefits from a low-salt diet have not been established by systematic investigations.\(^11\) Because lower levels of urinary Na\(^+\) excretion were predictive of mortality rates from cardiovascular disease,\(^12\) the regulation of Na\(^+\) accumulation appears to be important.

Sodium overloading transforms the state of endothelial cells from Na\(^+\) releasing to Na\(^+\) absorbing.\(^13\) This attenuates Na\(^+\)/K\(^-\) ATPase, leading to an increase in Na\(^+\) in the subplasma membrane of arterial smooth muscle.\(^14\) Na\(^+\)/K\(^-\) ATPase is a heteromeric enzyme composed of \(\alpha\) and \(\beta\)-polypeptide subunits.\(^15\) The \(\alpha\)-isoform is expressed in nearly all tissues: \(\alpha\)-2 is found in skeletal muscle, smooth muscle, heart, and brain (glial cells) and \(\alpha\)-3 in the brain (neurons) and ovaries. In our study, the Na\(^+\)/K\(^-\)-ATPase-\(\alpha\)-2 efflux pump was downregulated in HSD/OVX and HSD/OVX/RL rats. Because estrogen increases Na\(^+\)/K\(^-\)-ATPase,\(^16\) the downregulation that we observed may have been partly affected by hormones. Furthermore, consistent with our previous data,\(^7\) the downregulation of ER\(\alpha\) was observed in HSD/OVX rats, suggesting the partial ER\(\alpha\)-dependent effects on increased aneurysm formation.

The incidence of aneurysms and the Na\(^+\)/water ratio were decreased, and ATP1a2 was increased in rats treated with olmesartan at a dose that did not affect the blood pressure. A study indicating the suppression of tubular sodium reabsorption by olmesartan supports our results.\(^13\) Although we cannot rule out other mechanisms underlying the protective effects of olmesartan on the vascular wall, the reduction in the Na\(^+\)/water ratio with upregulation of the Na efflux pump in addition to RAS inhibition may have played a role in the observed suppression of cerebral aneurysm formation in our rats.

**Perspectives**

We here first demonstrated that the increased rate of cerebral aneurysm formation in oophorectomized rats fed a high-salt diet is associated with the increase in the Na\(^+\)/water ratio independent of hypertension.

Our study has some limitations. Ours was an experimental model, and we applied selected conditions. We do not know whether, and by how much, the amount of water-free Na\(^+\) was increased in the vascular wall of our rats, and our current findings do not prove a functional relationship between differences in renal electrolyte handling and aneurysm formation. We are pursuing studies using new methods\(^15\) to determine Na\(^+\) content directly.

Our study alerts to the importance of suppressing the accumulation of water-free Na\(^+\) because of excessive salt intake even under normotensive conditions. Especially, postmenopausal women should be warned of the accumulation of water-free Na\(^+\) and of the dangers of excessive salt intake.

---

**Figure 4.** mRNA level and protein expression of ATP1α2, an Na\(^+\)/K\(^-\)-ATPase subtype. **A**, The mRNA level was measured by quantitative real-time polymerase chain reaction and normalized by the GAPDH mRNA level. *P<0.05 vs sham and †P<0.05 vs high-salt diet (HSD) rats by ANOVA followed by Scheffe test (n=8, mean±SD). **B**, Total protein in tissue was measured by Western blot analysis, and images were analyzed with image software from Image J and quantified as the relative increase over the controls after normalization with β-actin. *P<0.05 vs sham rats by Student t test (n=6, mean±SD). **C**, Elastic van Gieson (EVG) and immunohistochemical staining for ATP1α2. HSD indicates high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation.
None.

Acknowledgment
We thank Daiichi Sankyo Pharmaceutical Company (Tokyo, Japan) for providing olmesartan.

Sources of Funding
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

Disclosures
None.

References
What Is New?

• We provide new evidence that, in oophorectomized rats fed a high-salt diet, the body Na+/water ratio is markedly increased irrespective of hypertension.

What Is Relevant?

• The increase in the Na+/water ratio with the downregulation of the Na+ efflux pump in the vascular wall was associated with the increased incidence of cerebral aneurysms.

Novelty and Significance

- Water-free Na+ accumulation may play an essential role in the formation of cerebral aneurysms. We alert to the importance of suppressing the accumulation of water-free Na+ even under normotensive conditions to prevent their development.

Summary
Increase in Body Na⁺/Water Ratio Is Associated With Cerebral Aneurysm Formation in Oophorectomized Rats

Nobuhisa Matsushita, Keiko T. Kitazato, Yoshiteru Tada, Manabu Sumiyoshi, Kenji Shimada, Kenji Yagi, Yasuhisa Kanematsu, Junichiro Satomi and Shinji Nagahiro

_Hypertension_. 2012;60:1309-1315; originally published online October 8, 2012;
doi: 10.1161/HYPERTENSIONAHA.112.198762

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/60/5/1309

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/10/08/HYPERTENSIONAHA.112.198762.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

INCREASE IN BODY $\text{Na}^+$-TO-WATER RATIO IS ASSOCIATED WITH CEREBRAL ANEURYSM FORMATION IN OOPHORECTOMIZED RATS

Nobuhisa Matsushita, Keiko T. Kitazato, Yoshiteru Tada, Manabu Sumiyoshi, Kenji Shimada, Kenji Yagi, Yasuhisa Kanematsu, Junichiro Satomi, Shinji Nagahiro

Department of Neurosurgery, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima City, Japan

Short title: Increase in $\text{Na}^+$/water ratio on cerebral aneurysms

Correspondence to: Nobuhisa Matsushita, MD and Keiko T Kitazato, MS
Department of Neurosurgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima 770-8503, JAPAN,
Phone :+81-88-633-7149, Fax :+81-88-632-9464. E-mail :nobuhisama@gmail.com and kitazato1117@yahoo.co.jp
Materials and Methods

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) Guide for the Care and Use of Laboratory Animals.

Before any procedures, all were anesthetized with 5% pentobarbital administered intraperitoneally. The estrogen level in oophorectomized rats was significantly lower than in the sham rats (13.6 ± 3.2 vs. 32.1 ± 11.2 pg/ml, p < 0.01). All rats were killed 12 weeks after the last procedure.

**Experimental study 1.**

We first subjected 13-week-old female Sprague-Dawley rats (n=128) to right common carotid artery ligation to induce hemodynamic stress on the left anterior cerebral artery-olfactory artery (ACA-OA) bifurcation. The rats were fed a high-salt diet (8% sodium chloride) and divided into 3 groups (each group n=28) without and with bilateral oophorectomy (HSD-, HSD/OVX rats), and rats subjected to additive bilateral posterior renal artery ligation (HSD/OVX/RL rats) to induce hypertension. These groups were compared with sham rats (n=28) that received a normal diet (0.3% sodium chloride). OVX was performed immediately after right common carotid artery ligation; additional RL was carried out 2 weeks after OVX to induce hypertension. We used 20 rats from each group to prepare vascular corrosion casts and 8 rats for PCR assay. Another 8 sham- and 8 HSD/OVX rats were used for Western blot analysis and immunohistochemical studies.

**Experimental study 2.**

Another set of HSD/OVX/RL rats (n=56) was randomized into 2 equal groups; one group served as the vehicle control (VC) and the other was treated with olmesartan, an angiotensin II (Ang II) type I receptor blocker (ARB, 0.3 mg/kg/day, perorally). We used 20 rats from each group to prepare vascular corrosion casts and the other 8 in each group for PCR assay. ARB treatment was started one month after RL and continued for 2 months. We chose the ARB dose based on our earlier findings that olmesartan at 0.3 mg/kg was neuroprotective without affecting the blood pressure.

**Supplemental study**

In preliminary study, we confirmed the effect of hypertension on the cerebral aneurysm formation. We first subjected 13-week-old female Sprague-Dawley rats (n=60) to right common carotid artery ligation to induce hemodynamic stress on the left ACA-OA bifurcation. Next, we subjected to bilateral posterior renal artery ligation to induce hypertension. They
were divided into 3 groups (n=20, in each group); one was undergone with (RL/OVX rats) and without (RL rats) bilateral oophorectomy and the 3rd group was fed a high-salt diet (RL/HSD rats). The procedure was performed as mentioned above. All experimental groups were compared with HSD rats of experimental study 1 (Figure S1 and Table S2).

To obtain their blood pressure, once a month unanesthetized rats were placed on a 37°C hot plate (NISSIN, Tokyo, Japan) and covered with a black blanket. After acclimatization, one blinded examiner recorded their average blood pressure based on 3 measurements obtained with the tail-cuff method (Softron, Tokyo, Japan). On the day of assessment, blood pressure measurements were taken in the morning and evening, there was no remarkable difference between the morning- and evening values. All rats were housed individually in metabolic cages (SHINANO, Tokyo, Japan) and their intake of food- and water (provided in 500-ml bottles) and their urine volume were recorded. Twelve weeks after the last procedure, urine and blood were withdrawn. Urine and plasma samples were stored at -80°C until use. The urinary and plasma levels of Na⁺ and K⁺ were determined by the electrode method, the plasma aldosterone level, renin activity, and the Ang II concentration by radioimmunoassay (SRL, Tokyo, Japan). Their body water, Na⁺ and K⁺ retention, and the Na⁺-to-water ratio were calculated from their food and water intake, the urinary Na⁺ and K⁺ concentration, and the urine volume using the formulae:

- Water retention (L/kg) = water intake(L/kg) - urine volume (L/kg)
- Na⁺ retention (mol/kg) = food intake (g/kg) x concentration of salt (%) / 58.5 (g/mol) - urine volume (L/kg) x urinary Na⁺ concentration (mol/L)
- K⁺ retention (mol/kg) = food intake (g/kg) x 0.9 (%) / 39.1 (g/mol) - urine volume (L/kg) x urinary K⁺ concentration (mol/L)

Preparation of vascular corrosion casts

Vascular corrosion casts were prepared as previously described. The rats were transcardially perfused with heparinized phosphate-buffered saline (PBS, 20 U/ml) and then Batson’s No. 17 plastic (Polyscience Inc., Warrington, PA, USA). The left ACA-OA bifurcation on the casts was inspected at 3 kV under a scanning electron microscope (VE8800, Keyence, Osaka, Japan). Based on our morphological findings we classified the vascular wall surface as normal, i.e. demonstrating normal endothelial cell imprints, as exhibiting endothelial damage, i.e. demonstrating irregularly-shaped cell imprints, and as harboring a cerebral aneurysm evidenced by changes such as moderate outward evagination or an obvious saccular aneurysm (Figure 1A). Three blinded examiners graded the aneurysmal changes by consensus. To determine the aneurysmal size, three blinded readers
manually outlined regions of interest corresponding to areas with aneurysmal involvement on each image using National Institutes of Health (Bethesda, MD) ImageJ software.

RNA isolation and quantitative RT-PCR

Samples from sham- (n=8), HSD- (n=8), HSD/OVX- (n=8), HSD/OVX/RL- (n=8), vehicle control treated HSD/OVX/RL- (n=8), and olmesartan-treated HSD/OVX/RL rats (n=8) were subjected to quantitative real-time PCR (qRT-PCR). At 12 weeks after RL, the rats were euthanized as described above. The left ACA-OA bifurcation was isolated and total RNA was extracted with the EZ1 RNA Universal Tissue Kit (QIAGEN, Tokyo, Japan) and placed in a MagNA lyser (Roche, Tokyo, Japan). For reverse transcription of total RNA to cDNA we used the transcriptor first-strand cDNA synthesis kit (Roche). qRT-PCR of each sample was in a LightCycler 2.0 instrument (Roche Diagnostics, Tokyo, Japan). LightCycler FastStart DNA master and SYBR green I (Roche) were used for ERα, ERβ, GPR30, NCX1, NHE1, NKCC1, ATP1α1, ATP1α2, ATP1α3, ACE, ACE2, ATR1a, ATR1b, ATR2, TNFα, and GAPDH. We used the following primers: for rat ERα, forward primer (F), 5’-TGC ACC ATC GAT AAG AAC C-3’, reverse primer (R), 5’-GTC TCC TGA AGT GCC CAT T-3’ (177 bp); for ERβ, F, 5’-CTG CAT GGC TGA GCG ACA A-3’; R, 5’-AGA GAC TCA TGG GAC TCA GAT-3’ (133 bp); for G-protein coupled receptor 30 (GPR30), F, 5’-CTC AAC CGC TTC TGC CAT-3’, R, 5’-CCA CCT GTG CTA GAG TGT GC-3’ (158 bp); for NCX1, F, 5’-TTC CCT CTA CCG TAA TCA TCA GCA-3’, R, 5’-ATT TCT GCA ATG CGT CA-3’ (91 bp); for NHE1, F, 5’-CTC CCC TCT GGA TTC TCC TG-3’, R, 5’-CAC CAG CAG CCC CAC TAC-3’ (113 bp); for NKCC1, F, 5’-CTG TAT TCC ATG CGT CA-3’, R, 5’-GTT TGT TAT GGT ACT TGA ACG CAC GAC G-3’ (63 bp); for ATP1α1, F, 5’-AAG CTC ATC ATC AGG CGA CGA-3’; R, 5’-CCA CAT GCT TTG GTG ATG CT-3’ (181 bp); for ATP1α2, F, 5’-GAA TGT ACC CAC TCA AGG TC-3’, R, 5’-CCT GTT CTG TTC TTT GTC C-3’ (162 bp); for ATP1α3, F, 5’-AAG CAG GAG CCT CTG ATG-3’, R, 5’-GTT CCT CCG GCA GGT AGT AA-3’ (115 bp), for ACE-2, F, 5’-CAG ACG TAT GGG TGA GTG AT-3’, R, 5’-GTT GGC TTA AGT GTT GGG TA-3’ (199 bp), for ATR1a, F, 5’-AGC GCA GCC TCT GAC TAA ATG-3’, R, 5’-AGA CGG CTT TGG TGG GTT ACT-3’ (145 bp), for ATR1b, F, 5’-GAC AGC AGA AGC CAG AGG AC-3’, R, 5’-CCC TTA AAG GAT TGC TGG AG-3’ (84 bp), for ATR2, F, 5’-TGT CTG TCC TAA TGG CCA ACA-3’, R, 5’-TTC ATT AAG GCA ATC CCA GCA GCA-3’ (131 bp), and for GAPDH, F, 5’-TAC ACT GAG GAC CAG GTT G-3’, R, 5’-CCC TGT TGC TGT AGC CAT A-3’ (145 bp). Primers and probe sets for ACE were from Roche and used according to the manufacturer’s directions. The amplified product was separated on 1.5% agarose gels containing EtBr solution (Wako, Osaka,
Japan) and visualized on an ultraviolet transilluminator. The results were quantified after normalization to the expression of GAPDH mRNA. The PCR conditions were 95°C for 10 min followed by 40 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 8 sec. We subjected samples from each group to 2 independent qRT-PCR assays. GAPDH was the internal control.

**Western blot analysis**

Total protein in the tissues was measured with the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein (100 µg) was separated by 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 2% skim milk in Tris-buffered saline solution Tween 20 (T-TBS) and then incubated with primary antibodies against ATP1α2 (Millipore, USA) and β-actin (Sigma, Steinheim, Germany) in T-TBS or Can Get signal Immunoreaction Enhancer Solution (Toyobo). After incubation with horseradish peroxidase-conjugated secondary antibodies, signals were detected by chemiluminescence using an ECL kit (GE Healthcare, Buckinghamshire, UK). Images were analyzed with image software from Image J and quantified as the relative increase over the controls after normalization with β-actin.

**Immunohistochemical Studies**

After perfusion with 4% paraformaldehyde, we inspected the arteries of the circle of Willis under a dissecting microscope. The left ACA-OA bifurcation was carefully dissected and immersed in 4%-paraformaldehyde. The arteries were rinsed with PBS, embedded in OCT compound (Tissue-Tek, Inc.) and 6-mm-thick serial sections from the ACA-OA bifurcation were cut with a cryotome (CM 1850; Leica). After 30-min serum-free protein blockade (Dako, Carpinteria, CA, USA), primary antibodies diluted with Canget signal immunostain (Toyobo, Osaka, Japan) were added for 1-hr incubation at room temperature (RT) or overnight at 4°C. Primary antibodies against ERα, Ang II, TNFα (Santa Cruz Biotechnology, CA, USA), ATP1α2 (Millipore, USA), CD31 (Chemicon, USA), and α-actin (Abcam, USA) were applied. Sections were then incubated for 1 hr at RT with the fluorescein-conjugated secondary antibodies Alexa Fluor 488 or 594 (Molecular Probes, CA, USA) in Canget signal immunostain (Toyobo), mounted with Vectashield (Vector Laboratories, CA, USA), and examined under a fluorescence microscope (Olympus IX71, Tokyo, Japan).
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. The incidence of cerebral aneurysmal changes was analyzed by the Fisher’s exact test. Statistical analyses were performed on a Windows computer running statistical software (StatView 5). Differences were considered statistically significant at p<0.05.
References


### Table S1  Characteristics in female rats fed a high salt diet

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sham</th>
<th>HSD</th>
<th>HSD/OVX</th>
<th>HSD/OVX/RL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>311.6±17.9</td>
<td>316.6±30.9</td>
<td>379.0±15.3*†</td>
<td>394.8±27.0*†</td>
</tr>
<tr>
<td>Food intake (g/kg)</td>
<td>46.4±1.2</td>
<td>47.4±4.9</td>
<td>52.0±15.8</td>
<td>52.1±8.0</td>
</tr>
<tr>
<td>Water intake (ml/kg)</td>
<td>80.2±5.2†</td>
<td>220.3±25.2*</td>
<td>207.3±22.4*</td>
<td>270.9±12.6*†</td>
</tr>
<tr>
<td>Urine Volume (ml/kg)</td>
<td>75.1±9.0†</td>
<td>178.7±39.1*</td>
<td>185.6±16.6*</td>
<td>253.5±14.7*†</td>
</tr>
<tr>
<td>Urinary Na (mmol/L)</td>
<td>49±24.6†</td>
<td>231±58.1*</td>
<td>202.5±16.5*</td>
<td>154.6±44.3*†</td>
</tr>
<tr>
<td>Urinary K (mmol/L)</td>
<td>83.5±27.1†</td>
<td>36.9±14.6*</td>
<td>21.7±9.9*</td>
<td>19.3±7.9*</td>
</tr>
<tr>
<td>Plasma Na (mmol/L)</td>
<td>161.0±1.4</td>
<td>159.8±1.6</td>
<td>163±1.89</td>
<td>160.4±2.6</td>
</tr>
<tr>
<td>Plasma K (mmol/L)</td>
<td>4.1±0.3</td>
<td>4.2±0.3</td>
<td>4.2±0.2</td>
<td>4.5±0.3</td>
</tr>
</tbody>
</table>

All rats were subjected to right common carotid artery ligation. Sham rats received a normal diet containing 0.3% sodium chloride. The HSD group was fed a high-salt diet containing 8% sodium chloride. HSD/OVX rats also underwent oophorectomy (OVX). HSD/OVX/RL rats ate a high-salt diet and were treated by OVX plus bilateral posterior renal artery ligation (RL). For details on the procedures, see Materials and Methods - *experimental study 1.* Data are the mean ± SD at 12 weeks after RL (each group n=28). *p < 0.05 vs sham, †p < 0.05 vs HSD by ANOVA followed by Scheffe’s test.

SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure
Table S2  Characteristics in female rats subjected to renal ligation

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HSD</th>
<th>RL</th>
<th>RL/OVX</th>
<th>RL/HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>316.6±30.9</td>
<td>304.6±17.1</td>
<td>380.3±22.6*</td>
<td>319.5±30.5</td>
</tr>
<tr>
<td>Food intake (g/kg)</td>
<td>47.4±4.9</td>
<td>44.7±10.7</td>
<td>53.6±7.9</td>
<td>45.7±3.0</td>
</tr>
<tr>
<td>Water intake (ml/kg)</td>
<td>220.3±25.2</td>
<td>147.6±8.2</td>
<td>125.9±16.0*</td>
<td>291.6±65.7</td>
</tr>
<tr>
<td>Urine volume (ml/kg)</td>
<td>178.7±39.1</td>
<td>103.2±10.7</td>
<td>109.2±29.5</td>
<td>249.0±61.5</td>
</tr>
<tr>
<td>Urinary Na (mmol/L)</td>
<td>231±58.1</td>
<td>89.3±41.5*</td>
<td>97.6±36.9*</td>
<td>153.7±26.3</td>
</tr>
<tr>
<td>Urinary K (mmol/L)</td>
<td>36.9±14.6</td>
<td>38.6±5.0</td>
<td>41.2±25.5</td>
<td>22.4±3.9</td>
</tr>
<tr>
<td>Plasma Na (mmol/L)</td>
<td>159.8±1.6</td>
<td>160.2±1.8</td>
<td>161.0±4.8</td>
<td>158.7±1.3</td>
</tr>
<tr>
<td>Plasma K (mmol/L)</td>
<td>4.2±0.3</td>
<td>4.4±0.3</td>
<td>4.3±0.4</td>
<td>4.0±0.1</td>
</tr>
</tbody>
</table>

All rats were subjected to right common carotid artery ligation. The RL group was subjected to bilateral posterior renal artery ligation. RL/OVX rats also underwent oophorectomy. RL- and RL/OVX rats received a normal diet containing 0.3% sodium chloride. RL/HSD rats were fed a high-salt diet containing 8% sodium chloride plus RL. For details on the procedures, see online supplement Materials and Methods - supplemental study. Data are the mean ± SD at 12 weeks after RL (each group n=28). *p < 0.05 vs HSD rats by ANOVA followed by Scheffe’s test.

SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure
Size of cerebral aneurysms. Each image under a scanning electron microscope was determined its size with Image J software (n=20)
HSD, high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation
Incidence of experimental cerebral aneurysms and systolic blood pressure changes in supplemental study.

A. Rat brain vascular corrosion casts were inspected at the anterior cerebral artery-olfactory artery bifurcation under a scanning electron microscope. Changes observed by 3 blinded examiners are presented in a bar graph. Data were analyzed with the Fisher’s exact test (each group, n=20).

B. The systolic blood pressure was measured by the tail-cuff method. Measurements were taken once a month. Data represent the mean ± SD for 20 rats in each group. †p<0.05 vs HSD rats by ANOVA followed by Scheffé’s test

HSD, high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation
$K^+$ retention (A), $K^+$-to-water ratio (B), and $Na^+ + K^+$-to-water ratio (C). They were calculated as described in Materials and Methods. Each bar represents the mean ± SD (n=28). *p<0.05 vs sham and †p<0.05 vs HSD rats by ANOVA followed by Scheffe’s test.

HSD, high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation.
Plasma biomarkers related to Na\(^+\) regulation and blood pressure. Plasma renin activity (A), angiotensin II (B) and aldosterone (C), (each group $n=8$) were measured by radioimmunoassay. Each bar represents the mean ± SD. *$p<0.05$ vs sham by ANOVA followed by Scheffe’s test.

HSD, high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation
Elastica van Gieson (EVG) and immunohistochemical staining for angiotensin II (ANG II) and TNFα in HSD/OVX- and sham rats. 

TNFα, tumor necrosis factor α; HSD, high-salt diet; OVX, oophorectomy
mRNA levels of Na⁺/K⁺-ATPase, ATP1-α1 (A) and ATP1-α3 (B). The mRNA level was measured by quantitative real-time PCR (each group n=8) and normalized by the GAPDH mRNA level. Each bar represents the mean ± SD. *p<0.05 vs sham by ANOVA followed by Scheffe’s test.

HSD, high-salt diet; OVX, oophorectomy; RL, bilateral posterior renal artery ligation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase
Elastica van Gieson (EVG) and immunohistochemical staining in sham rats. ATP1α2-positive cells (red) in the vascular wall were found in CD31 positive endothelium; α-actin (green) positive smooth muscle cells were also observed.

ATP1α2, Na⁺/K⁺-ATPase subtype
Changes of systolic blood pressure in rats treated with and without olmesartan.
Each data represents the mean ± SD (n=20).
Water retention (A), Na$^+$ retention (B), K$^+$ retention (C), and Na$^+$+K$^+$-to-water ratio (D) in rats treated with and without olmesartan. They were calculated as described in Materials and Methods. Each bar represents the mean ± SD (n=28). *p<0.05 vs VC by Student’s t-test. VC, vehicle control; olm 0.3, HSD/OVX/RL rats treated with olmesartan 0.3 mg/kg per day.