

Renal Denervation Prevents Stroke and Brain Injury via Attenuation of Oxidative Stress in Hypertensive Rats

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Background—Although renal denervation (RD) is shown to reduce blood pressure significantly in patients with resistant hypertension, the benefit of RD in prevention of stroke is unknown. We hypothesized that RD can prevent the incidence of stroke and brain injury in hypertensive rats beyond blood pressure lowering.

Methods and Results—High-salt-loaded, stroke-prone, spontaneously hypertensive rats (SHRSP) were divided into 4 groups: (1) control; (2) sham operation; (3) bilateral RD; and (4) hydralazine administration to examine the effect of RD on stroke and brain injury of SHRSP. RD significantly reduced the onset of neurological deficit and death in SHRSP, and this protection against stroke by RD was associated with the increase in cerebral blood flow (CBF), the suppression of blood–brain barrier disruption, the limitation of white matter (WM) lesions, and the attenuation of macrophage infiltration and activated microglia. Furthermore, RD significantly attenuated brain oxidative stress, and NADPH oxidase subunits, P67 and Rac1 in SHRSP. On the other hand, hydralazine, with similar blood pressure lowering to RD, did not significantly suppress the onset of stroke and brain injury in SHRSP. Furthermore, RD prevented cardiac remodeling and vascular endothelial impairment in SHRSP.

Conclusions—Our present work provided the first experimental evidence that RD can prevent hypertensive stroke and brain injury, beyond blood pressure lowering, thereby highlighting RD as a promising therapeutic strategy for stroke as well as hypertension. (*J Am Heart Assoc.* 2013;2:e000375 doi: 10.1161/JAHA.113.000375)

Key Words: hypertension • oxidative stress • renal denervation • stroke • sympathetic nerve

Multiple lines of evidence indicate that renal nerves, composed of efferent sympathetic nerves and afferent sensory nerves, are involved in the pathophysiology of hypertension and chronic kidney disease.^{1–5} Renal efferent sympathetic nerve activation enhances volume retention and sodium reabsorption in the kidney, reduces renal blood flow, and activates renin-angiotensin-aldosterone system.^{4,5} On the other hand, renal afferent sensory nerves transmit important

sensory information to the paraventricular nucleus of hypothalamus (PVN), and in turn PVN transmits the information to the rostral ventrolateral medulla (RVLM), the vasomotor center that determines basal sympathetic nerve activity.^{2,3,6}

A proof-of-concept study⁷ and a subsequent randomized-controlled trial⁸ have demonstrated that catheter-based bilateral renal denervation (RD) can cause a significant and sustained reduction of blood pressure (BP) in patients with treatment-resistant hypertension. Furthermore, it has been demonstrated that muscle sympathetic nerve activity and systemic norepinephrine spillover are significantly reduced after RD in a single patient with resistant hypertension.⁹ RD reduces left ventricular hypertrophy and improves cardiac function¹⁰ in patients with resistant hypertension. Furthermore, RD provides rate control and reduces susceptibility to arterial fibrillation (AF) in patients with permanent AF.¹¹ These previous reports support the notion that reduction of renal afferent nerve traffic caused by RD might elicit inhibition of central sympathetic activity, thereby suggesting the possibility that RD may have the benefit in prevention of cerebrovascular and cardiac events independently of BP.

The present study, using a rat model of hypertensive stroke, was undertaken to demonstrate our hypothesis that

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RD can prevent stroke and brain injury independently of BP-lowering effect. We obtained the first experimental evidence that RD can prevent hypertensive stroke and brain injury beyond BP-lowering effect.

Methods

Experimental Animals

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kumamoto University. Male stroke-prone spontaneously hypertensive rats (SHRSP) were purchased from Japan SLC.

Experiment I: Effect of RD on Stroke and Survival Rate in SHRSP

To examine the effect of bilateral renal denervation (RD) on incidental stroke in SHRSP, 9-week-old SHRSP were fed an 8% NaCl diet (high-salt), and were randomly assigned to 4 groups, including (1) high-salt only, (2) high-salt+sham operation, (3) high-salt+RD, and (4) high-salt+hydralazine treatment (5 mg/kg per day). Hydralazine was given orally to rats as the drinking water. SHRSP fed a 0.3% NaCl diet (low-salt) served as the control. The appearance of major stroke-associated signs, including paralytic gait, reduced motor activity, and sudden death, was carefully monitored every day for 50 days, as previously described.¹² When 1 or more of these signs occurred in SHRSP, they were regarded as stroke sign-positive.

Experiment II: Effect of RD on Brain Injury in SHRSP

To examine the effect of RD on brain injury in SHRSP, 9-week-old SHRSP fed an 8% NaCl diet (high-salt) were assigned to the same 4 groups as the above-mentioned Experiment I. At 3 weeks after RD, sham operation, or hydralazine treatment, all groups of SHRSP were anesthetized with 2% isoflurane, blood was collected by cardiac puncture, and the rats were perfused with phosphate-buffered saline. The brain, heart, carotid arteries, and kidneys were rapidly excised from the rats for estimation of various parameters, including cerebral blood flow, white matter lesion, blood-brain barrier disruption, inflammatory cell infiltration, oxidative stress, vascular endothelial function, cardiac hypertrophy and fibrosis, renal norepinephrine content, and plasma renin activity etc, as described below.

Experiment III: Effect of RD and Hydralazine on Blood Pressure in SHRSP

To examine the comparative effect of RD and hydralazine on blood pressure in SHRSP, 6-week-old SHRSP (n=15) were

subjected to implantation of miniaturized telemetry devices to measure direct BP. At 9 weeks of age, they were fed an 8% NaCl (high-salt) in the same manner as Experiments I and II, and were divided into 3 groups, including (1) high-salt+sham operation, (2) high-salt+RD, and (3) high-salt+hydralazine treatment (5 mg/kg per day). Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) of these rats were measured over 14 consecutive days after these treatments, as described below.

Renal Denervation (RD) and Sham Surgery

To perform RD, SHRSP were placed in a prone position, under general anesthesia with isoflurane. To minimize invasive approach to the kidney, the incision was made in the dorsal midline. The traction of the erector spinae muscles exposed the kidney. Thereby, the renal arteries and veins were identified. After isolating the vessels from connective tissue, all visible nerves along the vessels were cut. Furthermore the vessels were painted with a solution of 10% phenol in absolute ethanol.¹³ Then the vessels were washed with saline and the skin was closed. This method ablates both the afferent and efferent renal nerves. In the sham-operation group of SHRSP, the kidneys were exposed in the same manner as the RD group but the vessels were not stripped or painted with phenol. The achievement of RD was confirmed by evaluating whether the renal tissue content of norepinephrine was <10% of the mean value in the sham-operated group.

Direct Measurement of Blood Pressure With Telemetry

We used a telemetry system (TA11PA-C40; Data Sciences International, St Paul, MN) to record arterial pressure in SHRSP, as described in detail.¹⁴ Six-week-old SHRSP were anesthetized with isoflurane and were surgically implanted with miniaturized telemetry devices in the abdominal cavity. The blood pressure catheter was placed into the abdominal aorta. BP signals and HR derived from pressure waves from abdominal aorta were measured in conscious and unrestrained animals. After 2 weeks of recovery, 24-hour online recordings were digitized (1 kHz) and stored for further analysis. BP and HR data were obtained from each implanted animal, and BP and HR data were recorded as 5-minute averages every 60 minutes using a computer system (DATA-QUEST ART4.2 Acquisition; Data Sciences International).

Measurement of Cerebral Blood Flow

After 3 weeks of RD, the cerebral blood flow (CBF) of SHRSP was recorded by a laser speckle blood flow imager (Omega Zone; Omegawave), as described previously.¹⁵ Briefly, the

rats were anesthetized with 2% isoflurane and the rectal temperature was kept at $37.0 \pm 0.5^\circ\text{C}$. After the rats were placed in the prone position, the skull was exposed by a midline scalp incision. Then, the surface of the region of bilateral cerebral hemispheres was diffusely illuminated by 780 nm semiconductor laser light. Color-coded blood flow images obtained in high-resolution mode (638×480 pixels; 1 image/second) were captured by a CCD camera positioned above head and transferred to a computer for analysis. The settings of CCD camera and color image program were kept the same among all the measurements. Images were analyzed by the color image program incorporated in the flowmetry system to obtain the average value of blood flow. The mean CBF of 10 measurements in each group was determined. The value of CBF was expressed as a percentage of low-salt group. All measurements were performed in a blinded fashion.

Measurement of White Matter Lesions

The brain samples were fixed with 4% (w/v) paraformaldehyde overnight, embedded in paraffin, cut into $5\text{-}\mu\text{m}$ thick sections. The samples were subjected to Klüver-Barrera staining for the measurement of white matter lesions. The severity of the white matter lesions in the corpus callosum were graded on a 4-point scale: normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and disappearance of myelinated fibers (grade 3).^{15,16} All measurements were performed in a blinded fashion.

Measurement of IgG Extravasation

Immunolocalization of brain IgG was evaluated in a blinded fashion, as described previously.^{17,18} Briefly, the sections were incubated in the presence of 0.3% H_2O_2 and with rabbit anti-rat IgG-HRP (Invitrogen) for 1 hour. After washing with PBS, the reaction product was visualized with diaminobenzidine. IgG immunoreactivity was quantified in whole brain with Lumina Vision version 2.2.0 analysis software (Mitani Corporation). The mean value in low-salt group was expressed as 1.0.

Immunohistochemical Staining of Macrophage and Activated Microglia and Astrocyte

The brain and heart samples were fixed with 4% (w/v) paraformaldehyde overnight, embedded in paraffin, cut into $5\text{-}\mu\text{m}$ thick sections. The samples were incubated with blocking solution for 30 minutes, and then incubated overnight at 4°C with the primary antibodies. For assessment of macrophage in the white matter and myocardial muscle, the samples were immunostained with anti-ED-1 antibody (working dilution 1:500; BMA Biomedicals AG), as described.¹⁵ Positive

staining was detected using horseradish peroxidase—conjugated secondary antibodies (Nichirei) by incubating the sections with diaminobenzidine (DAKO). The number of ED-1-positive cells was counted in 4 fields of the bilateral white matter and in 10 fields of the myocardial muscle.

For assessment of activated microglia and activated astrocyte in the white matter, the samples were immunostained with anti-ionized calcium binding adaptor molecule-1 (Iba-1, 1:200; Abcam) and anti-glial fibrillary acidic protein (GFAP, 1:200), in the same manner described above. All measurements were performed in a blinded fashion and expressed as the mean number of the positive cells/ mm^2 .

Western Blot Analysis

Our detailed method was described previously.¹⁹ Briefly, protein extracts of brain cortex were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrically transferred to a polyvinylidene difluoride membrane. The membranes were probed with specific antibodies. The antibodies used were as follows: anti-occludin ($\times 30000$, Life Technologies Co), anti-p67^{phox} ($\times 1000$, BD Transduction Laboratories), anti-Rac1 ($\times 30000$, Upstate Biotechnology, Inc) and anti-GAPDH ($\times 30000$, Santa Cruz Biotechnology). The intensity of the bands was quantified using Image J (NIH Image analysis software v1.46) in a blinded fashion. In individual samples, each value was corrected for that of GAPDH.

Measurement of Brain Superoxide

The brains, removed from SHRSP, were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek) and sectioned ($8\ \mu\text{m}$) with a cryostat directly onto chilled microscope slides. Dihydroethidium (DHE; Sigma) was used to evaluate superoxide levels in the brain cortex, white matter, and paraventricular nucleus (PVN) in situ, as described.²⁰ DHE fluorescence was visualized by FLUOVIEW FV 1000 (OLYMPUS) using Alexa Fluor 594. DHE fluorescence of the sections from brain cortex, white matter, and PVN was quantified in a blinded fashion, using Lumina Vision version 2.2 analysis software. The mean fluorescence in each group was expressed relative to values obtained for low-salt group.

Vessel Ring Preparation and Organ Chamber Experiment

Isometric tension studies were performed as previously described.²¹ All measurements were performed in a blinded fashion. In brief, carotid arteries from SHRSP were cut into 5-mm rings with special care to preserve the endothelium and mounted in organ baths filled with modified Tyrode's buffer (pH 7.4; 121 mmol/L NaCl, 5.9 mmol/L KCl, 2.5 mmol/L CaCl_2 ,

1.2 mmol/L $MgCl_2$, 1.2 mmol/L NaH_2PO_4 , 15.5 mmol/L $NaHCO_3$, and 11.5 mmol/L D-glucose) aerated with 95% O_2 and 5% CO_2 at 37°C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were precontracted with L-phenylephrine (10^{-7} mol/L). After a plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (ACh) and sodium nitroprusside to obtain cumulative concentration-response curves.

Histological Evaluation of Cardiac Fibrosis

Hearts were fixed in 4% (wt/vol.) paraformaldehyde, embedded in paraffin, sectioned at 5 μm , and stained with Sirius Red F3BA (0.5% wt/vol. in saturated aqueous picric acid; Aldrich Chemical Company) for the measurement of collagen volume fraction. The positive area of fibrosis per field area was assessed by examining at least 10 fields per rat using Lumina Vision version 2.2 analysis software.

Other Analytic Procedures

Renal norepinephrine levels were quantified by using the commercial ELISA kit (Labor Diagnostika Nord). Measurement of plasma electrolyte concentrations and plasma renin activity was performed at SRL Inc.

Statistical Analysis

All assays and measurements in this study were performed in a blinded fashion. The method of statistical analysis used in each experiment is described in all figure legends. Results were expressed as mean \pm SEM. The onset of stroke symptom and survival rate were analyzed by the standard Kaplan–Meier analysis with a log rank test and χ^2 analysis, using GraphPad Prism version 5 for Windows (GraphPad Software). The data on SBP and DBP over 14 consecutive days after RD measured by telemetry, and vascular relaxation were analyzed by 1-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's post-hoc test for multiple comparisons. The data on SBP, DBP, HR, and locomotor activity during 24 hours (12-hour dark period and 12-hour light period) measured by telemetry were analyzed by 2-way ANOVA with repeated measures, followed by Bonferroni's post-hoc test for multiple comparisons. The other normal distribution data were analyzed by 1-way ANOVA with Bartlett's test for equal variances, followed by Bonferroni's Multiple Comparison test. Otherwise non-normal distribution data were analyzed by nonparametric test with Kruskal-Wallis test, followed by Dunn's Multiple Comparison test. In all tests, differences were considered statistically significant at a value of $P < 0.05$.

Results

Blood Pressure of Salt-Loaded SHRSP

As shown in Figure 1, direct BP measurement with telemetry over 14 consecutive days demonstrated that RD and hydralazine treatment slightly reduced SBP and DBP in SHRSP compared with sham group. There was no significant difference in SBP or DBP over 14 consecutive days between RD and hydralazine groups. As shown in Figure 2, no significant difference was noted between RD and hydralazine groups, regarding hourly averaged SBP and DBP during 24 hours (12-hour dark period and 12-hour light period). Furthermore, as shown in Figure 3, hourly averaged HR and locomotor

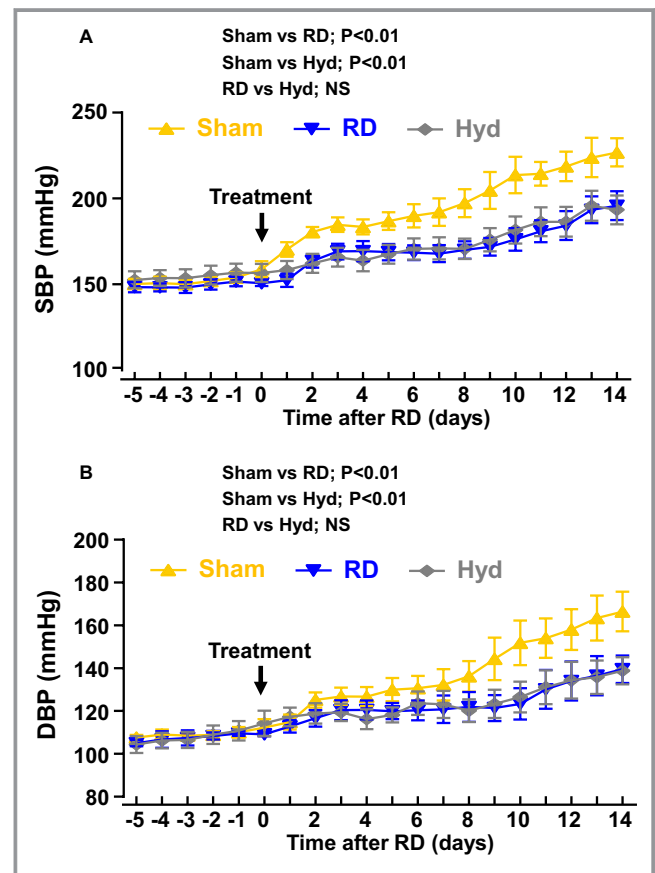


Figure 1. Twenty-four-hour averaged systolic BP (A) and diastolic BP (B) in SHRSP over 14 consecutive days after sham operation, RD, or hydralazine initiation. Values are the mean \pm SEM ($n=5$ in each group). Statistical analysis was performed by 1-way ANOVA with repeated measures followed by a post-hoc Bonferroni's Multiple Comparison test. Both SBP and DBP were significantly influenced by group ($P < 0.01$). The arrow indicates the initiation of each treatment. ANOVA indicates analysis of variance; BP, blood pressure; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; NS, not significant; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

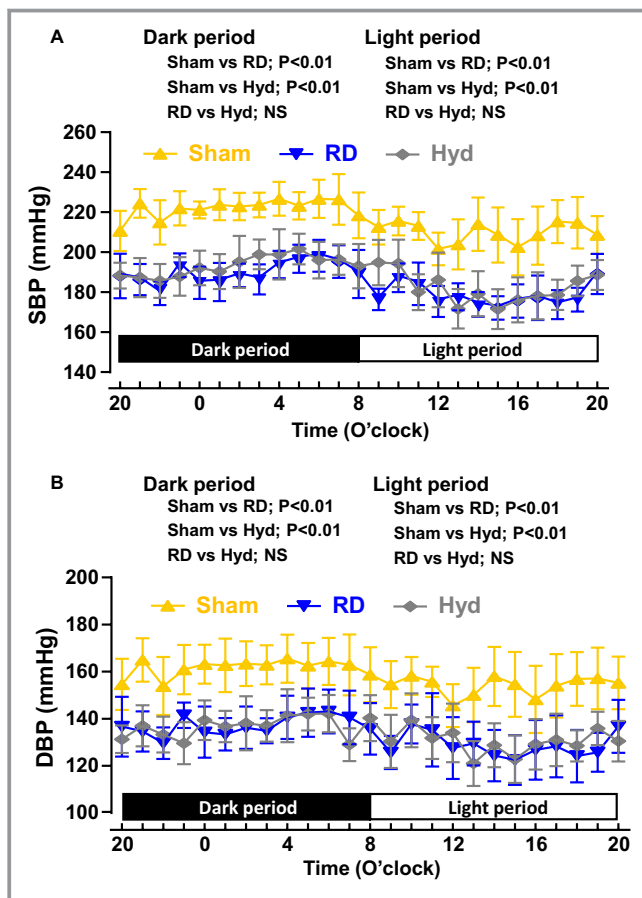


Figure 2. Hourly averaged systolic BP (A) and diastolic BP (B) in SHRSP during 24 hours (12-hour dark and 12-hour light periods) at 2 weeks after the treatment. Values are the mean \pm SEM ($n=5$ in each group). Statistical analysis was performed by 2-way ANOVA with repeated measures followed by a post-hoc Bonferroni's Multiple Comparison test. Both SBP and DBP were significantly influenced by group ($P<0.01$) and period ($P<0.01$). ANOVA indicates analysis of variance; BP, blood pressure; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; NS, not significant; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

activity were also comparable between RD and hydralazine groups during 12-hour dark period and 12-hour light period, except for the difference in HR during the light period.

Effects of RD on Brain Neurological Deficit and Death of Salt-Loaded SHRSP

The appearance of neurological deficit and death was carefully monitored every day over 50 days after RD, sham operation, or hydralazine treatment. As shown in Figure 4, a high-salt diet markedly deteriorated the neurological deficit ($P<0.01$) and survival rate ($P<0.01$) of SHRSP compared with a low-salt diet. Compared with sham operation, RD significantly prevented the onset of neurological deficit ($P<0.01$) and

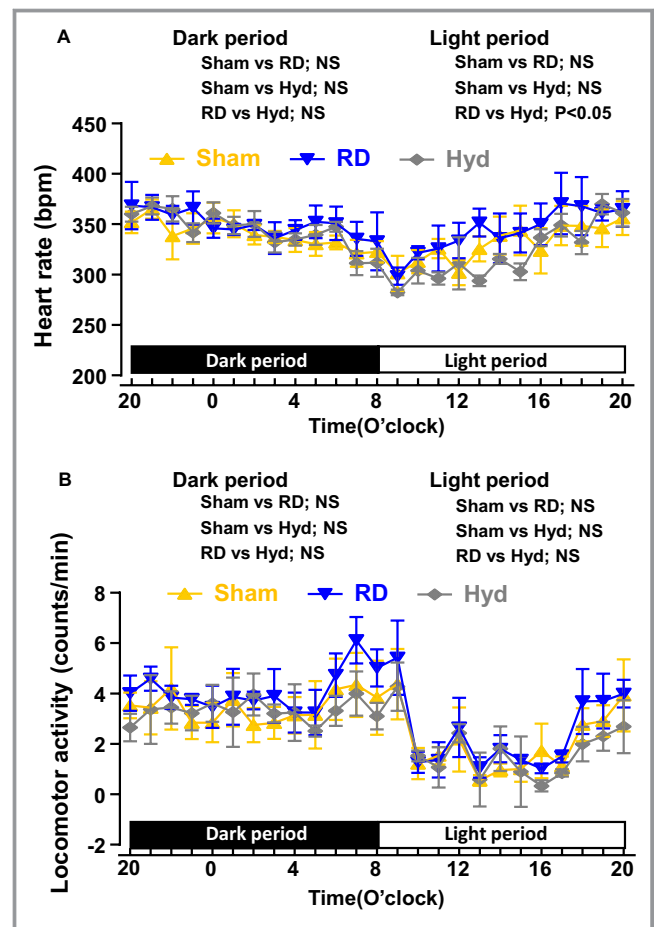


Figure 3. Hourly averaged heart rate (A) and locomotor activity (B) in SHRSP during 24 hours (12-hour dark and 12-hour light periods) at 2 weeks after the treatment. Values are the mean \pm SEM ($n=5$ in each group). Statistical analysis was performed by 2-way ANOVA with repeated measures followed by a post-hoc Bonferroni's Multiple Comparison test. Heart rate was significantly influenced by group ($P<0.05$) and period ($P<0.01$). Locomotor activity was significantly influenced by period ($P<0.01$) but not by group. ANOVA indicates analysis of variance; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; NS, not significant; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

improved survival rate ($P<0.01$) of salt-loaded SHRSP. On the other hand, hydralazine did not significantly prevent the deterioration of neurological deficit and death of salt-loaded SHRSP, despite comparative blood pressure lowering between hydralazine and RD.

Effects of RD on Cerebral Blood Flow and White Matter Lesions

As shown in Figure 5A, a high-salt diet significantly reduced CBF of SHRSP compared with a low-salt diet ($P<0.05$). RD significantly suppressed renal this reduction of CBF in salt-loaded

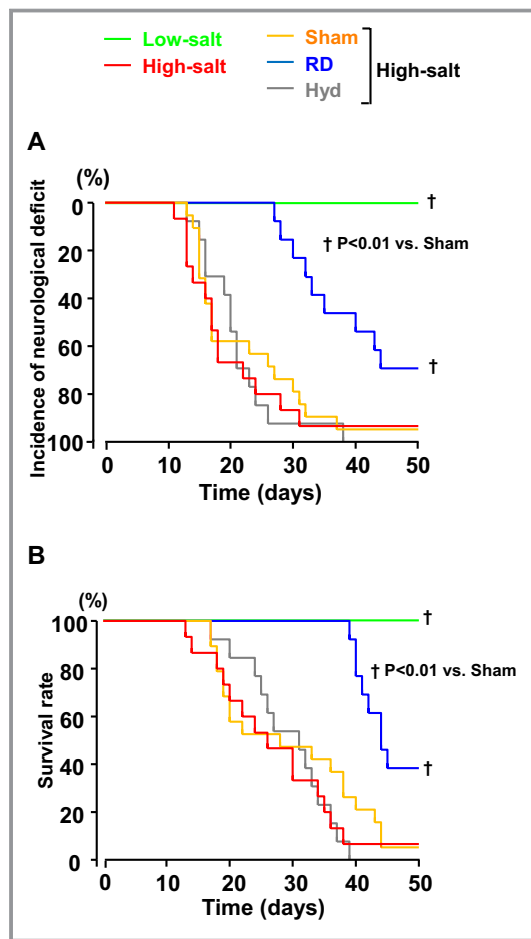


Figure 4. Effect of RD on neurological deficit (A) and survival rate (B) in SHRSP. Low-salt, SHRSP fed a low-salt (0.3% NaCl) diet; High-salt, SHRSP fed a high-salt (8% NaCl) diet. Values are mean \pm SEM (n=15 in High-salt; n=19 in Sham; n=13 in RD; n=13 in Hyd; n=5 in Low-salt). Statistical analysis was performed by the standard Kaplan-Meier analysis with a log rank test and χ^2 analysis. ANOVA indicates analysis of variance; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

SHRSP ($P<0.05$), but sham operation or hydralazine treatment failed to suppress it. As shown in Figure 5B, a high-salt diet caused marked progression of white matter lesion compared with a low-salt diet ($P<0.01$). RD significantly prevented the development of white matter lesions in salt-loaded SHRSP ($P<0.01$), while sham operation or hydralazine treatment did not prevent it.

Effects of RD on Blood–Brain Barrier (BBB) Disruption

As shown in Figure 6A, the density of IgG staining in the brain was greater in high-salt-loaded SHRSP than a low-salt-fed

SHRSP ($P<0.05$). RD significantly reduced this increase in IgG leakage ($P<0.05$) in salt-loaded SHRSP, in contrast to no significant attenuation of IgG leakage by sham-operation or hydralazine. As shown in Figure 6B, Western blot analysis showed that the expression of cerebral occludin was significantly decreased in high-salt-loaded SHRSP than in a low-salt-fed SHRSP ($P<0.05$). RD, but not sham operation or hydralazine treatment, suppressed this downregulation of occludin in high-salt-loaded SHRSP ($P<0.05$).

Effects of RD on Macrophage Infiltration, Microglia Activation, and Astrocyte Activation in Cerebral Cortex, White Matter (WM), and Paraventricular Nucleus (PVN)

A high-salt diet significantly increased the number of ED-1 positive cells ($P<0.01$) (Figure 7A), activated microglia ($P<0.01$) (Figure 7B), and activated astrocyte ($P<0.01$) (Figure 7C) in the brain cortex, white matter, and PVN in SHRSP, compared with a low-salt diet. RD significantly ameliorated the increases in number of ED-1 positive cells, activated microglia, and activated astrocyte in the brain cortex, white matter, and PVN in high-salt-loaded SHRSP. On the other hand, neither sham operation nor hydralazine treatment attenuated the increases in these inflammatory cells in cerebral cortex, white matter, and PVN in high-salt-loaded SHRSP.

Effects of RD on Brain Superoxide Levels and NADPH Oxidase Subunit

As shown in Figure 8, a high-salt diet increased superoxide levels of the brain cortex ($P<0.05$), white matter ($P<0.05$), and PVN ($P<0.05$) in SHRSP compared with a low-salt diet. RD significantly ameliorated the enhancement of superoxide in cortex ($P<0.01$), white matter ($P<0.01$), and PVN ($P<0.01$) of high-salt-loaded SHRSP, while sham operation or hydralazine failed to ameliorate them.

As shown in Figures 9A and 9B, Western blot analysis showed that cerebral expression of p67^{phox} ($P<0.01$) and Rac 1 ($P<0.01$) were significantly upregulated in high-salt-loaded SHRSP compared with a low-salt-fed SHRSP. RD significantly ameliorated the increase in cerebral p67^{phox} ($P<0.01$) and Rac 1 ($P<0.01$) proteins in high-salt-loaded SHRSP, while sham operation or hydralazine failed to reduce them.

Effects of RD on Renal Norepinephrine, Plasma Renin Activity, and Plasma Electrolytes

As shown in Figure 10A, renal norepinephrine contents in high-salt-loaded SHRSP tended to be higher than those in low-salt-fed SHRSP. Renal norepinephrine contents in RD group of SHRSP were significantly smaller than those in sham-opera-

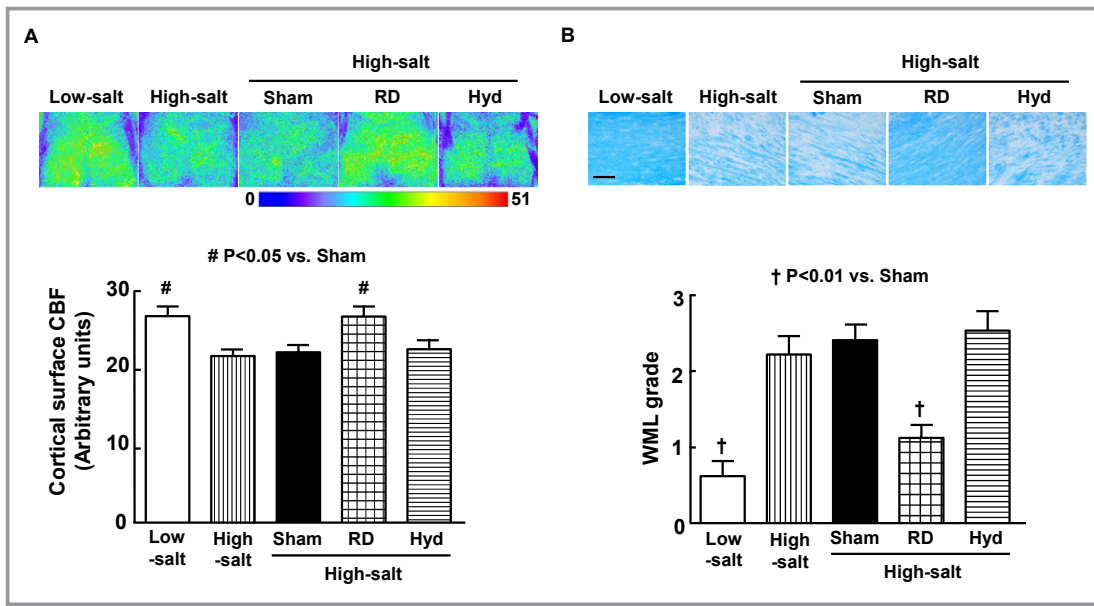


Figure 5. Effects of RD on cerebral blood flow (CBF) (A) and white matter lesions (B) in SHRSP. Upper panels in (A) indicate representative CBF image of each group, as assessed by laser speckle flowmetry. Upper panels in (B) indicate representative photomicrographs of Klüver-Barrera-stained white matter from each group. Scale bar=100 μ m. Values are mean \pm SEM (n=8 in each group). In both (A) and (B), statistical analysis was performed by 1-way ANOVA followed by a post-hoc Bonferroni's Multiple Comparison test. ANOVA indicates analysis of variance; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats; WML, white matter lesion.

tion group (12.5 \pm 1.9 ng/g versus 184.0 \pm 12.0 ng/g tissue; P <0.01). As shown in Figure 10B, plasma renin activity in RD group of SHRSP was significantly smaller than that in sham-

operated group of SHRSP (P <0.05). RD did not affect plasma sodium, potassium, or chloride concentrations in SHRSP (Table).

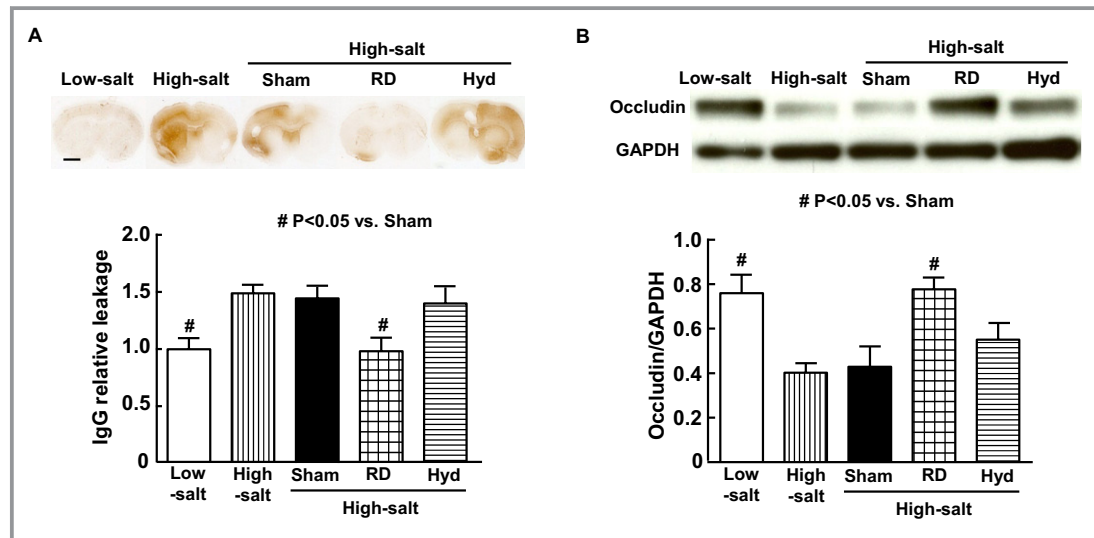


Figure 6. Effects of RD on cerebral IgG extravasation (A) and occludin protein levels (B) in SHRSP. Upper panels in (A) indicate representative photomicrographs of the whole brain immunostained with IgG from each group. Scale bar=5 mm. Upper panels in (B) show representative Western blots of occludin in the brain of each group. Values are mean \pm SEM (n=8 in each group). In both (A) and (B), statistical analysis was performed by 1-way ANOVA followed by a post-hoc Bonferroni's Multiple Comparison test. ANOVA indicates analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; IgG, immunoglobulin G; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

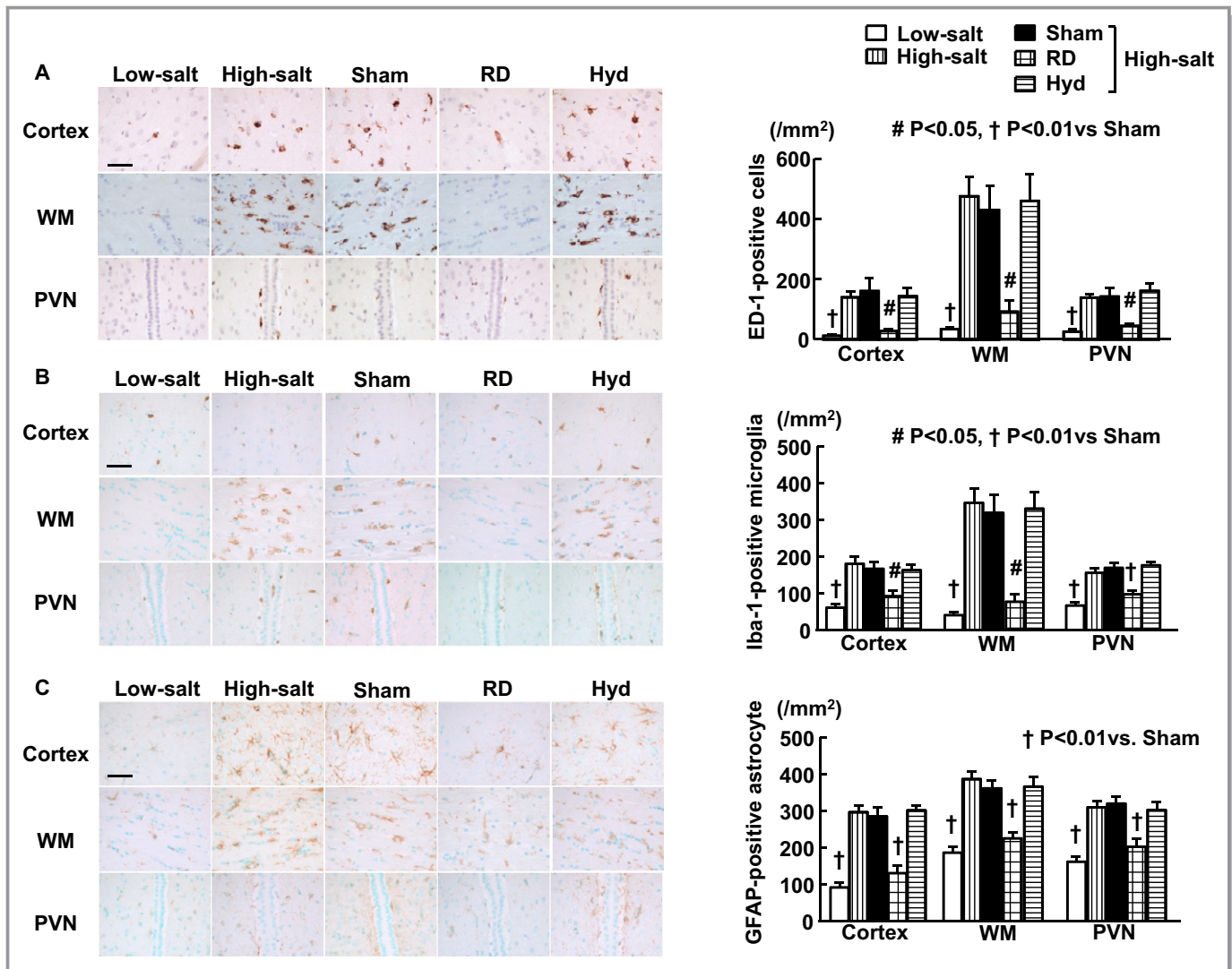


Figure 7. Effects of RD on the number of ED-1 positive cell (A), Iba-1-positive microglia (B), and GFAP-positive astrocyte (C) in the cortex, white matter, and paraventricular nucleus of SHRSP. Left panels in (A through C) indicate representative photomicrographs of the sections immunostained with ED-1, Iba-1, and GFAP, respectively, from brain cortex, white matter (WM), and paraventricular nucleus (PVN). Scale bar=50 μ m. Each bar represents mean \pm SEM (n=8 in each group). In (A), statistical analysis was performed by the Kruskal-Wallis test followed by a post-hoc Dunn's Multiple Comparison test. In (B and C), statistical analysis was performed by the 1-way ANOVA followed by a post-hoc Bonferroni's Multiple Comparison test. ANOVA indicates analysis of variance; ED-1, CD68 Antibody; GFAP, glial fibrillary acidic protein; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; Iba-1, ionized calcium binding adaptor molecule-1; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

Hydralazine treatment did not significantly affect renal norepinephrine content or plasma renin activity in SHRSP.

Effects of RD on Cardiovascular Injury

As shown in Figure 11A through 11C, a high-salt diet significantly increased left ventricular weight ($P<0.01$), cardiac ED-1 positive cell (macrophage) numbers ($P<0.01$), and cardiac interstitial fibrosis ($P<0.01$) in SHRSP, compared with a low-salt diet. RD significantly decreased left ventricular weight ($P<0.01$), cardiac ED-1 positive cell numbers ($P<0.01$), and cardiac

fibrosis ($P<0.01$) in high-salt-loaded SHRSP. On the other hand, sham operation or hydralazine did not attenuate them in high-salt-loaded SHRSP.

As shown in Figure 11D, a high-salt diet significantly impaired vascular endothelium-dependent relaxation with acetylcholine in SHRSP, compared with a low-salt diet. RD, but not sham operation or hydralazine, significantly ameliorated the impairment of vascular endothelial function in salt-loaded SHRSP. There was no significant difference in sodium nitroprusside-induced endothelium-independent vascular relaxation among each group (data not shown).

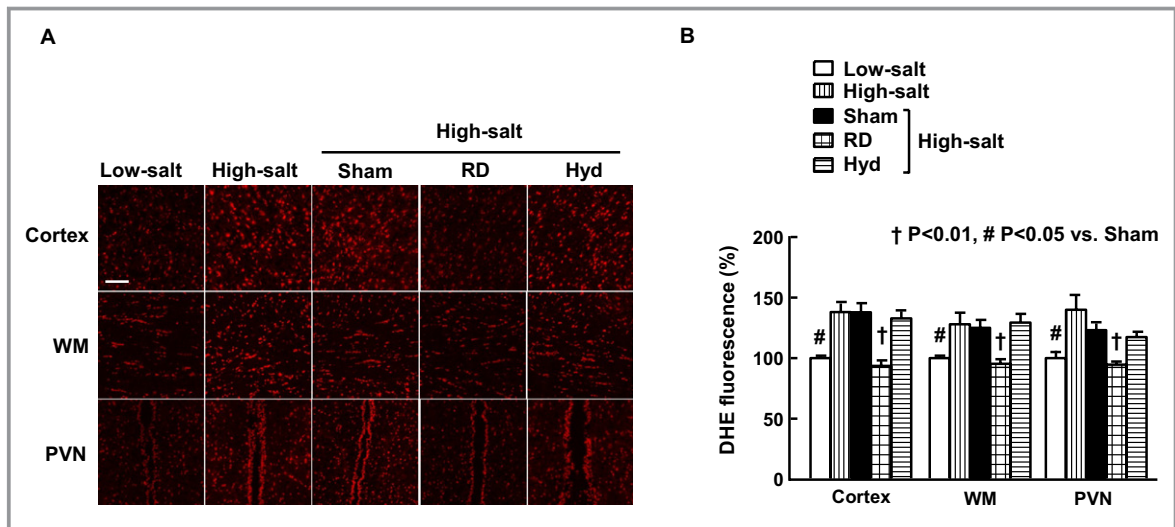


Figure 8. Effects of RD on superoxide levels in brain cortex, white matter, and paraventricular nucleus of SHRSP. Left panels show representative confocal images of DHE fluorescence in each region of the brain from each group. Scale bar=100 μ m. Values are mean \pm SEM (n=8 in each group). Statistical analysis was performed by the Kruskal–Wallis test followed by a post-hoc Dunn’s Multiple Comparison test. ANOVA indicates analysis of variance; DHE, dihydroethidium; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; PVN, paraventricular nucleus; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats; WM, white matter.

Discussion

Although RD significantly reduces BP in patients with resistant hypertension,^{5,7–9,22} it is unknown whether RD can prevent cerebrovascular events beyond BP-lowering effect. The major findings of our present work were that RD significantly prevented the onset of stroke and the progression of brain injury in hypertensive rats, and these brain-protective effects of

RD were at least in part, mediated by BP-independent effects, including attenuation of oxidative stress and inflammation, and suppression of BBB disruption. Therefore, our present work provided the first evidence supporting that RD seems to be a promising therapeutic strategy for stroke in hypertension.

In the present study, to determine the effectiveness of RD on stroke in hypertension, we used high-salt-loaded SHRSP, since SHRSP is regarded as an established and popular model

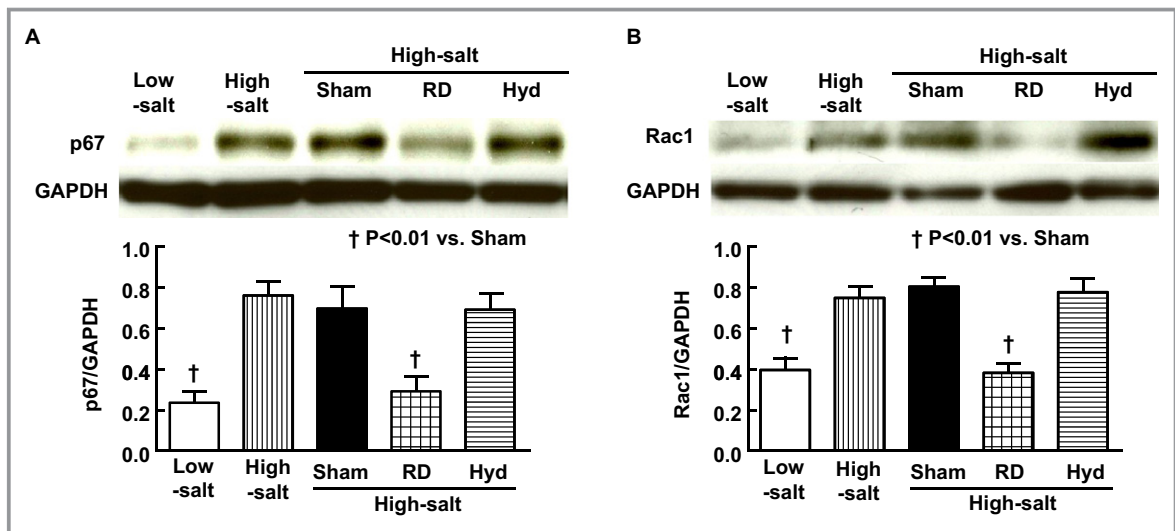


Figure 9. Effects of RD on brain p67^{phox} (A) and Rac1 (B) in SHRSP. Upper panels in (A) and (B) show representative Western blots of p67^{phox} and Rac1 in the brain of each group. Values are mean \pm SEM (n=8 in each group). In both (A) and (B), statistical analysis was performed by 1-way ANOVA followed by a post-hoc Bonferroni’s Multiple Comparison test. ANOVA indicates analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

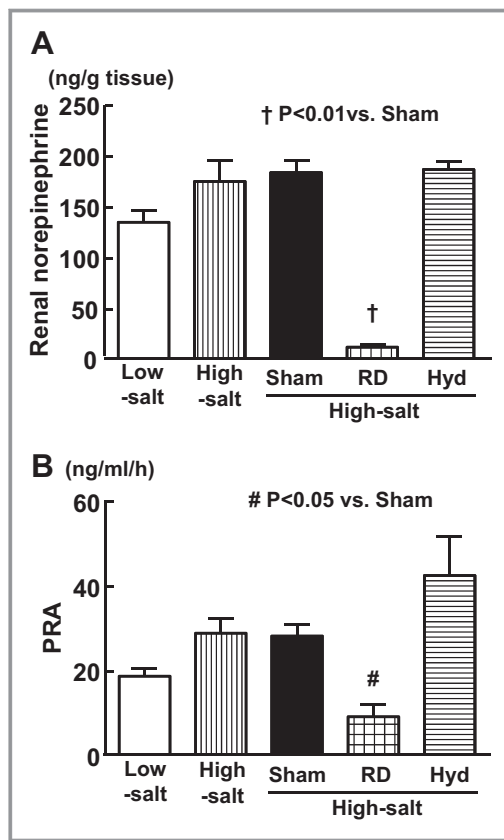


Figure 10. Effects of RD on renal norepinephrine contents (A) and plasma renin activity (PRA) (B) in SHRSP. Values are mean \pm SEM (n=8 in each group). In both (A) and (B), statistical analysis was performed by the Kruskal–Wallis test followed by a post-hoc Dunn’s Multiple Comparison test. ANOVA indicates analysis of variance; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

of hypertensive stroke.^{12,15,20,23} In agreement with our previous reports,^{12,15,20,23} high-salt intake significantly accelerated stroke incidence and brain injury in SHRSP, which is

consistent with clinical evidence^{24–28} that excessive salt intake is an important risk factor for stroke. Of note, RD significantly reduced the incidence of neurological deficit and death in high-salt-loaded SHRSP, despite the very small BP-lowering effect of RD in SHRSP. On the other hand, hydralazine treatment with similar small BP-lowering effect to RD did not significantly prevent stroke in SHRSP. These results demonstrate that the mechanism underlying prevention of stroke by RD in SHRSP is mediated by BP-independent effect. Furthermore, RD significantly attenuated the decrease in cerebral blood flow and significantly prevented the progression of white matter lesion in high-salt-loaded SHRSP. On the contrary, hydralazine failed to attenuate these changes induced by high-salt in SHRSP. These results indicate that RD prevented the disturbance of cerebral blood flow and progression of white matter lesion independently of BP. Furthermore, RD significantly limited disruption of BBB in high-salt-loaded SHRSP, being accompanied by the suppression of downregulation of occludin,^{29,30} a key tight junction protein involved in intact BBB function, while hydralazine treatment failed to limit these changes in high-salt-loaded SHRSP. Therefore, the protective effect of RD against stroke in high-salt-loaded SHRSP seems to be at least partially mediated by the improvement of cerebral blood flow and inhibition of BBB disruption.

In the present work, it is worthy to note that RD significantly ameliorated the increase in inflammatory cells such as macrophage, and activated microglia and astrocyte in cortex, white matter, and PVN regions in high-salt-loaded SHRSP, while hydralazine did not attenuate them. These findings imply that RD exerts anti-inflammatory effects in the brain of SHRSP. Accumulating evidence^{31–34} supports the notion that oxidative stress and inflammation play a key role in the pathogenesis of stroke and brain injury, through formation of a vicious cycle. Previously, we have reported that antioxidant significantly slows the incidence of stroke in high salt-loaded SHRSP, and this is associated with attenuation of cerebral inflammation,

Table. Body Weight, Plasma Sodium, Potassium, and Chloride Concentrations, and Osmotic Pressure at 3 Weeks After Renal Denervation, Sham Operation, or Hydralazine Treatment

	Low-Salt	High-Salt	High-Salt		
			Sham	RD	Hyd
BW (g)	268 \pm 3 [†]	203 \pm 9	213 \pm 11	248 \pm 5 [#]	203 \pm 7
Na (mEq/L)	141.9 \pm 0.6	140.5 \pm 1.8	141.5 \pm 1.6	141.4 \pm 0.7	142.5 \pm 2.5
K (mEq/L)	4.6 \pm 0.4	4.4 \pm 0.2	4.1 \pm 0.2	3.6 \pm 0.1	4.4 \pm 0.3
Cl (mEq/L)	101.9 \pm 0.7	96.4 \pm 2.3	98.5 \pm 1.7	102.6 \pm 1.1	96.6 \pm 3.3
Osm (mOsm/kg H ₂ O)	305.3 \pm 1.2	307.6 \pm 3.4	309.6 \pm 4.5	303.8 \pm 0.9	308.4 \pm 5.0

Values are mean \pm SEM (n=8 in each group). Na, K, and Cl indicates the respective plasma concentrations. BW indicates body weight; high-salt, SHRSP fed a high-salt diet; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; low-salt, SHRSP fed a low-salt diet; RD, SHRSP fed a high-salt diet and subjected to renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

[#]P<0.05, [†]P<0.01 vs Sham.

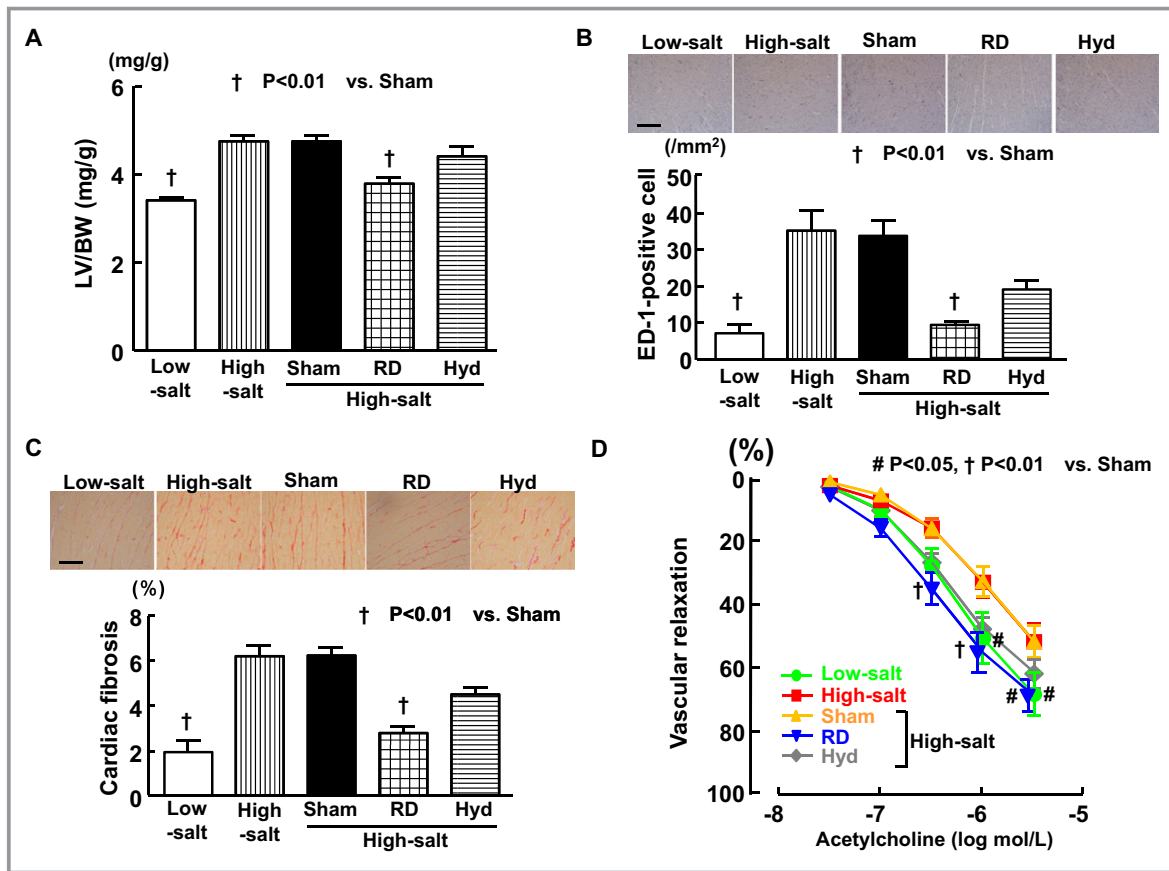


Figure 11. Effects of RD on cardiac weight (A), cardiac macrophage infiltration (B), cardiac fibrosis (C), and vascular endothelium-dependent relaxation with acetylcholine (D) in SHRSP. LV/BW indicates left ventricular weight corrected for body weight. Upper panels in (B and C) indicate representative photomicrographs of ED-1-immunostained cardiac sections and Sirius red-stained cardiac sections, respectively. Scale bar=100 μ m. Values are mean \pm SEM ($n=8$ in each group). Statistical analysis was performed by the Kruskal-Wallis test followed by a post-hoc Dunn's Multiple Comparison test in (A), 1-way ANOVA followed by a post-hoc Bonferroni's Multiple Comparison test in both (B and C), and 1-way ANOVA with repeated measures followed by a post-hoc Bonferroni's Multiple Comparison test in (D). ANOVA indicates analysis of variance; ED-1, CD68 Antibody; Hyd, SHRSP fed a high-salt diet and orally given hydralazine; RD, SHRSP fed a high-salt diet and subjected to bilateral renal denervation; SEM, standard error of the mean; Sham, SHRSP fed a high-salt diet and subjected to sham operation; SHRSP, stroke-prone spontaneously hypertensive rats.

thereby indicating the critical role of brain oxidative stress in the mechanism of stroke in high-salt-loaded SHRSP.²⁰ Therefore, in the present study, we examined the effect of RD on brain oxidative stress in SHRSP. RD, but not hydralazine, significantly reduced oxidative stress in cortex, white matter, and PVN of SHRSP. Furthermore, RD, but not hydralazine, significantly prevented the increase in NADPH oxidase subunits, P67 and Rac1 in SHRSP. These results show that RD attenuated brain oxidative stress beyond BP-lowering effect. Collectively, our present work supports the notion that the attenuation of brain oxidative stress by RD is involved in the prevention of stroke in high-salt-loaded SHRSP.

A growing body of clinical evidence^{35,36} and experimental work including SHRSP^{15,20,23} show that renin-angiotensin system participates in the pathophysiology of stroke and brain injury. In the present study, plasma renin activity was significantly reduced by RD in high-salt-loaded SHRSP,

indicating the suppression of circulating renin-angiotensin system by RD. Previously we have found that angiotensin II is directly involved in the progression of stroke in high-salt-loaded SHRSP.^{15,20,23} Therefore, not only attenuation of brain oxidative stress but also suppression of circulating renin-angiotensin system appears to account for the protective effects of RD against stroke and brain injury.

The method of RD used in the present work causes denervation of afferent renal sensory nerves as well as efferent renal sympathetic nerves. Substantial evidence indicates that afferent renal sensory nerves project directly to various areas in the central nervous system involved in the regulation of cardiovascular system.⁴ Previous experimental studies³⁷⁻³⁹ show that renal injury such as ischemia, through afferent renal nerve, significantly activates the central sympathetic nervous system, thereby leading to the enhancement of systemic sympathetic nerve activity. Furthermore, patients with chronic

renal failure are characterized by the enhancement of muscle sympathetic nerve activity through renal afferent nerves.^{40,41} Thus, it is proposed that renal afferent nerves are a key regulator of peripheral sympathetic nerve activity as well as the central sympathetic nervous system. Recent clinical studies show that RD significantly attenuates muscle sympathetic nerve activity⁴² and improves cardiac hypertrophy and function,¹⁰ atrial fibrillation,¹¹ glucose intolerance⁴³ in patients with resistant hypertension, thereby supporting the concept that RD can have the benefit in prevention of cardiovascular injury through afferent renal nerve ablation. However, it remains to be determined whether these potential organ protective effects of RD in hypertensive patients are secondary to BP-lowering or not. In the present work, notably, RD, but not hydralazine, significantly attenuated cardiac hypertrophy, cardiac macrophage infiltration, and cardiac fibrosis and also prevented the impairment of vascular endothelial function in high-salt-loaded SHRSP. These results suggest that BP-independent protective effects of RD against stroke and cardiovascular injury observed in the present work might be attributed, at least partially, to the ablation of renal afferent nerves.

Study Limitation

The present work did not allow us to define the direct mechanism underlying brain protection by RD in SHRSP and to determine how various pleiotropic effects of RD observed in this study are linked to each other. As described above, we have previously shown that renin-angiotensin system and oxidative stress both are directly involved in the progression of stroke and brain injury in SHRSP, through induction of inflammation, ischemia, and blood-brain barrier disruption.^{12,15,20,23} Therefore, it is likely that the suppression of

oxidative stress and renin-angiotensin system by RD might be the main mechanisms leading to brain protection (Figure 12). However, further study is required to demonstrate our proposal.

In conclusion, our present work provided the first experimental evidence indicating that RD ameliorated the incidence of stroke and brain injury in hypertensive rats beyond BP-lowering effect and these brain-protective effects of RD were attributed to the limitation of vicious cycle of oxidative stress and inflammation and the amelioration of renin-angiotensin system. Therefore, we propose that RD seems to be a promising therapeutic strategy for stroke in hypertensive patients. However, future clinical study is needed to elucidate our proposal.

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Disclosures

None.

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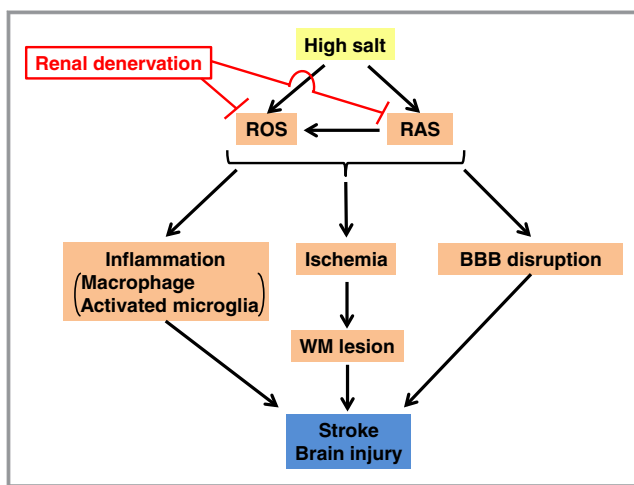


Figure 12. Proposed mechanism underlying the protection of stroke and brain injury by renal denervation. BBB indicates blood-brain barrier; RAS, renin-angiotensin system; ROS, reactive oxygen species; WM, white matter.

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