Role of Mineralocorticoid Receptor on Experimental Cerebral Aneurysms in Rats

Yoshiteru Tada, Keiko T. Kitazato, Tetsuya Tamura, Kenji Yagi, Kenji Shimada, Tomoya Kinouchi, Junichiro Satomi, Shinji Nagahiro

Abstract—Activation of the renin-angiotensin (Ang)-aldosterone system is involved in the pathology of vascular diseases. Although the blockade of the mineralocorticoid receptor protects against vascular diseases, its role in cerebral aneurysms remains to be elucidated. We treated female rats subjected to renal hypertension, increased hemodynamic stress, and estrogen deficiency for 3 months with the mineralocorticoid receptor blocker eplerenone (30 or 100 mg/kg per day) or vehicle (vehicle control). Eplerenone reduced the incidence of cerebral aneurysms and saline intake without lowering the blood pressure. In the aneurysmal wall, the production of Ang II and nitrotyrosine was increased. The mRNA levels of Ang-converting enzyme 1 and NADPH oxidase subunits NOX4, Rac1, monocyte chemoattractant protein 1, and matrix metalloproteinase 9 were increased. Eplerenone brought about a reduction in these molecules, suggesting that mineralocorticoid receptor blockade suppresses cerebral aneurysm formation by inhibiting oxidative stress, inflammatory factors, local renin-Ang system activation, and saline intake. Other female rats implanted with pellets of the mineralocorticoid receptor agonist deoxycorticosterone acetate manifested a high incidence of cerebral aneurysm formation and the upregulation of molecules related to oxidative stress, inflammatory factors, and the local renin-Ang system; their saline intake was increased. We demonstrate that mineralocorticoid receptor activation at least partly contributes to the pathogenesis of cerebral aneurysms. (Hypertension. 2009;54:552-557.)

Key Words: cerebral aneurysm inflammation mineralocorticoid receptor oxidative stress

Rupture of cerebral artery aneurysms results in catastrophic subarachnoid hemorrhage and a high risk for morbidity and mortality.1 On the basis of epidemiological data showing a high incidence of cerebral aneurysms in postmenopausal women, we subjected female rats to increased hemodynamic stress, hypertension, and estrogen deficiency (by oophorectomy); in these animals, the incidence of cerebral aneurysms was high.2 Treatment with 17β-estradiol or an angiotensin (Ang) II type 1 receptor blocker reduced this incidence.2 Elsewhere we suggested that endothelial injury is an initial event in the pathogenesis of cerebral aneurysms and that an increase in Ang II and NADPH oxidase subunits is involved.2 Also, inflammation and degradation of the extracellular matrix in the vascular wall play a role in the development of cerebral aneurysms.3-5

The renin-Ang-aldosterone system is involved in the pathophysiology of cardiovascular and kidney diseases, activation of the mineralocorticoid receptor (MR) is especially highlighted in recent studies.6-10 The identification of a new site of MR expression in nonpithelial tissues, such as the heart,11 vasculature,12 and brain,13 suggested the presence in these tissues of potential new MR target genes with unexpected biological functions. In aortic endothelial cells, aldosterone increased the expression of Ang-converting enzyme (ACE) genes that may be involved in the development of vascular injury.14 Eplerenone, a selective MR antagonist, performs beneficial actions, such as antihypertensive, anti-inflammatory effects,15 the prevention of cardiac fibrosis,16 and the suppression of NADPH oxidase activity8,15 and matrix metalloproteinase (MMP).17 However, the relationship between MR and cerebral aneurysms remains unclear.

We show that, in female rats, MR blockade by eplerenone inhibited the progression of cerebral aneurysms via its antioxidant and anti-inflammatory effects, suppressed local renin-Ang system (RAS) activation, and decreased their salt intake in a blood pressure–independent manner. We also demonstrate that MR activation by the MR agonist deoxycorticosterone acetate (DOCA) contributed to the formation of cerebral aneurysms.

Materials and Methods
For detailed descriptions of Materials and Methods, please see the online Data Supplement at http://hyper.ahajournals.org.

Results
MR Blockade Suppresses the Progression of Experimentally Induced Cerebral Aneurysms
Based on our morphological findings, we classified the left anterior cerebral artery-olfactory artery (ACA-OA) bifurca-
MR Blockade Reduces Oxidative Stress and Inflammatory Reaction in the Cerebral Vascular Wall

Next, we investigated the molecular mechanisms of cerebral aneurysm formation in rats. A, Aneurysmal changes at the left ACA-OA bifurcation of rats treated with VC (n=17) or eplerenone (30 mg/kg per day, Epl-30 group; 100 mg/kg per day, Epl-100 group, n=11) were morphologically evaluated by scanning electron microscopy using vascular corrosion casts. Data were analyzed with the Fisher's exact test. Epl indicates eplerenone. B, To determine the aneurysm size, on each image we outlined regions of interest corresponding with areas with aneurysmal involvement. Analysis was with National Institutes of Health image software. Data (mean±SD) were analyzed by 1-way ANOVA followed by Scheffe's test. *P<0.05 vs VC. C, Time course of SBP changes in VC (n=17), Epl-30 (n=11), and Epl-100 rats (n=11). OXY indicates oophorectomy. D, Saline intake of VC and eplerenone-treated rats (each group n=8). Data (mean±SD) were analyzed by 1-way ANOVA followed by Scheffe's test. *P<0.05 vs VC.

MR Blockade Reduces Activation of the Local RAS in the Cerebral Vascular Wall

We examined the effect of MR blockade on the local RAS in cerebral aneurysms. Immunohistochemically, Ang II was highly expressed in the endothelium and smooth muscle cells of aneurysms and parent arteries (Figure S3A). We detected the MR gene in cerebral arteries from VC rats by quantitative real-time PCR (Figure S3B). Eplerenone reduced the protein and mRNA level of MR (Figure S3A and S3B).

DOCA Salt Induces Cerebral Aneurysms Despite a Moderate Blood Pressure Increase

To test whether the activation of MR contributes to cerebral aneurysm formation, we used DOCA, an MR agonist, instead of inducing renal hypertension (rHT) by ligation. The incidence of cerebral aneurysms in DOCA rats was similar to that in rHT rats (69% versus 59%; Figure 4A). The aneurysm size was not different between the 2 groups (Figure 4B). SBP in
DOCA rats was moderately elevated compared with sham-operated rats, and it was lower than in rHT rats \( (P<0.05; \text{Figure 4C}) \). The saline intake of DOCA rats was significantly increased (Figure 4D) compared with that of rHT rats. The plasma aldosterone level was significantly lower in DOCA rats than in sham-operated rats (Figure S4); plasma aldosterone was not different between DOCA and rHT rats. Based on our observation that, in DOCA rats, the incidence of cerebral aneurysms was almost the same as in rHT rats, we posit that MR activation is involved in the pathogenesis of cerebral aneurysms.

**MR Activation Induces the Upregulation of Molecules Related to Oxidative Stress, Inflammation, and the Local RAS in the Aneurysmal Wall**

We examined whether oxidative stress, inflammation, and the local RAS contribute to the pathogenesis of cerebral aneurysms in DOCA rats. The mRNA levels of NOX4, p22phox, MCP-1, and MMP-9 were significantly higher in DOCA rats than in sham-operated rats (Figure 5A through 5D); the ACE1 mRNA level was also higher in DOCA rats (Figure 5E). On the other hand, the level of ACE2 mRNA was lower in DOCA rats than in sham-operated rats (Figure 5F).

**Discussion**

We report 2 new insights. First, blockade of MR activation by eplerenone suppressed the formation of cerebral aneurysms in rats. Second, the inhibition mechanisms may involve attenuation of oxidative stress and vascular inflammation, as well as inhibition of the activation of the local RAS. The reduced salt intake of rats subjected to MR blockade may be associated with the lower incidence of cerebral aneurysms.

Human studies have shown a link between elevated plasma aldosterone and stroke risk, especially the risk for hemorrhagic stroke.\(^{19,20}\) A comparison of patients with essential hypertension and primary hyperaldosteronism suggested that the increased incidence of cerebral hemorrhage in the latter group is blood pressure–independent.\(^{21}\) In stroke-prone spontaneously hypertensive rats fed a high-salt diet, MR antagonists prevented spontaneous hemorrhagic stroke without lowering the blood pressure.\(^{22,23}\) Moreover, autosomal-dominant polycystic kidney disease was strongly associated with the development of cerebral aneurysms; these patients exhibited significantly higher plasma aldosterone concentrations in the supine and upright positions and after ACE inhibition.\(^{24}\) These studies imply that aldosterone or MR activation is linked to hemorrhagic stroke or cerebral aneurysm development.

Estrogen deficiency induced reactive oxygen species generation and endothelial damage leading to cerebral aneurysm formation; in combination with hypertension, their effects were enhanced.\(^{3}\) Here we demonstrate that the protein and gene expression of NADPH oxidase subunits was increased in parallel with the increased expression of nitrotyrosine in the aneurysmal wall. We also found that inflammatory molecules and macrophage infiltration were increased in the aneurysmal wall. MCP-1 is a major factor promoting the accumulation of macrophages in atherosclerosis\(^ {25}\) and abdominal aortic aneurysms,\(^ {26}\) and it is involved in the patho-
activation.27 We found that Ang II production was increased in rHT and DOCA rats. MR blockade reduced the mRNA level of ACE1. Our results suggest that MR activation partly induced Ang II production of Ang II and the mRNA level of ACE1. In the aneurysm wall and that the mRNA level of ACE1 was increased in rHT and DOCA rats. MR blockade reduced the protein intake of rHT and DOCA rats (each group, n=8). Data (mean±SD) were analyzed by the Mann–Whitney U test. *P<0.05; †P<0.01 vs VC.

Figure 3. The mRNA levels of NOX4 (A), Rac1 (B), MCP-1 (C), MMP-9 (D), ACE1 (E), and ACE2 (F) were analyzed by quantitative real-time PCR (each group, n=7). Values were corrected by GAPDH and expressed as the mean±SD. *P<0.05; †P<0.01 vs sham, #P<0.01 vs VC.

Figure 4. A, Aneurysmal changes of rHT (n=17) and DOCA rats (n=13). They were morphologically evaluated by scanning electron microscopy using vascular corrosion casts. Data were analyzed with the Fisher’s exact test. B, Size of the aneurysms. Data (mean±SD) were analyzed by the Mann–Whitney U test. C, Time-course of SBP changes in sham-operated (n=15), rHT (n=17), and DOCA rats (n=13). Data (mean±SD) were analyzed by 1-way ANOVA followed by Scheffe’s test. †P<0.01 vs sham, #P<0.01 vs rHT. D, Saline intake of rHT and DOCA rats (each group, n=8). Data (mean±SD) were analyzed by the Mann–Whitney U test. *P<0.05 vs rHT.

Genesis of cerebral aneurysms.5 Macrophage-derived MMP-9 can degrade components of the extracellular matrix in vascular walls,3,4 thereby promoting the progression of cerebral aneurysms. Our results, that MR blockade reduced the protein and gene expression of these molecules, suggested that MR blockade suppressed cerebral aneurysm development by inhibiting oxidative stress and inflammatory factors.

We demonstrated that MR activation with DOCA resulted in a high incidence of cerebral aneurysms, and the mRNA levels of oxidative stress and inflammatory genes were increased despite a moderate increase in blood pressure. Oxidative stress and inflammation are associated with RAS activation.27 We found that Ang II production was increased in the aneurysm wall and that the mRNA level of ACE1 was increased in rHT and DOCA rats. MR blockade reduced the production of Ang II and the mRNA level of ACE1. Our results suggest that MR activation partly induced (and that MR blockade partly reduced) local RAS activation.

rHT is related to the activation of systemic RAS. In the DOCA-salt model, hypertension is generated by plasma volume expansion secondary to an increased sodium load and is associated with a dramatic reduction of plasma renin and Ang II concentrations.28 In preliminary studies, plasma renin activity was one tenth lower in DOCA rats than in rHT rats (data not shown), suggesting that the SBP response to DOCA is listless compared with the response in the presence of rHT. Although it had no marked effect on blood pressure, MR blockade reduced the incidence of aneurysmal changes and suppressed increases in the aneurysmal size. Therefore, the mechanisms underlying the suppression of cerebral aneurysm development would be independent of the antihypertensive effect of MR blockade.

MR blockade did not normalize the expression of genes, suggesting that the residual elevation of genes may be attributable to the increased blood pressure. We do not know what portion of the increase in gene expression is attributable
Values were corrected by GAPDH and expressed as the mean ± SD. *P<0.05; †P<0.01 vs sham.

Figure 5. The mRNA levels of NOX4 (A), p22phox (B), MCP-1 (C), MMP-9 (D), ACE1 (E), and ACE2 (F) assayed by quantitative real-time PCR (sham, n=7; DOCA, n=6). The mRNA levels of genes except ACE2 were significantly higher in DOCA than in sham-operated rats.

Perspectives
We first demonstrated that, in female rats, MR blockade reduced the incidence of cerebral aneurysm formation concomitant with a decrease in oxidative stress, inflammation, and the local RAS and that MR activation plays an important role in the formation of cerebral aneurysms. There are some limitations in our study. Because we did not use aged rats, our results do not reflect the possible effects of ageing. MR blockade was not sufficiently strong to completely prevent aneurysmal growth. Because the development of cerebral aneurysms involves multiple factors, other pathogenic mechanisms must be investigated. It remains unclear whether MR activation is associated with the rupture of cerebral aneurysms, because there are no reliable animal models of aneurysmal growth. In future studies, we need to establish a reliable experimental subarachnoid hemorrhage model and investigate the efficacy of MR blockade in preventing subarachnoid hemorrhage.

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Disclosures

None.

References

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**Role of mineralocorticoid receptor on experimental cerebral aneurysms in rats**

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Materials and Methods

Induction of experimental cerebral aneurysms

Cerebral aneurysms were induced as described by Jamous et al.\(^1\) All surgical procedures were performed under isofluorane (2-4%) inhalation. For aneurysm induction, 7-week-old female Sprague-Dawley rats were subjected to the simultaneous ligation of the right common carotid artery (CCA) to increase hemodynamic stress and the posterior branches of both renal arteries to induce hypertension. One week later we substituted their drinking water with a 1.0% saline solution and one month post-ligation they underwent bilateral oophorectomy (OVX). Immediately before the operation and again immediately before sacrifice we measured their blood pressure by the tail-cuff, auto-pickup method without any anesthesia. At 19 weeks of age, the rats were perfused transcardially with heparinized phosphate-buffered saline (PBS) and samples for vascular corrosion casts, histological study, and the determination of the mRNA level in the vascular wall were prepared. Age-matched female rats were sham-operated (sham group).

All experimental protocols were conducted in accordance with the Guiding Principles in the Care and Use of Animals of the American Physiological Society and were approved by the Animal Care Committee of the University of Tokushima.
Treatment regimens

**Treatment 1: MR antagonist**

Immediately after the ligation of the right CCA and the posterior branches of both renal arteries, the rats received eplerenone (30 mg/kg/day, Epl-30 group; 100 mg/kg/day, Epl-100 group) or vehicle (0.5% methylcellulose; VC group) in their food for 3 months. Eplerenone was kindly supplied by Pfizer Inc. (New York, NY).

**Treatment 2: DOCA**

We subjected 7-week-old female rats to ligation of the right CCA and at the same time we subcutaneously implanted 90-day-release pellets containing 200 mg deoxycorticosterone acetate (DOCA) (Innovative Research of America, Sarasota, FL) instead of performing renal artery ligation. One week later their drinking water was substituted with a 1.0% saline solution and one month post-ligation they underwent bilateral OVX. Two months after OVX they were euthanized as described above and samples for vascular corrosion casts and for the determination of aneurysmal mRNA were prepared.

**Preparation and study of vascular corrosion casts**

Vascular corrosion casts of control- (n=17), Epl-30- (n=11), Epl-100- (n=11), and DOCA rats (n=13) were prepared by transcardial infusion with Boston No. 17
plastic (Polyscience Inc., Warrington, PA). As our previous studies had indicated that cerebral aneurysms tended to be induced preferentially at the left anterior cerebral artery - olfactory artery (ACA-OA) bifurcation, this site was inspected at 3 kV under a scanning electron microscope (SEM) (VE8800, Keyence, Osaka, Japan). Based on our morphological findings we classified the left ACA-OA bifurcation as normal, indicating that there is neither arterial dilation nor are there irregular cell shapes, as exhibiting endothelial damage, indicating that the endothelial surface was rough and irregular without marked luminal dilation, and as harboring a cerebral aneurysm evidenced by changes such as moderate outward evagination or an obvious saccular aneurysm (Figure S1). To determine the aneurysmal size, 3 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.

**Measurement of plasma aldosterone levels and saline intake**

Twelve weeks after renal ligation the rats were anesthetized; plasma samples were preserved at -80°C until use. The plasma aldosterone concentration was measured by radioimmunoassay using an aldosterone RIA kit II (SRL, Tokyo, Japan). To examine the relationship between saline intake and aneurysm formation and mineralocorticoid receptor (MR), the saline intake in the course of 24 hr was recorded weekly throughout
the study period.

**Immunohistochemistry and cell counting**

Sham-operated- (n=15), VC- (n=15), and Epl-100 rats (n=13) were used for immunohistochemical study. At 12 weeks after renal ligation, the rats were deeply anesthetized and perfused transcardially with physiological saline followed by the injection of 4% paraformaldehyde. The left ACA-OA bifurcation was stripped, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Ted Pella, Inc. and PELCO International, Redding, California, USA), and cut into 5-μm sections. Sections from each sample were stained with elastica van Giesson (EvG) stain to detect the formation of cerebral aneurysms. After 30-min exposure to serum-free protein block (Dako, Carpinteria, CA) the slides were incubated overnight at 4°C with primary antibodies and then for 1 hr at room temperature with fluorescein-conjugated secondary antibodies in 1% BSA/PBS or Canget immunostain (Toyobo, Osaka, Japan) (Alexa Fluor594 and 488 donkey anti-mouse-, donkey anti-goat-, or goat anti-rabbit IgG; Molecular Probes, Eugene, OR). To identify cells, we used simultaneous 4’,6-diamino-2-phenylindole (DAPI) staining. Visualization was under a fluorescent microscope (IX71SIF-2, Olympus, Tokyo, Japan). Normal mouse- (Dako), goat- (Santa-Cruz, California, USA), or rabbit IgG (Dako) was the negative control. In all
staining procedures we referred to positive controls.

The primary antibodies we used were polyclonal anti-nitrotyrosine (1:200 dilution with Canget signal immunostain (CG stain), Upstate Biotechnology, Lake Placid, NY, USA), goat polyclonal anti-NOX4 (1:50 dilution with PBS, Santa-Cruz), rabbit polyclonal anti-Rac1 (1:50 dilution with PBS, Santa-Cruz), rabbit polyclonal anti-matrix metalloproteinase-9 (MMP-9) (1:50 dilution with PBS, Chemicon, California, USA), goat polyclonal anti-monocyte chemoattractant protein-1 (MCP-1) (1:50 dilution with PBS, Santa-Cruz), goat polyclonal anti-angiotensin II (1:50 dilution with PBS, Santa-Cruz), mouse monoclonal anti-CD68 (1:200 dilution with CG stain, AbD Serotec, Kidlington, UK), mouse monoclonal anti-smooth muscle α actin (1:200 dilution with PBS, Laboratory Vision, California, USA), and mouse monoclonal anti-MR (1:50 dilution with PBS, Abcam, Cambridge, UK). Areas containing CD68-positive cells in 150 x 150-μm fields around the aneurysm were assessed.

RNA isolation and quantitative RT-PCR

Samples from sham-operated- (n=7), VC- (n=7), Epl-100- (n=7), and DOCA rats (n=6), were subjected to quantitative real-time PCR (qRT-PCR). At 12 weeks after OVX, the rats were euthanized as described above. The left ACA-OA bifurcation was isolated and total RNA was extracted using the EZ1 RNA Universal Tissue Kit (QIAGEN,
Tokyo, Japan) and a MagNA lyser (Roche, Tokyo, Japan). Extracted RNA was treated with DNase (DNA-free; Ambion, Austin, TX) to remove genomic DNA. For reverse transcription of total RNA to cDNA we used the transcriptor first-strand cDNA synthesis kit (Roche). qRT-PCR of each sample was on a LightCycler 2.0 (Roche Diagnostics, Tokyo, Japan). LightCycler FastStart DNA master hybridization probes (Roche) were used for MR, NOX4, Rac1, MMP-9, MCP-1, p22phox, ACE1, and GAPDH. Primers and probe sets for MCP-1, ACE1, and GAPDH were from Roche and used according to the manufacturer’s directions. The other primers were:

5’-cttccccactgtcaatac-3’ and 5’-aagctctcatctccacaca-3’ for MR,
5’-acactctacggatgactgga-3’, and 5’-tctgtatccatctgtccact-3’ for NOX4,
5’-gaagctgactcccattacctac-3’ and 5’-cagcaggcatatcttccttc-3’ for Rac1, and
5’-ggctaggctcagagttaa-3’ and 5’-gacgtgtggtgtgtagtccag-3’ for MMP-9, and
5’-gccattggcagtgatcta-3’ and 5’-gacctcatgtccactgga-3’ for p22phox. The fluorescein/red-640 probes were:

5’-ggtgacctgtcagagaatgtggg-5’-tccgctctagactacattcagaaacgtg-3’ for MR,
5’-agactcattcattcagtgtgtgagcagac-3’-5’-tgctctatgtgctgatgaatggcaaa-3’ for NOX4,
5’-ctgctttaggctctgctgtgctg-3’-5’-gcactccaggatattcagcagcagcagcagcagct-3’ for Rac1, and
5’-ttcaatcctttccagatacgtgctccg-3’-5’-tgatcagcagagcctcctcaca-3’ for MMP-9, and
5’-cttcttgcggacctccgc-3’/5’-tgcctcctcttcggcctcactt-3’ for p22\textsuperscript{phox}. The PCR conditions were 95\textdegree C for 10 min, followed by 50 cycles at 95\textdegree C for 10 sec, 58\textdegree C (for MR, NOX4 and Rac1) or 61\textdegree C (for MMP-9 and p22\textsuperscript{phox}) for 15 sec, and 72\textdegree C for 7 sec. Fluorescence measurements were taken at the end of the annealing phase. FastStart DNA master plus SYBR Green I (Roche) was used for ACE2. The primers were 5’-cagacgtatgggtgagtgat-3’/5’-ggtggcttaagtgttgggta-3’. The PCR conditions were 95\textdegree C for 10 min followed by 50 cycles at 95\textdegree C for 10 sec, 60\textdegree C for 10 sec, and 72\textdegree C for 8 sec. We subjected samples from each group of rats to 2 independent qRT-PCR assays. GAPDH was the internal control.

**Statistical analysis**

Data (mean ± SD) were analyzed with the Mann-Whitney \textit{U} 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 with the Fisher exact test. P values of < 0.05 were considered to indicate statistical significance.
References


Figure S1. Representative images of the anterior cerebral artery - olfactory artery (ACA-OA) bifurcation on corrosion casts from each stage are shown. Bar = 50 μM
Figure S2. Plasma aldosterone levels determined by radioimmunoassay using an aldosterone RIA kit II in vehicle-control (VC)- and eplerenone-treated rats (30 mg/kg/day, Epl-30; 100 mg/kg/day, Epl-100) (each group n=7). Eplerenone significantly increased the plasma aldosterone level. Data (mean ± SD) were analyzed by one-way ANOVA followed by Scheffe’s test. †p < 0.01 vs VC. Epl indicates eplerenone; VC, vehicle control.
**Figure S3.**

A. Elastica van Gieson (EvG) and immunohistochemical staining for mineralocorticoid receptor (MR) (red). MR was expressed in the endothelium and smooth muscle cells of the aneurysm and parent arteries; eplerenone treatment reduced their expression. Bar = 50 μM

B. The mRNA level of MR was analyzed by quantitative real-time PCR in VC- and Epl-100 rats (each group n=7). The mRNA expression of MR was detectable in VC rats. Eplerenone significantly decreased the mRNA level of MR. Each mRNA level was normalized by GAPDH mRNA. Data (mean ± SD) were analyzed by the Mann-Whitney U test (n = 7 for each group). *p < 0.05 vs VC.
Figure S4.
Plasma aldosterone levels in sham-operated- (sham), renal hypertensive- (rHT), and DOCA rats (each group=7). The plasma aldosterone level was significantly lower in DOCA- than sham rats. Data (mean ± SD) were analyzed by one-way ANOVA followed by Scheffe’s test: *p < 0.05 vs sham.