Possible Inhibition of Focal Cerebral Ischemia by Angiotensin II Type 2 Receptor Stimulation

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Background—The role of angiotensin II receptor subtypes was investigated in focal brain ischemia induced by middle cerebral artery (MCA) occlusion.

Methods and Results—In Agtr2+/− (wild-type) mice, MCA occlusion induced focal ischemia of ≈20% to 30% of the total area in coronal section of the brain. The ischemic area was significantly larger in angiotensin II type 2 receptor–deficient (Agtr2−/−) mice than in Agtr2+/− mice. The neurological deficit after MCA occlusion was also greater in Agtr2−/− mice than in Agtr2+/− mice. The decrease in surface cerebral blood flow after MCA occlusion was significantly exaggerated in the peripheral region of the MCA territory in Agtr2−/− mice. Superoxide production and NADPH oxidase activity were enhanced in the ischemic area of the brain in Agtr2−/− mice. An AT2 receptor blocker, valsartan, at a nonhypotensive dose significantly inhibited the ischemic area, neurological deficit, and reduction of cerebral blood flow as well as superoxide production and NADPH oxidase activity in Agtr2−/− mice. These inhibitory actions of valsartan were weaker in Agtr2+/− mice.

Conclusions—These results suggest that AT2 receptor stimulation has a protective effect on ischemic brain lesions, at least partly through the modulation of cerebral blood flow and superoxide production. (Circulation. 2004;110:843-848.)

Key Words: stroke ■ angiotensin ■ ischemia ■ receptors ■ stress

Angiotensin II (Ang II) is a potent vasoactive substance in the renin-angiotensin system (RAS), which has a variety of physiological actions, including vasoconstriction, aldosterone release, and cell growth.1 Previous articles on the brain RAS studied primarily the regulatory mechanism of blood pressure.2–5 Recent clinical studies, such as PROGRESS, SCOPE, and LIFE, indicated that blockade of RAS is important to prevent stroke.6–8 Accumulating results of basic research also indicate the possible involvement of the central RAS in ischemic brain damage. Maeda et al9 reported that the core lesion area after middle cerebral artery (MCA) occlusion as well as mean arterial blood pressure was significantly reduced in angiotensinogen-knockout (AT2 KO) mice. Walter et al10 also reported that a smaller lesion area was observed after MCA occlusion in Ang II type 1 (AT1) receptor–deficient mice. Moreover, Nishimura et al11 reported that blockade of the AT1 receptor by an AT1 receptor blocker (ARB), candesartan, reduced the ischemic area after MCA occlusion in genetically hypertensive rats. These results suggest that AT1 receptor stimulation is involved in the development of brain ischemic lesions and that blockade of the AT1 receptor decreases ischemia at least partly through lowering of blood pressure. The major cardiovascular actions of Ang II have been reported to be mediated by the AT1 receptor, whereas a second receptor subtype, known as the Ang II type 2 (AT2) receptor, in brain ischemic lesions is still an enigma.

It has been reported that AT2 receptor stimulation antagonizes the effects of AT1 receptor stimulation in most tissues.12–14 In the brain, AT2 receptors are expressed not only in the vascular wall but also in the thalamus, hypothalamus, and specific brainstem nuclei.15,16 When the AT1 receptor is blocked by an ARB, unbound Ang II acts preferentially on the AT2 receptor. These results point to the pathophysiological importance of the AT2 receptor in the clinical use of ARBs, which are widely used in patients with hypertension and cardiovascular disease. We have previously reported that AT2 receptor stimulation is involved in the beneficial effects of an ARB on vascular injury and cardiac remodeling.17–19 However, the function of AT2 receptor stimulation in the brain is not yet fully understood. Previous reports suggest that AT2 receptor stimulation is involved in axonal regeneration20 and in memory and behavior.21–23 In the present study, we tried to
clarify the roles of the AT2 receptor in vascular remodeling, including blood flow in the penumbra and oxidative stress in ischemic brain damage after MCA occlusion.

Methods

Animals
Adult male AT receptor–deficient mice (Agtr2−/−; based on C57BL6J strain bred in our laboratory) and wild-type mice (Agtr2+/+; age, 10 to 12 weeks; weight, 25 to 30 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. They were given a standard diet (MF, Oriental Yeast Co Ltd) and water ad libitum. The Animal Studies Committee of Ehime University approved the experimental protocol.

MCA Occlusion
Focal cerebral ischemia was induced by occlusion of the MCA by use of an intraluminal filament technique according to a method previously described.24,25 Briefly, after a midline neck incision had been made, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (Ethilon; Ethicon) coated with silicon resin (Xantopren; Bayer Dental) was introduced through a small incision into the common carotid artery and advanced to a position 9 mm distal from a carotid bifurcation for occlusion of the MCA. A selective ARB, valsartan (provided by Novartis Pharma AG),26 was administered via an osmotic minipump (model 1002, Alza) implanted intraperitoneally 10 days before MCA occlusion. 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. They were given a standard diet (MF, Oriental Yeast Co Ltd) and water ad libitum. The Animal Studies Committee of Ehime University approved the experimental protocol.

Laser-Doppler Flowmetry
Cerebral blood flow was determined in the territory of the MCA by laser-Doppler flowmetry using a flexible fiberoptic extension to the master probe (Omegaflo FLO-C1, Omegawave). The tip of the probe was fixed to the intact skull over the supplying territory of the MCA. A selective ARB, valsartan, at a dose of 3 mg · kg−1 · d−1 for 10 days before MCA occlusion significantly suppressed mortality, neurological score, and the ischemic area in Agtr2−/− mice (Figures 1 and 2). Valsartan also inhibited the reduction of cerebral blood flow in the periphery (Figure 3). However, cerebral blood flow in the peripheral region was significantly attenuated in Agtr2−/− mice (Figure 3).

Detection of Superoxide Anion in Brain Sections
Histological detection of superoxide anion was performed as described previously.30 In brief, frozen, enzymatically intact, 10-μm-thick sections were prepared from mouse brain 24 hours after MCA occlusion and incubated immediately with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. DHE 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 use of computer-imaging software (Densitograph, ATTO Corp).

NADPH Oxidase Activity
The activity of NADPH oxidase in brain was measured according to the method of Pagano et al.31 Briefly, contralateral and ipsilateral cortices of the brain were obtained 24 hours after MCA occlusion. They were homogenized in 10 volumes of ice-cold Tris-sucrose buffer, and the enzyme activity was determined by use of the cytochrome c assay with or without superoxide dismutase.31

Statistical Analysis
Values are expressed as mean±SEM in the text and figures. The data were analyzed by 2-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. A value of P<0.05 was considered to be statistically significant.

Results

Focal Ischemic Injury of Brain After MCA Occlusion in Agtr2−/− Mice
Focal brain ischemia was induced by MCA occlusion with the intraluminal filament technique. In Agtr2−/− mice, the maximal ischemic area was ≈25% of the total area around section 3, which is in the territory of the MCA (Figure 1). The ischemic area of the brain was significantly larger in Agtr2+/+ mice. Neurological score at 24 hours after MCA occlusion was higher in Agtr2−/− mice than in Agtr2+/+ mice (Figure 2). Systolic blood pressure 24 hours after MCA occlusion was not significantly different between Agtr2−/− and Agtr2+/+ mice (Table).

Changes in Cerebral Blood Flow After MCA Occlusion in Agtr2−/− Mice
Cerebral surface blood flow was measured in the core region and peripheral region of the MCA territory (Figure 3). Cerebral blood flow decreased just after MCA occlusion to ≈10% of the basal level in the core region and to ≈60% in the periphery in Agtr2−/− mice (Figure 3). This reduction of cerebral blood flow continued for at least 24 hours after MCA occlusion in Agtr2−/− mice. The decrease in cerebral blood flow in the core was not significantly different between Agtr2−/− and Agtr2+/+ mice (Figure 3). However, cerebral blood flow in the peripheral region was significantly attenuated in Agtr2−/− mice (Figure 3).

Oxidative Stress in Mouse Brain After MCA Occlusion
To assess the involvement of oxidative stress in the exaggeration of focal brain ischemia, superoxide anion production was evaluated (Figure 4). Superoxide anion production was increased in the occluded side but not in the nonoccluded side. Superoxide production in the ischemic area was enhanced in Agtr2−/− mice. Moreover, the activity of NADPH oxidase was measured in brain cortices 24 hours after MCA occlusion (Figure 5). NADPH oxidase activity was increased after MCA occlusion. This increase was enhanced in AT1KO mice (Figure 5).

Effect of ARB on Focal Brain Ischemia After MCA Occlusion
Administration of an ARB, valsartan, at a dose of 3 mg · kg−1 · d−1 for 10 days before MCA occlusion significantly suppressed mortality, neurological score, and the ischemic area of the brain in Agtr2−/− mice (Figures 1 and 2), with no apparent change in blood pressure (Table). Valsartan also inhibited the reduction of cerebral blood flow in the peripheral region (Figure 3) and the increase in superoxide anion production as well as NADPH oxidase activity in the infarcted side of the brain after MCA occlusion in Agtr2−/− mice (Figures 4 and 5). These effects of valsartan were signifi-
cantly weaker in Agtr2<sup>-</sup> mice than in Agtr2<sup>+</sup> mice (Figures 1 to 5).

**Discussion**

In the present study, focal cerebral ischemia was induced by a silicon-coated microfilament guided into the MCA. The present study showed that the focal ischemic area induced by MCA occlusion was exaggerated in Agtr2<sup>-</sup> mice. These results suggest that the AT<sub>2</sub> receptor plays an important role in cerebral ischemia, showing an antagonistic action against AT<sub>1</sub> receptor–mediated effects. Moreover, it was suggested that such antagonism of AT<sub>2</sub> receptor–mediated actions are involved in the effects of ARB, because the inhibitory effect of valsartan on the ischemic area was significantly weaker in Agtr2<sup>-</sup> mice. The antagonistic action of AT<sub>2</sub> receptor stimulation seems to be mediated, at least in part, by modulation of cerebral blood flow and oxidative stress in the ischemic region.

As reported previously, 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.<sup>24</sup> The penumbra is considered to be a border zone between the region in which electrical activity of neurons ceases and that exhibiting membrane depolarization. In the present study, we measured surface cerebral blood flow in the core and periph-

**Blood Pressure Change After MCA Occlusion in WT and AT<sub>2</sub>KO Mice**

<table>
<thead>
<tr>
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<th>Systolic Blood Pressure, mm Hg</th>
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<tr>
<td></td>
<td>WT</td>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Before MCA</td>
<td>90.1±2.7</td>
</tr>
<tr>
<td>24 h after MCA</td>
<td>84.7±2.1</td>
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WT indicates wild type; AT<sub>2</sub>KO, AT<sub>2</sub> receptor knockout. Systolic blood pressure was measured by the indirect tail-cuff method as described in Methods. Valsartan was administered via an osmotic minipump implanted intraperitoneally 10 days before MCA occlusion. n=5 to 6 for each group.
eral regions of the MCA territory. The decrease in cerebral blood flow in the peripheral region (penumbra) was exaggerated in Agtr2−/− mice, and the inhibitory effect of valsartan on blood flow reduction was smaller in Agtr2−/− mice than in Agtr2+/+ mice (Figure 3). These results indicate that the AT1 and AT2 receptors are involved in cerebral blood flow distribution. The reduction in the focal ischemic area in valsartan-treated mice may be caused by inhibition of blood flow reduction in the peripheral region (Figure 3). Therefore, blockade of the AT1 receptor and stimulation of the AT2 receptor show a protective effect on ischemic brain lesions. In receptor gene−deficient mice, it seems possible that the change of microcirculation, including collaterals, may have already developed under basal conditions before MCA occlusion, because the reduction of cerebral blood flow immediately after MCA occlusion in the peripheral region tended to be exaggerated in Agtr2−/− mice (Figure 3). Treatment of Agtr2−/− mice with valsartan inhibited a reduction of surface cerebral blood flow in the periphery. This inhibitory action of valsartan on reduction of cerebral blood flow was also weaker in Agtr2−/− mice (Figure 3). These results also indicate the importance of AT1 receptor blockade to improve brain ischemia and the involvement of AT2 receptor stimulation in the action of ARBs. A previous report indicated that AT2 receptor stimulation promoted axonal regeneration in the optic nerve.32 These results, together with those in the present study, suggest the potential neural protective action of AT2 receptor stimulation.

Oxidative stress is considered to be involved in various pathological processes.33−35 It has been reported that brain ischemia enhances oxidative stress.35,36 As reported previously, Ang II increases NADPH oxidase activity and stimulates production of reactive oxygen species, including the superoxide anion, through AT1 receptors.37−39 However, the role of the AT2 receptor in oxidative stress is not yet fully understood. The present study demonstrated an inhibitory effect of AT2 receptor stimulation on superoxide production using a combination of gene-deficient mice and an ARB (Figure 4). MCA occlusion increased oxidative stress in the ischemic area (Figures 4 and 5). Production of superoxide anion and NADPH oxidase activity in the ischemic area was exaggerated in Agtr2−/− mice, whereas valsartan attenuated both superoxide production and NADPH oxidase activity in Agtr2−/− mice. Moreover, the inhibitory effect of valsartan was smaller in Agtr2−/− mice. These results suggest that modulation of local superoxide production by AT1−/− and AT2-receptor stimulation is involved in the development of ischemic brain lesions. A previous article also indicated that stimulation of the AT1 receptor decreased the expression of NADPH oxidase subunit.19 Thus, the regulatory action of AT1−/− and AT2-receptor stimulation on superoxide production may ap-

Figure 3. Change in cerebral blood flow after MCA occlusion in AT1 receptor−deficient mice. Surface cerebral blood flow was determined immediately and at 1 hour and 24 hours after MCA occlusion by laser-Doppler flowmetry as described in Methods. Blood flow change was expressed as a percentage of basal flow rate. Some mice were treated with valsartan (3 mg · kg−1 · d−1) for 10 days via an osmotic minipump before MCA occlusion as described in Methods. Agtr2−/−, wild-type; Agtr2−/−, AT2 receptor−deficient. n=6 to 8 for each group. *P<0.05 vs Agtr2−/−, †P<0.05 vs without valsartan. Values are mean±SEM.

Figure 4. Detection of superoxide anion production in mouse brain 24 hours after MCA occlusion. Freshly frozen sections of brain were prepared and superoxide anion production was detected with DHE (10 μmol/L) as described in Methods. Some mice were treated with valsartan (3 mg · kg−1 · d−1) for 10 days via an osmotic minipump before MCA occlusion as described in Methods. a. Photos are reproducible staining of brain sections (cortex) from nonischemic and ischemic areas. b. Intensity analysis of superoxide production stained with DHE. Intensity of fluorescence was determined by use of computer-imaging software as described in Methods. Agtr2−/−, wild-type; Agtr2−/−, AT2 receptor−deficient. n=6 to 8 for each group. *P<0.05 vs nonischemic area. †P<0.01 vs without valsartan. ‡P<0.05 vs Agtr2−/− without valsartan. §P<0.05 vs without valsartan. Values are mean±SEM.
possible that it may have affected the ischemic brain area
receptor stimulation on neural cells and glial cells. 42,43 It is
tailed expression pattern of AT1 and AT2 receptors in cerebral
H11006
SEM.

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Figure 5. NADPH oxidase activity in brain cortices 24 hours
after MCA occlusion. Protein samples were prepared from brain
cortices, and enzyme activity was assayed as described in
Methods. Some mice were treated with valsartan (3 mg · kg
· d−1) for 10 days via an osmotic minipump before MCA occlu-
sion. Agtr2−/−, wild-type; Agtr2−/+, AT2 receptor-deficient. n=6 to 7
for each group. *P<0.05 vs nonischemic area. †P<0.05 vs with-
out valsartan. 4P<0.05 vs Agtr2−/− without valsartan. Values are
mean±SEM.

pear, at least in part, through the synthesis and activity of the
NADPH oxidase subunit. Further examinations are needed to
clarify the signal transduction pathways in the AT2 receptor–
mediated response.

Because valsartan was administered intraperitoneally, it is
possible that it may have affected the ischemic brain area
through indirect actions on peripheral hemodynamics. How-
ever, this may not be the case in our study, because we used
a nonhypotensive dose of valsartan, as described previously.
18,19 In addition, systolic blood pressure was not signifi-
cantly different after MCA occlusion between Agtr2−/− and
Agtr2−/+ mice (Table). It is not yet clear how peripherally
administered valsartan is effective in reducing the ischemic
brain region. However, as reported previously, peripherally
applied candesartan, an ARB, effectively inhibited the cen-
trally mediated effects of Ang II.40,41 Therefore, it seems
probable that valsartan administered peripherally in the pres-
ent study also was able to enter the ischemic brain lesion and
block central AT1 receptors.

Further investigations are necessary to examine the de-
tailed expression pattern of AT1 and AT2 receptors in cerebral
arteries and the possible direct action of AT1− and AT2−
receptor stimulation on neural cells and glial cells.52,53 It is
also necessary to determine the detailed localization of
oxidative stress in brain blood vessels, inflammatory cells,
and neural tissues in the brain after ischemia; this should also
be examined in future experiments.

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