Temporary Pretreatment With the Angiotensin II Type 1 Receptor Blocker, Valsartan, Prevents Ischemic Brain Damage Through an Increase in Capillary Density

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Background and Purpose—We investigated the effect of temporary treatment with a nonhypotensive dose of valsartan on ischemic brain damage in C57BL/6 mice.

Methods—We separated the mice into 3 groups of valsartan treatment before middle cerebral artery (MCA) occlusion: (1) for 4 weeks: Val (2W, 2W); (2) for 2 weeks followed by its cessation for 2 weeks: Val (2W, -); and (3) no treatment for 4 weeks: Val (-, -).

Results—Ischemic volume, DNA damage, superoxide production, and mRNA levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α on the ipsilateral side after 24 hours of MCA occlusion were significantly reduced in both Val (2W, 2W) and Val (2W, -) mice compared with those in Val (-, -) mice, whereas these parameters were larger in Val (2W, -) mice than in Val (2W, 2W) mice. Moreover, mice in both the Val (2W, 2W) and Val (2W, -) groups exhibited an increase in cerebral blood flow in the peripheral territory of the MCA 1 hour after MCA occlusion, with increases in endothelial nitric oxide synthase activation and nitric oxide production. Before MCA occlusion, treatment with valsartan did not influence superoxide production or mRNA levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α in the brain. However, the capillary density in the brain in both Val (2W, 2W) and Val (2W, -) mice was increased before MCA occlusion.

Conclusions—Our results suggest that temporary valsartan treatment could protect against ischemic brain damage even after its cessation, at least in part due to an increase in capillary density. *(Stroke. 2008;39:2029-2036.)*

Key Words: angiotensin II type 1 receptor blocker ■ capillary density ■ cerebral blood flow ■ oxidative stress ■ stroke

Angiotensin (Ang) II is a potent vasoactive substance that has a variety of physiological actions, including vasconstriction, aldosterone release, and cell growth.1 Recent clinical studies such as LIFE, MOSES, and the Jikei Heart Study demonstrated that an Ang II receptor blocker (ARB) prevented the onset of stroke beyond blood pressure-lowering effects.2-4 Consistent with clinical results, accumulating evidence from preclinical studies also indicates that ARB could prevent the onset of stroke.5,6 Moreover, the possible involvement of the central renin–angiotensin system in ischemic brain damage has been reported. Maeda et al reported that the core lesion area after middle cerebral artery (MCA) occlusion was significantly reduced in angiotensinogen-knockout mice.7 Walter et al and our group reported that a smaller ischemic lesion area was observed after MCA occlusion in Ang II type 1 (AT₁) receptor-deficient mice.5,8 We previously demonstrated that Ang II type 2 receptor null mice exhibited a larger ischemic size and greater neurological deficit after subjection to MCA occlusion.5 In addition, pretreatment with an ARB has been reported to prevent ischemic brain damage in various animal models.5,9 These results suggest that AT₁ receptor stimulation is involved in the development of brain ischemic damage, whereas Ang II type 2 receptor stimulation could prevent it.

Despite the accumulating evidence, the detailed mechanisms of the protective effects of pretreatment with an ARB on brain ischemic brain damage are still not well understood. A recent clinical trial, TROPHY, showed that treatment of prehypertension with an ARB, candesartan, reduced the incidence of hypertension even after cessation of ARB administration.10 This clinical study led us to examine the possibility that temporary pretreatment with an ARB could decrease brain ischemic damage after MCA occlusion in mice. Moreover, we tried to explore the possible mechanisms involved in this ARB’s potential protective effects on ischemic brain damage. Accordingly, we investigated the effects
of pretreatment with an ARB, valsartan, focusing on oxidative stress, inflammation, and vascular remodeling before and after MCA occlusion in mice.

**Materials and Methods**

Adult male C57BL/6 wild-type mice (CLEA, Tokyo, Japan) from 6 weeks (weight, 20 g) were used in this study. The mice were housed in a room in which lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at 25°C. The Animal Studies Committee of Ehime University approved the experimental protocol.

**Experimental Protocol**

Mice were given a standard diet (MF; Oriental Yeast Co Ltd, Tokyo, Japan) and water ad libitum. An ARB, valsartan (provided by Novartis Pharma AG), was administered through an intraperitoneally implanted osmotic minipump (model 1002; Alza) at a dose of 3 mg/kg per day. This dose of valsartan is a nonhypotensive dose, as previously reported.12 The mice were separated into 3 groups based on the treatment with valsartan before MCA occlusion (1): valsartan (3 mg/kg per day) treatment for 4 weeks: Val (2W, 2W); (2) valsartan treatment for 2 weeks followed by cessation of its administration for 2 weeks: Val (2W, -); and (3) no treatment with valsartan for 4 weeks: Val (-, -).

**Middle Cerebral Artery Occlusion**

Focal cerebral ischemia was induced by occlusion of the MCA by an intraluminal filament technique according to a method previously described.7,12 Brain samples were obtained 24 hours after MCA occlusion, and coronal sections of 1-mm thickness were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride as previously described.12 Neurological deficit was evaluated 24 hours after MCA occlusion by use of neurological scores developed by Huang et al.14

**Evaluation of Capillary Density**

We evaluated capillary density using a recently developed microangiographic technique,15 or immunofluorescence in the cerebral cortex of the perifrontal zone before and after MCA occlusion. Capillary density was evaluated in the cerebral cortex of the perifrontal zone before and after MCA occlusion. In the brain without MCA occlusion (ie, “before” MCA occlusion), we have evaluated the capillary density in a similar region of the brain. First, bovine desiccate albumin–fluorescein isothiocyanate (Sigma-Aldrich, St Louis, Mo; 10 mg/mL) was injected through the tail vein at 200 μL per mouse just before sampling the brain. The number of vessels was analyzed with a Leica DMI 6000B microscope (Leica Microsystems, Wetzlar, Bensheim, Germany) at ×100 magnification. Capillary density in a cortical area of 1 mm² was calculated from 5 sections in each mouse. Approximately 100 to ~700 capillaries were included in the area of 1 mm². Second, brain sections were fixed with 10% formalin and permeabilized with 0.1% saponin before incubation with goat polyclonal anti-PECAM-1 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and goat polyclonal anti-GLUT-1 antibody (Santa Cruz Biotechnology, Inc). The secondary antibody was rabbit antigoat antibody (Molecular Probes, Carlsbad, Calif). Cells were counterstained with DAPI (Vectorshied; Vector Laboratories, Inc, Burlingame, Calif). Images were viewed at ×10 magnification with an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) using image analysis software (Atto Densitograph, Tokyo, Japan).

**Detection of Superoxide Anion**

Histological detection of superoxide anion was performed as described previously.17 In brief, frozen, enzymatically intact, 10-μm-thick sections were prepared from mouse brain 24 hours after MCA occlusion and incubated immediately with dihydroethidium (10 μmol/L) in phosphate-buffered saline for 30 minutes at 37°C in a humidified chamber protected from light. Dihydroethidium is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For detection of ethidium, samples were examined with an Axioskop microscope (Axioskop 2 plus with AxioCam; Carl Zeiss) equipped with a computer-based imaging system. The intensity of the fluorescence was analyzed and quantified by means of computer imaging software (Densitograph; ATTO Corp, Tokyo, Japan).

**Real-Time Reverse Transcriptase–Polymerase Chain Reaction**

Real-time quantitative reverse transcriptase–polymerase chain reaction was performed with a SYBR kit (MJ Research, Inc, Waltham, Mass). Polymerase chain reaction primers are shown in the supplemental Table I, available online at http://stroke.ahajournals.org.

**Quantification of Damaged DNA**

DNA damage was quantified with a DNA Damage Quantification Kit (Dojindo, Kumamoto, Japan) based on the detection of abasic sites in genomic DNA with an aldehyde reactive probe reagent that reacts specifically with the open ring form of the abasic sites. We performed this assay according to the manufacturer’s instructions.

**Western Blot**

Total protein was prepared from the brain, and Western blotting was performed as previously described.18 Antibodies against phosphoendothelial nitric oxide synthase and total endothelial nitric oxide synthase was purchased from Cell Signaling Technology, Inc (Danvers, Mass).

**Detection of Nitric Oxide Production**

Brain segments were loaded with 10 μmol/L diaminofluorescein-2 diacetate (Daichi Pure Chemicals Co, Ltd, Tokyo, Japan) for 30 minutes at 37°C in HEPES buffer (pH 7.4). 1-arginine (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at a dosage of 100 μmol/L was put into the chamber during measurements to ensure adequate substrate availability for nitric oxide synthase. The production of nitric oxide was analyzed with a Leica DMI 6000B microscope at ×100 magnification.

**Statistical Analysis**

Values are expressed as mean±SEM in the text and figure. Data were analyzed using analysis of variance followed by Newman-Keuls test for multiple comparisons. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. Values of P<0.05 were considered statistically significant.

**Results**

**Focal Ischemic Injury of the Brain After Middle Cerebral Artery Occlusion in Mice Treated With Valsartan**

Focal brain ischemia was induced by MCA occlusion with the intraluminal filament technique. To examine the possible protective effects of temporary treatment with valsartan on brain ischemia, we separated C57BL/6J mice into 3 groups (Val [2W, 2W], Val [2W, -], and Val [-, -]) as described in “Materials and Methods.” As we previously reported,4 treatment with valsartan just before MCA occlusion (Val [2W, 2W]) decreased the ischemic area and its volume (Figure...
Interestingly, treatment with valsartan for 2 weeks followed by its cessation for 2 weeks (Val [2W, -]) before MCA occlusion significantly decreased the ischemic volume, although its protective effect seemed to be less than that in Val (2W, 2W) mice (Figure 1B). We further assessed the effects of valsartan on the DNA damage after MCA occlusion by quantification of apurinic sites in genomic DNA. We observed that MCA occlusion increased apurinic sites on the ipsilateral side and that treatment with valsartan for 4 weeks decreased apurinic sites on the ipsilateral side (Val [2W, 2W]) with less but significant effects of treatment with valsartan for 2 weeks (Val [2W, -]) even after its cessation (Figure 1C). Treatment with valsartan did not change apurinic sites before MCA occlusion (Figure 1C). Neurological deficit after MCA occlusion evaluated by neurological scores was also improved in both Val (2W, -) and Val (2W, 2W) mice with more marked effects in Val (2W, 2W; Figure 1D). Systolic blood pressure before and 24 hours after MCA occlusion was not significantly different between these 3 groups.

Effects of Valsartan on Oxidative Stress and Inflammatory Response in Mouse Brain Before and after Middle Cerebral Artery Occlusion

To further assess the effects of temporary treatment with valsartan on ischemic brain damage, we examined superoxide anion production and inflammatory responses. Superoxide anion production in the brain cortex before and 24 hours after MCA occlusion was determined by dihydroethidium staining (Figure 2A–B). We observed that superoxide anion production was enhanced on the ipsilateral side after MCA occlusion, and treatment with valsartan for 4 weeks before MCA occlusion decreased this (Val [2W, 2W]) and temporary treatment with valsartan also decreased superoxide anion production (Val [2W, -]), whereas treatment with valsartan did not influence superoxide production before MCA occlusion. Next, we examined mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) using real-time reverse transcriptase–polymerase chain reaction (Figure 2C–D). mRNA expression of MCP-1 and TNF-α was increased on the ipsilateral side but not on the contralateral side after MCA occlusion or before MCA occlusion. Their expression in the ischemic area was decreased in Val (2W, -) mice and further decreased in Val (2W, 2W) mice.

Valsartan Increased Capillary Density and Cerebral Blood Flow After Middle Cerebral Artery Occlusion

Cerebral surface blood flow was measured in the core region and peripheral region of the MCA territory. Cerebral blood flow decreased just after MCA occlusion to approximately 10% of the basal level in the core region and to approximately...
50% in the periphery in Val (2W, -) mice (Figure 3). This reduction of cerebral blood flow continued for at least 24 hours after MCA occlusion. The decrease in cerebral blood flow in the core was not significantly different between these groups, whereas cerebral blood flow in the peripheral region was significantly attenuated in Val (2W, -) and Val (2W, 2W) mice (Figure 3). Cerebral blood flow in the peripheral region was increased by valsartan treatment, but without a significant difference.

To examine the possibility that administration of valsartan could increase vascular bed flow in response to MCA occlusion, we examined capillary density. Using confocal microscopy, capillary density in the brain was calculated per surface area. Administration of valsartan for 4 weeks just before MCA occlusion (Val [2W, 2W]) increased capillary density in the nonischemic and ischemic brain, as shown in Figure 4A–C. Interestingly, an increase in capillary density in the brain was also observed by temporary administration of valsartan (Val [2W, -]) before and after MCA occlusion (Figure 4A–C).

Effect of Valsartan on Endothelial Nitric Oxide Synthase and Nitric Oxide Production

To further explore the vasoprotective effects of valsartan in terms of preventing ischemic brain damage, we examined the possibilities that treatment with valsartan could increase endothelial nitric oxide synthase (eNOS) expression, eNOS activation, and nitric oxide production (Figure 5). Figure 5A shows that serine phosphorylation of eNOS was induced on the ipsilateral side but not on the contralateral side or before MCA occlusion. This increase in the ischemic area was enhanced in Val (2W, -) mice and further enhanced in Val (2W, 2W) mice (Figure 5B–C).
Discussion

A recent clinical trial, TROPHY, showed that treatment of prehypertension with an ARB, candesartan, reduced the incidence of hypertension even after its cessation. In this study, 2 years after cessation of candesartan, the incidence of hypertension in the candesartan group remained lower than that in the placebo group. Therefore, we examined the possibility that temporary treatment with an ARB, valsartan, could reduce ischemic brain damage after its cessation in a mouse MCA occlusion model. In the present study, focal cerebral ischemia was induced by a silicon-coated microfilament guided into the MCA. As we reported previously, administration of valsartan at a nonhypotensive dose attenuated the focal ischemic area induced by MCA occlusion. Interestingly, temporary administration of valsartan significantly decreased the ischemic area, even after its cessation, suggesting that valsartan could imprint some factors that protect against ischemic brain damage.

The ischemic region can be separated into the infarct core, in which oxygen supply is too low to sustain cell viability and the ischemic penumbra. The penumbra is considered to be a border zone between the region in which electric activity of neurons stops and the region exhibiting membrane depolarization. In the present study, we measured surface cerebral blood flow in the core and peripheral regions of the MCA territory. The decrease in cerebral blood flow in the peripheral region (penumbra) was smaller in mice with continuous treatment with valsartan before MCA occlusion (Val 2W). Of note, temporary treatment with valsartan until 2 weeks before MCA occlusion increased cerebral blood flow in the peripheral region. Treatment with valsartan could increase the microcirculation, including formation of collaterals, thereby contributing to an increase in the vascular bed and flow in response to MCA occlusion. To examine this possibility, we examined the effects of valsartan on capillary density in the brain before MCA occlusion. In the present study, we observed that capillary density in the brain was increased in valsartan-treated mice even before MCA occlusion and that even temporary treatment with valsartan increased capillary density. Recent studies indicate an inhibitory role of the AT1 receptor in capillary density. Li et al reported that capillary density in the heart was reduced in untreated C57BL/6 mice after nephrectomy; however, it was significantly restored in both AT1 receptor null mice and valsartan-treated mice. Their findings suggest that the reduced myocardial capillary density is likely to be related to augmented oxidative stress, which is thought to be mediated by the AT1 receptor. Moreover, an AT1 receptor blocker, losartan, has been shown to increase myocardial capillary density in a rat model of myocardial infarction. Furthermore, Maxfield et al demonstrated that in streptozocin-diabetic rats, endothelial capillary density, which was unaffected by diabetes, was increased by an AT1 receptor antagonist. Xie et al also indicated that AT1 receptor blockade by candesartan improved capillary density of the left ventricular wall in stroke-prone spontaneously hypertensive rats. Interstingly, expression of vascular endothelial growth factor and angiopoietin were increased by prostaglan-
din F2-induced luteolysis in sheep to maintain vascular structure, during which expression of the AT1 receptor was decreased. Therefore, we speculate that valsartan pretreatment before MCA occlusion might have inhibited the AT1 receptor or increased vascular endothelial growth factor and angiopoietin mRNA expression, resulting in an increase in capillary density. In the recent clinical trial, the Jikei-Heart study demonstrated that the addition of valsartan to conventional treatment reduced the onset of stroke to 40% less than supplementary conventional treatment, indicating that treatment with valsartan has a beneficial effect on stroke onset beyond an antihypertensive effect. Although preventive effects of ARBs on stroke have been discussed, our results also suggest that hypertensive patients with ARB can be prevented from stroke partly through increasing capillary densities even after temporary treatment. Further studies to investigate the detailed mechanisms are needed in the future.

Oxidative stress is involved in various pathological processes, and brain ischemia enhances oxidative stress. As reported previously, Ang II increases NADPH oxidase activity and stimulates the production of reactive oxygen species, including the superoxide anion, through the AT1 receptor. MCA occlusion increased superoxide production in the ischemic area of the brain, and temporary treatment with valsartan (Val [2W, -]) attenuated superoxide production, whereas continuous treatment with valsartan (Val [2W, 2W]) decreased oxidative stress. Moreover, inflammatory responses are closely associated with ischemic brain damage. The mRNA expression of MCP-1 and TNF-α was also increased in response to MCA occlusion, and treatment with valsartan attenuated the inflammatory response assessed by MCP-1 and TNF-α mRNA expression. However, treatment with valsartan (Val [2W, -], Val [2W, 2W]) did not apparently influence superoxide production or mRNA expression of MCP-1 and TNF-α in the brain before MCA occlusion. It was recently reported that in stroke-prone spontaneously hypertensive rats, eNOS and nitric oxide concentration were increased by valsartan, contributing to vasodilation. Jiang et al also demonstrated that eNOS expression and nitric oxide production were significantly increased after hypoxia in endothelial progenitor cells. In the present study, we observed that even temporary treatment with valsartan increased activation of eNOS and nitric oxide production on the ischemic side after MCA occlusion. We speculate that the increase in capillary density and consequent increase in cerebral blood flow by temporary treatment with valsartan even after its cessation could attenuate oxidative stress and inflammatory responses and increase nitric oxide production. However, we did not observe such changes in oxidative stress, inflammatory responses, and nitric oxide production after the increases of capillary density and cerebral blood flow. In fact, as reported recently, the change in superoxide production does not closely parallel the change in capillary density. It has been demonstrated that in untreated C57BL/6 mice after nephrectomy, capillary density in the heart was reduced, whereas oxidative stress was markedly increased, indicating that the change in oxidative stress does not parallel the change in capillary density. Recent data from D’Ascia et al demonstrated that myocardial tissue from patients revealed a significant decrease in TNF-α expression.

Figure 5. Expression of phosphorylated-eNOS and nitric oxide production in the brain before and 24 hours after MCA occlusion. A, Western blot analysis of serine phosphorylation of eNOS. B, Representative photographs of the staining in brain cortexes. C, Intensity analysis of nitric oxide production reacted with diaminofluorescein-2 diacetate. n=8 for each group. Val, valsartan (3 mg/kg per day). *P<0.05, **P<0.01 versus Val (-, -) before MCA; #P<0.01 versus Val (-, -) ipsilateral; #P<0.05 versus Val (2W, -) ipsilateral. Values are mean±SEM.
with increased capillary density after 6 months of cardiac resynchronization therapy. Moreover, endotoxemia tended to decrease intestinal functional capillary density, whereas it elevated TNF-α and interleukin levels in rats. It is suggested that an increase in capillary density is not followed by an increase in inflammatory responses. Furthermore, in a peritonitis model of sepsis in the rat, nitric oxide level increased and contributed to decreased functional capillary density in remote organs. Moreover, for evaluating the effects of ARB on oxidative stress and inflammation before MCA occlusion, it has to be taken in account that we used a nonhypotensive mouse model in this experiment, which showed low basal levels of oxidative stress and inflammation. Therefore, it is possible that we cannot observe significant changes in superoxide anion production and MCP-1 and TNF-α expression by valsartan treatment before MCA occlusion. In addition, it is also possible that Ang II could exert diverse and biphasic effects depending on Ang II concentrations, the levels of AT1 and Ang II type 2 receptors, and cell types in situ. Therefore, to understand further the protective effects of pretreatment with ARB on ischemic brain, more detailed analysis of Ang II production and expression of Ang II receptors in situ have to be evaluated.

In the present study, we demonstrated that temporary treatment with an ARB, valsartan, for 2 weeks followed by its cessation for 2 weeks attenuated brain damage after stroke. This beneficial effect was induced partly due to increase in capillary density before MCA occlusion; decrease in oxidative stress, inflammation, and DNA damage; and increase in cerebral blood flow with an increase in nitric oxide production. In conclusion, these results provide new insights in the protective effects of temporary ARB treatment on the ischemic brain even after cessation, at least in part due to improvement of vascular remodeling, although further studies are needed to explore the detailed mechanisms of the effects of temporary treatment with valsartan concerning its protective effects in the ischemic brain.

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Disclosures
None.

References


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