Attenuation of Brain Damage and Cognitive Impairment by Direct Renin Inhibition in Mice With Chronic Cerebral Hypoperfusion

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Abstract—The role of the renin-angiotensin system in cognitive impairment is unclear. This work was undertaken to test our hypothesis that renin-angiotensin system may contribute to cognitive decline and brain damage caused by chronic cerebral ischemia. C57BL/6J mice were subjected to bilateral common carotid artery stenosis with microcoil to prepare mice with chronic cerebral hypoperfusion, a model of subcortical vascular dementia. The effects of aliskiren, a direct renin inhibitor, or Tempol, a superoxide scavenger, on brain damage and working memory in these mice were examined. Chronic cerebral hypoperfusion significantly increased brain renin activity and angiotensinogen expression in C57BL/6J mice, which was attributed to the increased renin in activated astrocytes and microvessels and the increased angiotensinogen in activated astrocytes in white matter. Aliskiren pretreatment significantly inhibited brain renin activity and ameliorated brain p67phox-related NADPH oxidase activity, oxidative stress, glial activation, white matter lesion, and spatial working memory deficits in C57BL/6J mice with bilateral common carotid artery stenosis. To elucidate the role of oxidative stress in brain protective effects of aliskiren, we also examined the effect of Tempol in the same mice with bilateral common carotid artery stenosis. Tempol pretreatment mimicked the brain protective effects of aliskiren in this mouse model. Posttreatment of mice with aliskiren or Tempol after bilateral common carotid artery stenosis also prevented cognitive decline. In conclusion, chronic cerebral hypoperfusion induced the activation of the brain renin-angiotensin system. Aliskiren ameliorated brain damage and working memory deficits in the model of chronic cerebral ischemia through the attenuation of oxidative stress. Thus, direct renin inhibition seems to be a promising therapeutic strategy for subcortical vascular dementia.

Key Words: vascular dementia • chronic cerebral ischemia • oxidative stress • renin • working memory • white matter lesion

Subcortical vascular dementia, one of the major subtypes of vascular dementia, is characterized by white matter (WM) changes and lacunar infarctions and is caused by a chronic decrease in cerebral blood flow attributed to small vessel disease. However, the precise mechanism of subcortical vascular dementia remains to be defined. A preventive or therapeutic strategy for subcortical vascular dementia has not been fully established.

Aliskiren is the first in a new class of direct renin inhibitors approved for the treatment of hypertension. Differing from angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers, aliskiren blocks the renin-angiotensin system (RAS) by directly inhibiting the enzymatic activity of renin and thereby suppressing the formation of angiotensin I from angiotensinogen. Aliskiren has been suggested to be at least as effective as the conventional RAS blockers in blood pressure (BP)–lowering effect on hypertensive patients, renoprotective effect against diabetic nephropathy, and improvement of biomarkers in heart failure patients. However, in contrast to many experimental reports showing the protective effects of aliskiren against cardiovascular and renal injuries, at present there is no available information on the potential effect of direct renin inhibition with aliskiren on cognitive function in any experimental and clinical work.

In the present work, using an established mouse model of subcortical vascular dementia, we investigated the effect of direct renin inhibition on cognitive impairment to test our hypothesis that RAS may be involved in cognitive impairment. We obtained the first evidence that brain RAS plays a pivotal role in the development of brain damage and cognitive impairment induced by chronic cerebral ischemia.

Received March 22, 2011; first decision April 14, 2011; revision accepted July 19, 2011.

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Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.111.173534

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Experiment I: Effects of Aliskiren on BP and Cerebral Blood Flow in C57BL/6J Mice With Chronic Cerebral Hypoperfusion

As shown in Figure S1, available in the online Data Supplement, aliskiren pretreatment did not significantly change BP and cerebral blood flow of C57BL/6J mice subjected to BCAS surgery.

Effects of Aliskiren on Spatial Working Memory in C57BL/6J Mice With Chronic Cerebral Hypoperfusion

As shown in Figure 1, the spatial working memory estimated by Y-maze test (spontaneous alternation) in mice with chronic cerebral hypoperfusion was significantly impaired at 2 (P<0.05) and 3 (P<0.05) weeks after the surgery as compared with mice with sham operation. Aliskiren pretreatment significantly prevented the impairment of spatial working memory at 2 (P<0.05) and 3 (P<0.05) weeks as compared with vehicle treatment. There was no significant difference in the number of arm entries among sham-operated mice, aliskiren-treated mice with chronic cerebral hypoperfusion, and vehicle-treated mice with chronic cerebral hypoperfusion (Figure 1).

Effects of Aliskiren on Cerebral Hypoxia, WM Lesions, and Glial Activation in C57BL/6J Mice With Chronic Cerebral Hypoperfusion

As shown in Figure 2A, chronic cerebral hypoperfusion induced by BCAS surgery in C57BL/6J mice caused significant cerebral hypoxia estimated by using hypoxyprobe (P<0.01) as compared with sham operation. Aliskiren pretreatment significantly attenuated cerebral hypoxia in mice with chronic cerebral hypoperfusion (P<0.05).

Chronic cerebral hypoperfusion induced significant WM lesions in the corpus callosum in mice (P<0.01), and the occurrence of WM lesions was significantly ameliorated by
aliskiren pretreatment ($P<0.05$; Figure 2B). The number of glial fibrillary acidic protein–positive astrocytes (Figure 2C) and Iba-1–positive microglia (Figure 2D) were significantly increased in the corpus callosum in mice with chronic cerebral hypoperfusion, and aliskiren pretreatment significantly attenuated the activation of astrocytes ($P<0.01$) and microglia ($P<0.05$).

**Effects of Aliskiren on Cerebral Nitrotyrosine, NADPH Oxidase, p67phox, Superoxide Dismutase Isoforms, Endothelial NO Synthase, Monocyte Chemoattractant Protein 1, and Tumor Necrosis Factor-α in C57BL/6J Mice With Chronic Cerebral Hypoperfusion**

As shown in Figure 3A, chronic cerebral hypoperfusion in mice significantly increased cerebral nitrotyrosine levels ($P<0.05$). Aliskiren pretreatment significantly attenuated the hypoperfusion-induced increase in cerebral nitrotyrosine levels ($P<0.01$). As shown in Figure 3B and 3C, chronic cerebral hypoperfusion significantly increased cerebral NADPH oxidase activity and its subunit p67phox in mice. Aliskiren pretreatment significantly prevented the increase in NADPH oxidase activity ($P<0.05$) and p67phox ($P<0.01$) in these mice. There was no significant difference in cerebral Cu/Zn superoxide dismutase (SOD), MnSOD, and extracellular SOD among sham, vehicle, and aliskiren groups (Figure S2). As shown in Figure 3D and 3E, cerebral mRNA expression of proinflammatory cytokines monocyte chemoattractant protein 1 and tumor necrosis factor-α was significantly increased in mice with chronic cerebral hypoperfusion. These increases in monocyte chemoattractant protein 1 and tumor necrosis factor-α were significantly prevented by aliskiren pretreatment.

**Brain Angiotensinogen and Renin Expressions in C57BL/6J Mice With Chronic Cerebral Hypoperfusion and the Effects of Aliskiren on Brain Renin**

As shown in Figure 4A, chronic cerebral hypoperfusion significantly increased the expression of angiotensinogen protein and mRNA levels in C57BL/6J mice brain. As shown in Figure 4B, this increase in angiotensinogen expression was associated with the increased angiotensinogen expression in glial fibrillary acidic protein–positive astrocytes (activated astrocytes) in the WM in mice ($P<0.01$).

As shown in Figure 4C, chronic cerebral hypoperfusion significantly enhanced renin expression in both glial fibrillary
Experiment II: Effects of Tempol on C57BL/6J Mice With Chronic Cerebral Hypoperfusion

To examine whether cerebral oxidative stress is involved in brain injury induced by BCAS surgery, we also examined the effect of Tempol on C57BL/6J mice with chronic cerebral hypoperfusion. Tempol pretreatment did not affect systolic BP (Figure S4) and cerebral blood flow (data not shown) of C57BL/6J mice with chronic cerebral hypoperfusion. As shown in Figure 5A, Tempol pretreatment significantly decreased cerebral nitrotyrosine levels in mice with chronic cerebral hypoperfusion (P<0.05). As shown in Figure 5B through 5F and Figure S5, Tempol pretreatment significantly ameliorated the impairment of spatial working memory (P<0.05), cerebral hypoxia (P<0.05), WM lesions (P<0.05), and the activation of astrocytes (P<0.01) and microglia (P<0.05).

Experiment III: Effect of Posttreatment With Aliskiren or Tempol After BCAS Surgery

We also examined the effect of posttreatment with aliskiren or Tempol at 24 hours after BCAS surgery on cognitive decline. As shown in Figure 6, the start of administration of aliskiren or Tempol at 24 hours after BCAS surgery significantly prevented the impairment of spatial working memory (spontaneous alteration) at 2 or 3 weeks in mice with chronic cerebral hypoperfusion, as estimated by Y-maze test.

Discussion

In the present work, we investigated for the first time the protective effect of direct renin inhibition with aliskiren against cognitive impairment and WM lesions. The 3 major findings of this study were as follows: (1) chronic cerebral hypoperfusion caused the significant activation of brain RAS as shown by the increased renin activity and the marked induction of angiotensinogen in brain; (2) direct renin inhibition, through inhibition of brain renin, directly prevented chronic cerebral hypoperfusion-induced WM lesions and cognitive impairment; (3) the underlying mechanism of prevention of cognitive impairment by aliskiren was mediated by the attenuation of NADPH oxidase–mediated nitrosative stress and subsequent inhibition of glial activation. Our present work provided the first evidence showing that renin plays a key role in the development of WM lesions and cognitive impairment caused by chronic cerebral hypoperfusion. Thus, our findings highlight direct renin inhibition as a promising therapeutic strategy for subcortical vascular dementia.

In the present work, to examine the role of renin in cognitive impairment, we used an established mouse model with chronic cerebral hypoperfusion, which was prepared by BCAS surgery with microcoil.11,12 Although it is well known that renin-/angiotensinogen- or angiotensin-converting enzyme-deficient mice showed a reduction in brain renin activity,11,12 we used aliskiren, a direct renin inhibitor, in the present study. Our findings in this study support the idea that renin is involved in the development of chronic cerebral hypoperfusion-induced WM lesions and cognitive impairment.
reports establish that this model specifically exhibits glial activation, WM lesions, and working memory deficits but does not display any apparent gray matter lesions. Therefore, this model is regarded as an appropriate model of subcortical vascular dementia. At present, there is only one available report 13 investigating the effect of a RAS blocker on this model of subcortical vascular dementia. In this previous report, telmisartan was used as an RAS blocker. However, importantly, it has been established that telmisartan has peroxisome proliferator-activated receptor-\(\gamma\)-modulating activity and inhibits angiotensin II type 1 receptor. Interestingly, the amelioration of working memory deficits in this model by telmisartan treatment was abolishe by cotreatment with GW9662, a peroxisome proliferator-activated receptor-\(\gamma\) antagonist, suggesting the critical role of peroxisome proliferator-activated receptor-\(\gamma\) activation rather than angiotensin II type 1 receptor blockade in brain protection of telmisartan in this model. Thus, the significant peroxisome proliferator-activated receptor-\(\gamma\) activation induced by telmisartan in the previous report hampered the determination of the precise role of RAS in cognitive function in this model.13

These findings encouraged us to investigate the precise involvement of RAS in this model by using a direct renin inhibitor aliskiren.

Our present work provided the first evidence that chronic cerebral hypoperfusion caused the increase in brain renin activity and angiotensinogen expression, and these increases were attributed to the enhancement of renin expression in activated astrocytes and microvessels and the enhancement of angiotensinogen in activated astrocytes. Hence, chronic cerebral hypoperfusion seems to cause the activation of brain RAS. Of note, aliskiren pretreatment significantly inhibited the increase in brain renin activity in this model with chronic cerebral hypoperfusion. This inhibition of brain renin by aliskiren might be explained by the fact that the blood-brain barrier is significantly disrupted in this mouse model, although blood-brain barrier disruption was not investigated in this study. Moreover, aliskiren significantly prevented glial activation, the development of WM lesions, and the disruption of spatial working memory in this model with chronic cerebral hypoperfusion. Taken together, our results showed that brain renin is involved in the development of cognitive decline induced by chronic cerebral ischemia.
Accumulating evidence indicates that oxidative stress generated by NADPH oxidase plays a key role in the pathophysiology of ischemia-induced cognitive decline and brain injury. Furthermore, angiotensin II, the biologically active final product of RAS, enhances oxidative stress through involvement of nitroxidative stress in aliskiren-induced brain injury. Furthermore, angiotensin II, the biologically active final product of RAS, enhances oxidative stress through activation of NADPH oxidase in cardiovascular and brain tissues. Therefore, in this study, we examined the effect of aliskiren on brain oxidative stress. Aliskiren pretreatment in the mouse model with chronic cerebral hypoperfusion significantly attenuated the increase in brain nitrotyrosine, a useful marker of peroxynitrite generated by the reaction of superoxide anion and NO, thereby suggesting the potential involvement of nitroxidative stress in aliskiren-induced brain protection. Furthermore, we also examined the effect of aliskiren on NADPH oxidase and SOD isoforms, because these enzymes are involved in the regulation of oxidative stress. Interestingly, despite no alteration of SOD isoforms, cerebral NADPH oxidase and its important subunit p67phox were significantly increased by chronic cerebral hypoperfusion, and aliskiren markedly prevented the increase in these parameters. Our results support the notion that the attenuation of oxidative stress by aliskiren pretreatment was at least partially attributed to the inhibition of NADPH oxidase. To confirm the critical role of oxidative stress in the brain protective effects of aliskiren, we examined the effect of Tempol, a superoxide scavenger, in this mouse model. We

**Figure 5.** Effects of Tempol on brain nitrotyrosine levels (A), spatial working memory after 2 weeks of bilateral common carotid artery stenosis (BCAS; B), cerebral hypoxia (C), white matter lesions (D), glial fibrillary acidic protein (GFAP)-positive astrocytes (E) and Iba-1-positive microglia (F) in C57BL/6J mice with chronic cerebral hypoperfusion. Sh indicates sham-operated C57BL/6J mice; Ve, vehicle-treated mice; Te, Tempol-treated mice. Values are mean±SEM (each group included n=6 to 8 in A, n=9 in B, n=8 in C, n=5 in D, n=8 in E, n=8 in F).

**Figure 6.** Posttreatment with aliskiren or Tempol after bilateral common carotid artery stenosis (BCAS) surgery prevents cognitive impairment. A indicates spontaneous alternation and number of arm entries at 2 weeks after BCAS surgery, and B indicates those at 3 weeks after BCAS surgery. Sh indicates sham-operated mice; Ve, vehicle (saline)-treated mice; Al, aliskiren-treated mice; Te (1), Tempol (1 mmol/L)-treated mice; Te (5), Tempol (5 mmol/L)-treated mice. Values are mean±SEM (n=8 to 10 in each group).
found that the attenuation of brain nitrooxidative stress with Tempol pretreatment in the mice with chronic cerebral hypoperfusion led to the prevention of WM lesions and spatial working memory deficits, accompanied by the prevention of glial activation. Collectively, our present observations demonstrated that brain oxidative stress is involved in the underlying mechanism of brain protection by aliskiren in this model.

**Study Limitation**

Several study limitations should be considered in this work. First, although we examined the effects of both pretreatment and posttreatment with aliskiren on the mouse model with chronic cerebral hypoperfusion, posttreatment was initiated at 24 hours after BCAS surgery. Therefore, the present work did not allow us to determine whether aliskiren can be effective on mice with established cognitive impairment. Second, the potential role of protein kinase C20 and cyclooxygenases,26 which play a critical role in oxidative stress-induced brain injury, was not clarified in the present study. Finally, another potential mechanism(s) than NADPH oxidase, responsible for amelioration of hypoxic damage by aliskiren, was not examined in this study. Particularly, in this study, the effect of Tempol was less than aliskiren in preventing BCAS-induced adverse effects. Therefore, it cannot be excluded that the brain-protective effects of aliskiren observed in this study might be partially attributed to other mechanisms than attenuation of oxidative stress. Further study is needed to elucidate the detailed molecular mechanism underlying the brain protective effects of aliskiren.

In conclusion, for the first time we demonstrated that chronic cerebral hypoperfusion caused the activation of brain RAS, and direct renin inhibition led to the prevention of WM lesions and cognitive impairment caused by chronic cerebral hypoperfusion. Therefore, brain renin is involved in the pathogenesis of cognitive decline induced by chronic brain ischemia. Furthermore, the mechanism of brain protection by renin inhibition was attributed to the attenuation of brain oxidative stress. Our present work highlights direct renin inhibition as a promising therapeutic strategy for subcortical vascular dementia, although future clinical study is warranted to demonstrate our hypothesis.

**Perspectives**

Hypertension is an important risk factor of cognitive decline or vascular dementia. Hypertension leads to chronic cerebral hypoperfusion via small vessel disease, including vascular endothelial dysfunction and vascular remodeling, thereby causing lacunar infarction and WM lesions. Thus, hypertension is regarded as one of the main causes of subcortical vascular dementia. By using an established model of human subcortical vascular dementia, we obtained the first experimental evidence that brain renin and angiotensinogen are enhanced in the WM under chronic cerebral hypoperfusion, and direct renin inhibition causes the suppression of brain damage and cognitive impairment in subcortical vascular dementia. Thus, our present work provided a novel insight into the molecular mechanism and the therapeutic strategy of subcortical vascular dementia in hypertension. However, future clinical study is required to demonstrate our proposal.

**Sources of Funding**

This work was supported by a grant from Novartis Pharmaceutical Corporation.

**Disclosures**

None.

**References**


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Hypertension. 2011;58:635-642; originally published online August 22, 2011; doi: 10.1161/HYPERTENSIONAHA.111.173534

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Attenuation of brain damage and cognitive impairment by direct renin inhibition in mice with chronic cerebral hypoperfusion

Short title: Renin and subcortical ischemic vascular dementia

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**Online methods**

**Animals and drugs**

All experiments on animals were approved by Committee for Laboratory Animal Care and Use in Kumamoto University. All experimental procedures were performed in accordance with Guidelines on Animal Experimentation released by Japanese Association for Laboratory Animal Science. Wild-type mice (C57BL/6J) (SLC, Shizuoka, Japan) were used for this study. All mice were housed in an animal facility with a 12-hour light-dark cycle and were given the standard chow and water ad libitum.

Aliskiren was kindly gifted from Novartis (Basel, Switzerland). The IC$_{50}$ for aliskiren against mouse renin is 4.5 nmol/L. Previous report demonstrated that administration of aliskiren at 2.5 mg/kg/day via osmotic minipump in mice gives plasma aliskiren concentrations which is 5-fold higher than IC$_{50}$ for mouse renin and significantly decreases plasma concentrations of angiotensin peptides including angiotensin II, indicating that this dose of aliskiren significantly inhibits renin-angiotensin system in vivo. Furthermore, this dose of aliskiren is known not to significantly lower blood pressure of mice. Therefore, 2.5 mg/kg/day of aliskiren is an appropriate dose to examine the effect of renin inhibition on brain injury and cognitive impairment in mice. Therefore, in this study, the dose of aliskiren used was 2.5 mg/kg/day.

Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl), a superoxide scavenger, was purchased from Sigma–Aldrich Co. In this study, tempol (1 or 5 mmol/L in drinking water) was orally given to mice. These doses of tempol has been demonstrated to significantly attenuate tissue oxidative stress in mice in vivo.

**Experimental protocol**

**Experiment I:** Eleven-week-old male C57BL/6J mice were divided into 2 groups and were continuously given (1) vehicle (saline) or (2) aliskiren (2.5 mg/kg/day). Aliskiren was subcutaneously infused to mice via ALZET® micro-osmotic pump (DURECT Co., Cupertino, CA). Five days after the start of aliskiren or saline infusion, mice were subjected to bilateral common carotid artery stenosis (BCAS) surgery, and aliskiren or saline infusion was continued for further 3 weeks. C57BL/6J mice with sham operation were served as control. Three weeks after the BCAS surgery, mice were deeply anesthetized by ether and arterial blood was collected by cardiac puncture. After that, phosphate-buffered saline was used to perform the cardiac perfusion and brain was rapidly excised from mice for the measurement of various parameters as described below. Brain samples were subjected to Klüver-Barrera staining for evaluation of white matter (WM) lesions. Brain astrocyte and microglia activation, and hypoxia were evaluated by immunostaining of glial fibrillary acidic protein (GFAP, a marker for astrocyte), ionized calcium binding adaptor molecule-1 (Iba-1, a marker for microglia), and Hypoxyprobe, respectively. Quantification of renin-positive astrocytes and angiotensinogen-positive astrocytes in the paramedian of corpus callosum were made by double immunofluorescent staining of GFAP and renin or GFAP and angiotensinogen.

Quantification of renin-positive microvessels was made by immunostaining of renin. Brain nitrotyrosine level and brain renin activity were measured using the commercial kits. Brain eNOS and angiotensinogen were detected by western blot. Brain monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) were detected by quantitative real time PCR.
Experiment II: Five days before BCAS surgery, eleven-week-old male C57BL/6J mice were divided into 2 groups and were given (1) vehicle or (2) tempol (1 mmol/L in drinking water). After 5 days of tempol treatment, mice were subjected to BCAS surgery and were continuously treated with tempol for further 3 weeks. Sham-operated C57BL/6J mice were served as control. Mice were sacrificed in the same manner as Experiment I.

Experiment III: We also examined the effect of post-treatment with aliskiren or tempol after BCAS surgery. C57BL/6J mice were divided into 4 groups, and treated with (1) vehicle, (2) aliskiren (2.5 mg/kg/day via osmotic minipump), (3) tempol (1 mmol/L in drinking water), or (4) tempol (5 mmol/L in drinking water). The administration of each drug was started at 24 hours after BCAS surgery. Sham-operated mice were served as the control. Mice were sacrificed in the same manner as Experiment I.

In Experiments I, II and III, systolic blood pressure (BP) was measured by tail-cuff plethysmography (BP-98A; Softron Co, Tokyo, Japan) before and 5 days after the start of drug treatment, and 1 week after the BCAS surgery. Cerebral blood flow (CBF) was measured before BCAS, and 10 minutes and 3 weeks after BCAS surgery. Spatial working memory was assessed by the Y-maze test at 2 or 3 weeks after BCAS surgery.

Surgical procedure of BCAS surgery

BCAS surgery was performed as previously described. In brief, mice were anesthetized with isoflurane (1.5% Vol.), held on a warming pad and thermostatically controlled at 37°C. Both common carotid arteries were exposed through a midline cervical incision, and a microcoil with an inner diameter of 0.16 mm was applied to the bilateral common carotid arteries.

Measurement of CBF

CBF of the mice was recorded by a laser speckle blood flow imager (Omega Zone; Omegawave, Tokyo, Japan) under anesthesia with isoflurane (1.5% Vol.). Mice were held on a warming pad and the body temperatures were thermostatically controlled at 37°C from the start of anesthesia and during the CBF measurement. Mice, under anesthesia with isoflurane, were placed in the prone position, the skull was exposed by a midline scalp incision, and the surface of the region of bilateral cerebral hemispheres was diffusely illuminated by 780 nm semiconductor laser light. The scattered light was filtered by a hybrid filter to detect only scattered light that had perpendicular polarization to the incident laser light so that stable and specific measurements were achieved. Color-coded blood flow images obtained in high-resolution mode (638×480 pixels; 1 image/sec) were captured by a CCD camera positioned above head and transferred to a computer for analysis. Image pixels were analyzed by the color image program incorporated in the flowmetry system to obtain the average perfusion values. 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 blood flow was expressed as a percentage of the baseline blood flow.

Y-maze test for spatial working memory assessment

The spatial working memory was evaluated by Y-maze test as described previously.
Briefly, Y-maze, consisting of 3 arms with 40 cm long, 13.5 cm high and 4 cm wide, was located in a dim room and a video camera was mounted on the ceiling above the apparatus (Muromachi Kikai, Tokyo, Japan). Each mouse was placed at the end of an arm and allowed to move freely through the maze for 8 minutes session. Spontaneous alteration was defined as the consecutive entry of a mouse into all three different arms to form a triplet of non-repeated components. The percentage of spontaneous alteration was calculated as the ratio of actual to possible alterations automatically by the Y-maze system (defined as the number of spontaneous alteration behavior / (the total number of arm entries - 2) × 100) (Muromachi Kikai, Tokyo, Japan). The total number of arms entered during the sessions, which reflect locomotor activity, was also recorded.

**Measurement of cerebral hypoxia**

Cerebral hypoxia was estimated with Hypoxyprobe™-1 (Hypoxyprobe, Burlington) according to the manufacturer’s instructions. In brief, Hypoxyprobe™-1 (60 mg/kg body weight) was intraperitoneally injected to mice before sacrifice. At 15 minutes after the injection, mice were anesthetized with ether, and then the brain was immediately perfused with phosphate-buffered saline and removed. Pimonidazole, which is an element of Hypoxyprobe™-1 and reductively activated in hypoxic cells, was stained with DAB (DAKO, Glostrop, Denmark). Photos of the immunostained brain samples were captured by a CCD camera (Nikon, Tokyo, Japan) using the same settings in all cases. The optical density of DAB signal was analyzed and quantified using Image-Pro Plus v6 analysis software (Media Cybernetics, Inc., USA). Data were expressed as the relative optical density to that of sham-operated mice.

**Brain renin activity**

Brain samples were homogenized on ice in 1×PBS, pH 7.4. Homogenates were sequentially centrifuged at 10,000×g at 4 °C for 20 min and the collected supernatants were used to measure brain renin activity without further dilution. Brain renin activity was assessed with a mouse renin assay kit (fluorimetric) (AnaSpec, Inc. Fremont, CA) according to the protocol attached to the kit. Briefly, by incubating a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide with the brain homogenate at 37 °C, the FRET peptide was cleaved into two separate fragments by mouse renin in the homogenate and the fluorescence of 5-FAM was recovered. Fluorescence was monitored at excitation/emission=490/520 nm and detected by a fluorescence microplate reader (CORONA ELECTRIC Co.,Ltd. Ibaraki, Japan). Fluorescence intensity was normalized per milligram of protein. Protein concentrations were measured by the method of Bradford. Renin activity was expressed as a relative percentage of that of sham-operated mice.

**Brain nitrotyrosine level**

Brain samples were collected to measure nitrotyrosine, a marker of peroxynitrite. Samples were homogenized on ice in 1×PBS, pH 7.4. Homogenates were sequentially centrifuged at 10,000×g at 4 °C for 20 min and 100,000×g at 4 °C for 15 min. Supernatants without further dilution were used for measurements of nitrotyrosine concentrations with a Nitrotyrosine ELISA Kit (Cell biolabs, Inc, San Diego, CA), according to a protocol attached to ELISA kit. Nitrotyrosine concentrations were normalized per milligram of protein. Protein concentrations were measured by the method of Bradford.
Brain NADPH oxidase activity

For measurement of brain NADPH oxidase activity, brain tissues were homogenized on ice in 1×PBS containing protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 1,000×g at 4 °C for 20 min. Supernatants were used for measurements of NADPH oxidase activity with the method of lucigenin chemiluminescence in the presence of modified Krebs/HEPES buffer (pH 7.4) equilibrated at 37 °C, 10 μM NADPH, and 10 μM lucigenin as electron acceptor, as described previously. The chemiluminescence was then recorded by a luminescence reader (BLR-201, Aloka) every 15 seconds for 10 minutes. Values of chemiluminescence were expressed as KCPM/mg protein. Protein concentrations were measured by the method of Bradford.

Assessment of WM lesions and glial activation

Brain samples were sliced into coronal sections. Samples were fixed in 4% (wt/vol.) paraformaldehyde overnight, embedded in paraffin and cut into 5 μm thick sections. The sections were subjected to Klüver-Barrera staining for the measurement of white matter (WM) lesions. The severity of the WM lesions was graded as normal (Grade 0), disarrangement of the nerve fibers (Grade 1), the formation of marked vacuoles (Grade 2), and the disappearance of myelinated fibers (Grade 3) in the corpus callosum.

For assessment of activated microglia and astrocytes, the sections were immunostained with goat anti-ionized calcium binding adaptor molecule-1 (Iba-1) antibody (1:200, Abcam) and goat anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Santa Cruz Biotechnology), respectively. Positive staining was detected with horseradish peroxidase-conjugated anti-goat secondary antibodies (Santa Cruz Biotechnology), visualized with 3,3′-diaminobenzidine (DakoCytomation, Carpinteria, CA, USA) and counter-stained with 1% methylgreen. Normal goat IgG (Santa Cruz Biotechnology) was used as negative control for Iba-1 or GFAP primary antibody. We counted the numerical density of the glial cell nuclei with immunopositive perikarya in the white matter, as described.

Renin and angiotensinogen expressions in the brain white matter

For double immunofluorescent staining of renin and GFAP, the sections were incubated with the primary antibodies [a mixture of rabbit anti-renin serum (1:200) and goat anti-GFAP (1:200)], followed by incubating with a mixture of Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated donkey anti-goat IgG (Invitrogen). For double immunostaining of angiotensinogen and GFAP, the sections were incubated with the primary antibodies [a mixture of rabbit anti-angiotensinogen IgG (1:200, Proteintech) and goat anti-GFAP (1:200)], followed by incubating with a mixture of Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated donkey anti-goat IgG (Invitrogen). Normal goat IgG (Santa Cruz Biotechnology), normal rabbit serum (Sigma) and normal rabbit IgG (DakoCytomation) were used as negative controls for GFAP, renin and angiotensinogen primary antibodies, respectively. We quantified renin-positive astrocytes and angiotensinogen-positive astrocytes in the white matter (the ratio of renin or angiotensinogen-positive astrocytes to GFAP-positive cells).

For quantification of renin-positive microvessels in the white matter, the sections were immunostained with rabbit anti-renin (1:200) primary antibody, followed by incubating with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (DAKO). The reactions were finally visualized with 3,3′-diaminobenzidine (Dako) and counterstained.
with haematoxylin. Normal rabbit serum (Sigma) was used as negative control for renin primary antibody. We counted the number of all renin-positive microvessels in the corpus callosum in at least two serial sections from the same mouse brain sample.

**Preparation of brain protein extracts and western blot analysis**

Our detailed method was described previously. Briefly, cerebral protein extracts were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and were electric transferred to polyvinyldene difluoride membrane. The membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho-eNOS (Ser-1177) (1:1000, BD Transduction Laboratories), anti-eNOS (1:2000, BD Transduction Laboratories), anti-angiotensinogen (1:1000, IBL), anti-p67phox (1:1000, Santa Cruz Biotechnology), anti-MnSOD (1:5000, Stressgen Bioreagents), anti-EcSOD (1:5000, Upstate), anti-Cu/ZnSOD (1:5000, Stressgen Bioreagents) and anti-GAPDH (1:10000, Santa Cruz Biotechnology). In individual samples, each value was correct for that of GAPDH.

**Quantitative real time PCR**

Total RNA was extracted from brain, according to the manufacturer’s instruction. 1μg of RNA sample was reverse transcribed to first-strand cDNA, using QuantiTect® Reverse Transcription Kit (QIAGEN, Tokyo, Japan), according to the manufacturer’s recommended protocol. Thermal Cycler Dice® Real Time System (Takara Bio Inc., Shiga, Japan) was used for 2-step RT-PCR. cDNA was amplified using SYBR® Premix Ex TagTM with specific oligonucleotide primers for target sequences of angiotensinogen (AGT, F 5’-TGACCCAGTTCTTGCCACTGAG-3’; R 5’-ACACCGAGATGCTTGTGTCACACGACCCAGTTCTTGCCACTGAG-3’), monocyte chemoattractant protein-1 (MCP-1, F 5’-GCATCCACGTGGCTTGGCTCA-3’; R 5’-CTCCAGCCTACTCTATTGGGATCA-3’) and tumor necrosis factor-α (TNF-α, ; F 5’-AAGCGTCTAGCCACCTGCTGAGG-3’; R 5’-GTCCTAGCTTGCTTGGTCTTTG-3’). Amplification conditions included 10 seconds at 95 oC and run for 40 cycles at 95 oC for 5 seconds and 60 oC for 30 seconds, and then dissociation 15 seconds at 95 oC and 30 seconds at 60 oC on Thermal Cycler Dice® Real Time System. Specificity of the SYBR® Premix Ex TagTM assays was confirmed by melting point analysis. Each threshold cycles (Ct) value was normalized to β-actin (F 5’-CATGGGTAAAGACCTCTATGCAACACCCAGTTCTTGCCACTGAG-3’; R 5’-ATGGGAGCCACCGTCCACA-3’) Ct value and a control sample. ΔΔCt method, according to the instruction of Thermal Cycler Dice® Real Time System, was used for relative quantization.

**Statistics**

Results were expressed as mean ± SEM. Statistical significance was determined by one way ANOVA, followed by least square differences analysis, using SPSS 11.5.0 (SPSS, Chicago, IL, USA). In all test, differences were considered statistically significant at the value of P less than 0.05.
References


Figure S1. Time course of systolic blood pressure (A) and cerebral blood flow (CBF) (B) in C57/BL6J mice before and after BCAS surgery

(A) Blood pressure was measured before, day 5 and day 12 (day 7 after BCAS surgery) after start of vehicle or aliskiren infusion. BCAS surgery did not affect systolic blood pressure in C57BL/6J mice throughout the experiment. Aliskiren pre-treatment did not significantly change blood pressure of C57BL/6J mice subjected to BCAS surgery, throughout the treatment. (B) The CBF of vehicle-treated mice and aliskiren-treated mice was decreased to 62.1 ± 2.6 % (P<0.01) and 58.0 ± 2.4 % (P<0.01), respectively, of baseline values at 10 minutes after BCAS surgery, and there was no difference between the two groups in the degree of decrease in CBF. Furthermore, the CBF of vehicle and aliskiren groups remained decreased to 74.7 ± 2.3 % (P<0.01) and 76.4 ± 2.3 % (P<0.01), respectively, of baseline values at 3 weeks after BCAS surgery, and there was no difference between the two groups, although a laser speckle blood flow imager used in this study provides only a relative measure and this method is highly dependent on exact placement.

Abbreviations used: Sh, sham-operated C57/BL6J mice; Ve, vehicle-infused C57/BL6J mice (saline); Al, aliskiren-infused C57/BL6J mice. Values are means ± SEM (n=12-15 in each group).
Figure S2

(A) Cu/ZnSOD

(B) MnSOD

(C) EcSOD

Figure S2 Effects of chronic cerebral hypoperfusion and aliskiren infusion on superoxide dismutase isoforms in brain of C57BL/6J mice

Abbreviations used are the same as in Figure S1. SOD, superoxide dismutase. Values are means ± SEM (n=8 in each group).
Figure S3. Effects of chronic cerebral hypoperfusion and aliskiren infusion on eNOS expression in brain of C57BL/6J mice

Abbreviations used are the same as in Figure S1. Upper panels are representative western blot of phospho-eNOS and total-eNOS. Values are means ± SEM (n=8 in each group).
Figure S4. Time course of systolic blood pressure in C57BL/6J mice treated with tempol, before and after BCAS surgery.

Blood pressure was measured before, day 5 and day 12 (day 7 after BCAS surgery) after start of vehicle or tempol administration.

Abbreviations used: Sh, sham-operated C57/BL6J mice; Ve, vehicle-treated mice; Te, tempol-treated mice. Values are means ± SEM (n=9-11 in each group).
Figure S5. The representative photomicrographs of cerebral hypoxia immunostained with Hypoxyprobe (A), Klüver-Barrera staining in the paramedian parts of the corpus callosum (B), immunostaining of GFAP (C) and Iba-1 (D) in the paramedian parts of the corpus callosum.

Abbreviations used are the same as in Fig. S4. Scale bar= 2 mm in (A), 200 μm in (B), and 100 μm in (C) and (D). Insets are enlarged images of oligodendrocytes (B), astrocytes (C) and microglia (D).