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Pretreatment with eplerenone reduces stroke volume in mouse middle cerebral artery occlusion model

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Abstract

Eplerenone, a mineralocorticoid receptor antagonist, is reported to be effective to prevent end-stage cardiovascular damage induced by aldosterone. However, the effect of eplerenone on brain damage is not fully understood. Here, we investigated whether pretreatment with eplerenone attenuates stroke size in mice subjected to middle cerebral artery occlusion. Middle cerebral artery occlusion with a microfilament technique induced focal ischemia, to approximately 25% of the total area in a coronal section of the brain. Treatment with eplerenone at a dose of 1.67 mg/g chow significantly reduced the ischemic area, ischemic volume, and neurological deficit, without a blood pressure-lowering effect. Laser-Doppler flowmetry analysis showed a decrease in surface cerebral blood flow in the peripheral region after 1 h of middle cerebral artery occlusion. This decrease was smaller in mice treated with eplerenone. Superoxide production evaluated by staining with dihydroethidium was attenuated in the ischemic area of the brain in eplerenone-treated mice. Taken together, our findings suggest that eplerenone has a protective effect on ischemic brain damage, at least partly due to improvement of cerebral blood flow in the penumbra and reduction of oxidative stress.

Keywords: Stroke; Aldosterone; Eplerenone; Blood flow; Oxidative stress

1. Introduction

The renin-angiotensin-aldosterone system is well known as a regulator of normal cardiovascular homeostasis and to play an important role in the pathogenesis of a variety of cardiovascular diseases. Aldosterone, a potent mineralocorticoid and a final substance of renin-angiotensin-aldosterone system, has attracted further interest in its role in the development and progression of cardiovascular disease, such as blood pressure elevation, cardiovascular remodeling and myocardial fibrosis (Brilla and Weber, 1992). The classical genomic action of aldosterone has been described as binding to its intracellular mineralocorticoid receptors, followed by translocation of the steroid-receptor complex to the nucleus, where it acts as a transcriptional regulator to promote gene expression and protein synthesis (Timmermans, 1999; Xiao et al., 2004). Moreover, the existence of rapid non-genomic actions of aldosterone is supported by recent accumulating experimental evidence (Losel et al., 2002; Verrey, 1999).

A mineralocorticoid receptor antagonist, spironolactone, has been used for the treatment of hypertension, hyperaldosteronism and edematous states, primarily in patients with uncompensated heart failure. The results of the Randomized Aldactone Evaluation Study (RALES) demonstrated that low-dose spironolactone therapy markedly reduced morbidity and mortality in patients with severe heart failure, independent of hemodynamic effects (Pitt et al., 1999). However, in spite of the beneficial effects of spironolactone, patients treated with spironolactone suffer from side effects caused by antagonistic effects on the androgen receptor due to the low selectivity of spironolactone.

Eplerenone is a newly developed mineralocorticoid receptor antagonist with expectation of a reduction of blood pressure and greater protective effects on end organ damage than spironolactone, because it can potentially block mineralocorticoid

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receptor signaling, and also appears to block the rapid actions of aldosterone (Michea et al., 2005). Recently, a major randomized trial, EPHESUS (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study), demonstrated that eplerenone also markedly reduces mortality in patients with heart failure and induces left ventricular hypertrophy regression and blood pressure control as effectively as enalapril (Pitt et al., 2003b). Therefore, eplerenone is expected to have preventive effects on cardiovascular events.

Mineralocorticoid receptor is reported to be expressed in the brain (Anderson and Fanestil, 1976; Gomez-Sanchez et al., 1990), and mineralocorticoid receptor activation leads to inward hypertrophic remodeling and an increase in the ischemic damage in the event of a stroke (Dorrance et al., 2006). Moreover, cardiovascular complications, especially stroke, are commonly observed in patients with primary aldosteronism (Nishimura et al., 1999), indicating that aldosterone appears to play a major role in the development of stroke. Rocha et al. have demonstrated that eplerenone treatment prevents naturally occurring strokes in stroke-prone spontaneous hypertensive rats (SHRSP) (Rocha and Stier, 2001). Furthermore, in SHRSP, blockade of aldosterone by spironolactone reduces stroke-related mortality (Rocha et al., 1998) and the size of cerebral infarcts via a reduction in the expression of epidermal growth factor (EGF) receptor mRNA (Dorrance et al., 2001). Therefore, long-term blockade of aldosterone probably contributes to preventing stroke onset; however, the effect of eplerenone on brain damage and stroke expansion after stroke is totally unknown. Therefore, we investigated the effect of eplerenone on focal brain ischemia in mice induced by middle cerebral artery occlusion and assessed the mechanism of the cerebro-protective effects of eplerenone.

2. Materials and methods

2.1. Animals and pretreatment with eplerenone

Male C57BL/6J mice (10 weeks old) were purchased from Nihon Clea (Tokyo, Japan). They were housed in an air conditioned room at 25 °C with a 12 h light, 12 h dark cycle. Mean weight of mice at the beginning of the experiments was 23 ± 2 g. Eplerenone treatment was performed as referred to the previous report (Qin et al., 2003; Suzuki et al., 2006). Mice received rodent pellet chow, AIN-76A, with or without eplerenone (provided by Pharmacia Corporation Peapack, NJ) at 1.67 g/kg for 2 weeks before middle cerebral artery occlusion. The daily dose of eplerenone was approximately 150 mg/kg body weight. The amount of daily chow showed no difference between two groups. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

2.2. Blood pressure measurement

Systolic blood pressure was monitored in conscious mice by the tail-cuff method (MK-1030, Muromachi Co. Ltd., Tokyo, Japan) twice after 2 weeks of treatment. Mice were held in a small plastic holder on a warming pad thermostatically controlled at 37 °C. In these experimental conditions, blood pressure was monitored before and after one week of eplerenone treatment, and just before and 24 h after middle cerebral artery occlusion.

2.3. Middle cerebral artery occlusion

Focal cerebral ischemia was induced by occlusion of the left middle cerebral artery, by applying a modified intraluminal filament technique as described previously (Maeda et al., 1999; Pitt et al., 2003a). Briefly, mice were anesthetized with 120 mg/kg ketamine and 6 mg/kg xylazine in saline. A midline neck incision was made, and the left common and external carotid arteries were isolated. A silicon (Xantopren; Bayer Dental, Osaka, Japan)-coated nylon microfilament (Ethilon, Ethicon, Norderstedt, Germany) was inserted into the left common carotid artery, and occluded the middle cerebral artery. Mice were sacrificed 24 h after middle cerebral artery occlusion, by administration of an overdose of anesthetic drug, and a brain sample was removed.

2.4. Neurological score

Neurological deficit was evaluated 24 h after middle cerebral artery occlusion using the neurological scores developed by Huang et al. (1994). Neurological scores were defined as follows: 0, no neurological deficit; 1, failure to extend right forelimb; 2, circling to the contralateral side; 3, falling to the contralateral side at rest; 4, no spontaneous motor activity.

2.5. Measurement of infarct size

To evaluate the ischemic area in the brain, the extracted brain was sliced into six coronal sections with 1 mm thickness and stained with 2% 2,3,5-triphenyltetrasodium chloride (TTC). The ischemic area was determined morphometrically from the TTC-stained area as the percentage of the total area. Ischemic volume, expressed in mm^3 was calculated by linear integration of the corrected lesion area.

2.6. Laser-Doppler flowmetry

Regional cerebral blood flow in the middle cerebral artery territory was determined just before and after 0, 1 and 24 h of middle cerebral artery occlusion by laser-Doppler flowmetry (Omegaflo. FLO-C1; Omegawave, Tokyo, Japan) as previously described (Iwai et al., 2004). Briefly, mice were anesthetized with 120 mg/kg ketamine and 6 mg/kg xylazine in saline, and maintained at 37 °C with a heated mattress. The tip of the probe was fixed with a stereotactic frame using a tissue adhesive (Aron Alpha; Toa, Tokyo, Japan) to the intact skull over the territory supplied by the proximal part (core; 2 mm caudal to bregma and 6 mm lateral to midline) and the peripheral part (periphery; 2 mm caudal to bregma and 3 mm lateral to midline) of the middle cerebral artery (Connolly et al., 1996). Blood flow was expressed as a percentage of the value before middle cerebral occlusion.

2.7. Detection of superoxide anion in brain sections

Detection of superoxide anion was carried out as described previously (Szocs et al., 2002). In brief, frozen, enzymatically intact, 10-μm-thick sections were prepared from mouse brain 24 h after middle cerebral artery occlusion, and immediately incubated in dihydroethidium (DHE) in PBS for 30 min at 37 °C in a humidified chamber protected from light. Superoxide anion production in regions of interest has been described previously (Mogi et al., 2006). DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. Samples were examined with an Axioskop microscope (Axioskop 2 Plus with AxioCam, Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. Fluorescence of ethidium was detected with a 590 nm long-pass filter (Axioskop). Intensity of the fluorescence was analyzed and quantified using computer-imaging software (Densitograph, ATTO Corporation, Tokyo, Japan).

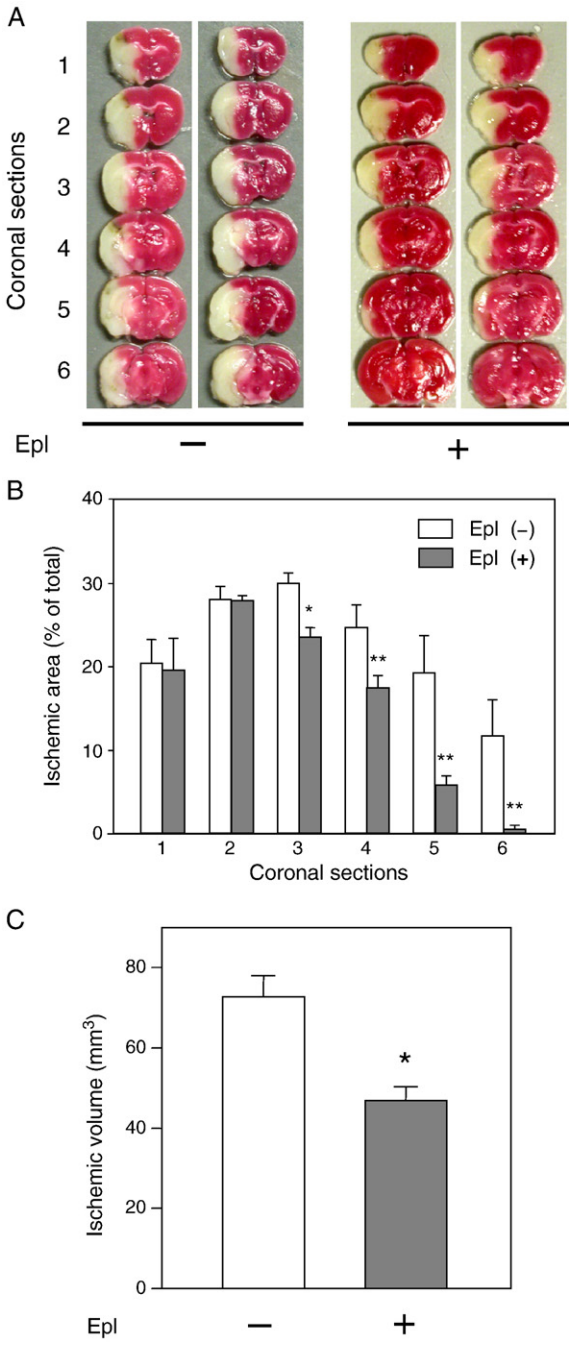

Fig. 1. Effects of eplerenone (1.67 mg/g chow) on focal ischemic area in mouse brain 24 h after middle cerebral artery occlusion. Staining of brain samples with 2,3,5-triphenyltetrasodium chloride (TTC) was performed as described in Materials and methods. (A) Representative photographs of brain samples with TTC staining of coronal sections of six parts of the brain. Each column represents an individual mouse. The ischemic area is shown as white, and the non-ischemic area as red. Epl, Eplerenone. Morphometric analysis of ischemic area in each section (B) and ischemic volume (C) in mice with or without eplerenone pretreatment (1.67 mg/g chow) for 2 weeks. *$P<0.05$ vs. no treatment, **$P<0.01$ vs. no treatment. Epl, eplerenone; $n=10$ per group.

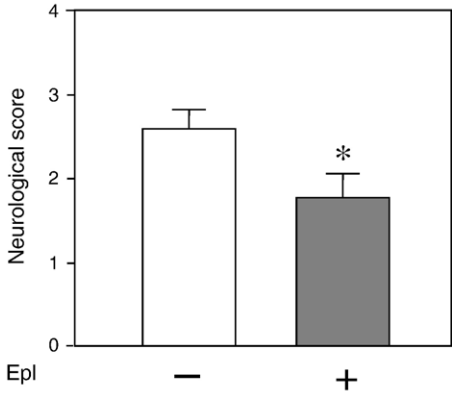

Fig. 2. Effects of eplerenone (1.67 mg/g chow) on neurological deficit 24 h after middle cerebral artery occlusion using neurological scores as described in Materials and methods. Score 0 indicates normal status. *$P<0.05$ vs. no treatment. Epl, eplerenone; $n=10$ per group.

2.8. Statistical analysis

All values were expressed as mean ± S.E.M. To determine the significance of differences between control and eplerenone-treated mice, Mann–Whitney rank sum test was used. A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Eplerenone pretreatment attenuated ischemic size after middle cerebral artery occlusion

The left middle cerebral artery was permanently occluded by placement of a nylon monofilament coated with silicon resin. Brain samples were obtained 24 h after middle cerebral artery occlusion and stained with TTC (Fig. 1A). In control mice without eplerenone treatment, the maximal ischemic area was found in section 3, and was about 30% of the total area (Fig. 1B). Administration of eplerenone at a dose of 1.67 mg/g chow attenuated the ischemic size by significantly reducing the ischemia area in the occipital region ((23% reduction in section 3 (especially in caudate putamen area); 32% reduction in section 4 (especially in caudate putamen area); 68% reduction in section

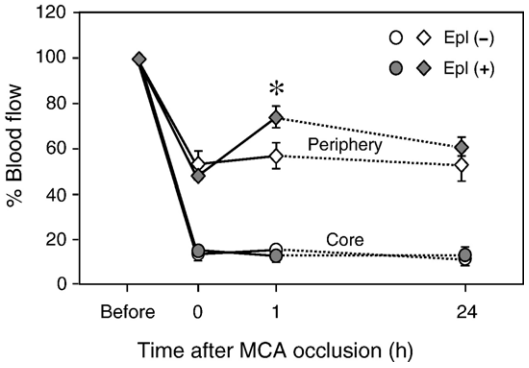

Fig. 3. Effects of eplerenone (1.67 mg/g chow) on cerebral blood flow. Cerebral blood flow was measured in the core and peripheral regions of the middle cerebral artery territory before and after middle cerebral artery occlusion as described in Materials and methods. *$P<0.05$ vs. no treatment. Epl, eplerenone; MCA, middle cerebral artery; $n=5$ per group.

5 (especially in caudate putamen and piriform cortex area); 91% reduction in section 6 (especially in lateral entorhinal cortex area)) (Paxinos and Franklin, 2004) (Fig. 1B). Pretreatment with eplerenone showed an approximately 36% reduction in the ischemic volume (72.7 mm^3 in control mice, 46.9 mm^3 in eplerenone-treated mice) (Fig. 1C). Mean systolic blood pressure of ten measurements in each group ($n=10$ per group) was not changed by treatment with eplerenone just before middle cerebral artery occlusion (control; 93.5 ± 1.5 mmHg vs. eplerenone treatment; 92.9 ± 3.9 mmHg, respectively) and 24 h after middle cerebral artery occlusion (control; 94.8 ± 3.5 mmHg vs. eplerenone treatment; 94.2 ± 2.6 mmHg, respectively).

3.2. Eplerenone pretreatment improved neurological deficit after middle cerebral artery occlusion

Mice usually exhibited some neurological deficit after middle cerebral artery occlusion, such as hemiplegia, loss of balance or no spontaneous motor activity. Neurological deficit was evaluated by neurological score 24 h after middle cerebral artery occlusion. Neurological score was lower in eplerenone-treated mice (average 1.8, range 1.5 to 2.1) compared with control mice (average 2.6, range 2.4 to 2.8), indicating that

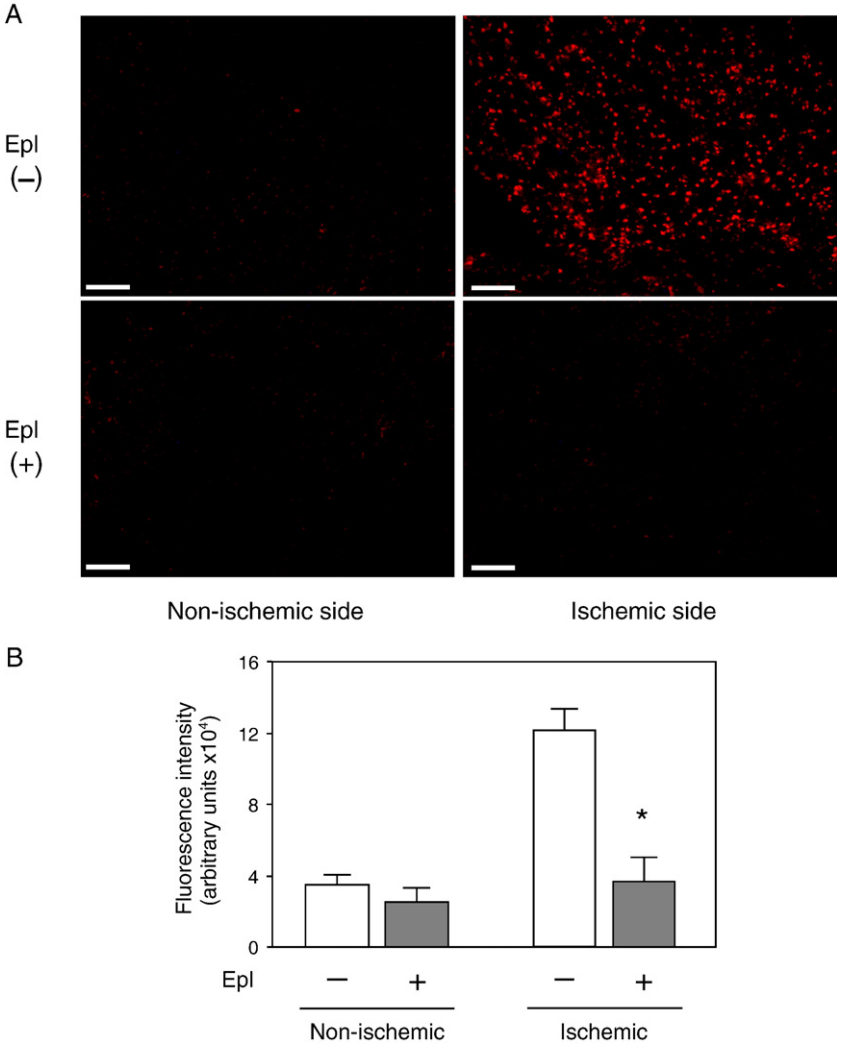

Fig. 4. Effects of eplerenone (1.67 mg/g chow) on superoxide anion production. Superoxide anion production was detected by staining with dihydroethidium. (A) Representative photographs of non-ischemic and ischemic regions in mice with or without eplerenone treatment. Scale bars: 250 μm. Epl: eplerenone. (B) Intensity analysis of superoxide production. *$P<0.01$ vs. ischemic side without eplerenone. Epl, eplerenone; $n=5$ per group.

pretreatment with eplerenone could prevent neurological deficit after focal brain ischemia (Fig. 2).

3.3. Eplerenone pretreatment ameliorated the reduction of cerebral blood flow after middle cerebral artery occlusion

To assess the mechanism of the reduction of infarct size, cerebral blood flow was measured in the core and peripheral regions of the middle cerebral artery territory before and after middle cerebral artery occlusion (Fig. 3). Just after middle cerebral artery occlusion, cerebral blood flow in both the core and periphery decreased, with an approximately 90% reduction of basal level in the core and a 40% reduction in the periphery. In our previous report (Iwai et al., 2004), cerebral blood flow at time zero of middle cerebral artery occlusion showed no significant difference between treatment with and without a cerebroprotective drug. Pretreatment with eplerenone did not affect cerebral blood flow in the core region; however, approximately 32% increased blood flow was observed in the peripheral region of the middle cerebral artery territory of eplerenone-treated mice after 1 h of middle cerebral artery occlusion (Fig. 3).

3.4. Eplerenone pretreatment suppressed superoxide production after middle cerebral artery occlusion

Next, to examine the effect of oxidative stress on stroke size, the production of superoxide anion was estimated by DHE staining. Superoxide production in the peripheral territory of middle cerebral artery was increased in the ipsilateral side, compared with the contralateral side (Fig. 4A). Eplerenone pretreatment markedly attenuated such production in the ischemic area. (Fig. 4A, B).

4. Discussion

Here, we demonstrated that a non-hypotensive dose of mineralocorticoid receptor antagonist, eplerenone, reduced the stroke size after middle cerebral artery occlusion in mice. We speculated that these inhibitory effects of eplerenone on ischemic size in the brain were at least partly due to improvement of the early phase of cerebral blood flow in the peripheral region of the ischemic area and prevention of superoxide production in the injured brain. Our findings showed evidence that eplerenone at a non-hypotensive dose for blood pressure reduces the ischemic size after middle cerebral artery occlusion. Blockade of aldosterone is known to be effective to reduce blood pressure. Indeed, the efficacy of eplerenone as an antihypertensive drug is well documented (Krum et al., 2002; Weinberger et al., 2002), and it prevents naturally occurring strokes in SHRSP (Rocha and Stier, 2001). It, as well as spironolactone, was reported to decrease blood pressure and result in a reduction of intracerebral hemorrhage in hypertensive rats (MacLeod et al., 1997). Moreover, very recent paper demonstrates that spironolactone improves structure and increases tone in the cerebral vasculature of male SHRSP (Rigsby et al., in press), indicating that long-term administration of spironolactone is effective to prevent stroke onset. Thereby, blockade of mineralocorticoid receptor signaling could be therapeutically useful to prevent both “infarct expansion” and “stroke onset”.

The cellular and molecular mechanisms underlying the protective effects of eplerenone have not been elucidated. Stroke enhances superoxide production (Chan, 2001; Lerouet et al., 2002). Administration of aldosterone increases macrophage superoxide production (Keidar et al., 2004), and affect peripheral blood monocytes (Ahokas et al., 2003) and vascular smooth muscle cells (Mazak et al., 2004) by production of reactive oxygen species. Aldosterone also causes vascular inflammation and vascular injury in several animal models through increasing oxidative stress (Joffe and Adler, 2005), resulting in impairment of cerebral blood flow. The expression of cell adhesion molecules induced by inflammation is associated with the cerebral infarct size in animal models of middle cerebral artery occlusion (Frijns and Kappelle, 2002). Superoxide production in the peripheral, but not central, territory of middle cerebral artery was increased in the ipsilateral side, compared with the contralateral side. Therefore, the reduction of superoxide production may be induced by the preventing effect of decrease in cerebral blood flow after stroke. Recently, we reported that eplerenone attenuates aldosterone-induced NADPH oxidase activity and inflammation in vascular smooth muscle cells and reduces the development of atherosclerotic lesion (Suzuki et al., 2006). In this study, we analyzed the brain ischemia in the acute phase, 24 h after middle cerebral artery occlusion. However, we did not observe an increase in NADPH activity in the brain, cultured mouse neurons or monocytes after aldosterone stimulation (data not shown), indicating that the beneficial effects of eplerenone may be caused by mainly acute vascular protective effects through the inhibition of superoxide production in cerebral vessels, but not by a neuroprotective mechanism. Further studies to address the pathological significance of the effect of treatment with eplerenone on oxidative stress and its effects on ischemic stroke have to be performed.

On the other hand, mineralocorticoid receptors “in neurons” have been reported to have diverse effects on stroke (Rigsby et al., 2005). For example, mineralocorticoid receptor activation protects neurons by an increase in an antiapoptotic gene, B-cell leukemia/lymphoma 2 (Almeida et al., 2000). Blockade of mineralocorticoid receptor in neurons could facilitate and inhibit the neuronal damage caused by stroke. Therefore, the effect of mineralocorticoid blockade on stroke appears to depend on a balance between the effect on the vasculature and neurons. Further studies are necessary to investigate neural damage after stroke in this model.

We recently reported cross-talk of growth-promoting signaling between aldosterone and angiotensin II in vascular smooth muscle cells. Aldosterone exerts a synergistic mitogenic effect with angiotensin II via non-genomic and genomic pathways of aldosterone (Min et al., 2005). Moreover, we also reported possible reduction of the ischemic area by angiotensin II type 1 receptor blocker treatment via angiotensin II type 2 receptor stimulation (Iwai et al., 2004). Taken together, these results indicate that blockade of both angiotensin II and aldosterone with a mineralocorticoid antagonist and an angiotensin

II type 1 receptor blocker has potential as a treatment to reduce the ischemic area as well as prevent cardiovascular disease. Further studies of combination therapy with eplerenone and an angiotensin II type 1 receptor blocker will be needed to clarify the synergistic advantage of multiple blockade of the renin-angiotensin-aldosterone system for stroke.

In conclusion, our findings add a new insight into the possibilities that pretreatment with eplerenone would reduce the severity of brain damage after ischemic stroke beyond its antihypertensive effect.

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The authors declare that they have no conflicts of interest.

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