Angiotensin II Type-2 Receptor Stimulation Prevents Neural Damage by Transcriptional Activation of Methyl Methanesulfonate Sensitive 2

Masaki Mogi, Jian-Mei Li, Jun Iwanami, Li-Juan Min, Kana Tsukuda, Masaru Iwai, Masatsugu Horiuchi

Abstract—The molecular mechanisms of the contribution of angiotensin II type-1 receptor blockers to neuronal protection are still unclear. Here, we investigated the effect of angiotensin II type-2 (AT2) receptor stimulation on neurons and cognitive function involving a new neuroprotective factor, methyl methanesulfonate sensitive 2 (MMS2). Angiotensin II treatment of neurospheres enhanced their differentiation and increased MMS2 expression. Knockdown of the MMS2 gene by small interference RNA (siRNA) significantly reduced the number of neurospheres, with loss of sphere formation. An angiotensin II type-1 receptor blocker, valsartan, enhanced such neurosphere differentiation and MMS2 induction, whereas an AT2 receptor antagonist, PD123319, inhibited them. After mice underwent permanent middle cerebral artery occlusion, AT2 receptor mRNA expression was significantly increased in the ischemic side of the brain. Passive avoidance rate to evaluate cognitive function was significantly impaired in AT2 receptor null (Agtr2/) mice compared with wild-type mice. Treatment with valsartan prevented the cognitive decline in wild-type mice, but this effect was weaker in Agtr2/– mice. In ischemic brain regions, MMS2 was increased in wild-type mice, but not in Agtr2/– mice. Valsartan also enhanced MMS2 expression to a greater degree in wild-type mice. Finally, intracerebroventricular administration of MMS2 siRNA showed more impaired avoidance rate after middle cerebral artery occlusion compared with that in control siRNA–transfected mice. These findings experimentally support the clinical evidence and indicate a unique mechanism of the AT2 receptor in brain protection. (Hypertension. 2006;48:141-148.)

Key Words: stroke ■ angiotensin II ■ receptors angiotensin II ■ antihypertensive agents ■ central nervous system ■ neuroregulators

Stroke is one of the leading causes of disability and death in the industrialized world; however, radical treatment for stroke is limited. Therefore, therapy to prevent stroke is based on treating an individual’s underlying risk factors for stroke, such as hypertension, atrial fibrillation, and diabetes. Recent major clinical research such as the Perindopril Protection Against Recurrent Stroke Study (PROGRESS),1 Study on Cognition and Prognosis in the Elderly (SCOPE),2 Losartan Intervention For Endpoint reduction in hypertension study (LIFE),3 and Acute Candesartan Cilextil Evaluation in Stroke Intervention For Endpoint reduction in hypertension study (LIFE),3 and Acute Candesartan Cilextil Evaluation in Stroke Survivors (ACCESS)4 studies indicates that blockade of the renin–angiotensin system (RAS) is effective to prevent a first or recurrent stroke, atrial fibrillation, and type 2 diabetes mellitus beyond blood pressure lowering. However, the detailed molecular mechanisms of preventing the onset of such pathologic conditions are still an enigma.

Angiotensin II (Ang II) is the principal vasoactive substance of the RAS, having a variety of physiological actions, including vasoconstriction, aldosterone release, and cell growth.5 Ang II binds 2 major receptors, the Ang II type-1 (AT1) receptor and type-2 (AT2) receptor. AT1 receptor blockers (ARBs) have been widely used as antihypertensive drugs, with the expectation of vascular protective effects. Although the majority of Ang II actions are mediated via the AT1 receptor, evidence has accumulated that the AT2 receptor not only opposes the AT1 receptor but also has unique effects beyond an interaction with AT1 receptor signaling. Recently, we reported the effects of the AT2 receptor on ischemic brain regions using a middle cerebral artery occlusion model in AT2 receptor-deficient (Agtr2/) mice.6 Agtr2/– mice displayed a larger ischemic area compared with wild-type mice, and pretreatment with a nonhypotensive dose of an ARB, valsartan, significantly reduced the ischemic area in wild-type mice but not in Agtr2/– mice through a reduction of oxidative stress in the ischemic brain and an increase in cerebral blood flow in the penumbral region, independent of blood pressure lowering, suggesting that activation of the AT1 receptor or blockade of the AT1 receptor attenuates brain injury.
Stroke is also one of the most important causes of cognitive impairment and dementia in the aging population. Poor cognitive performance significantly impairs social interaction and the quality of life of patients. However, once there is cognitive decline, little can be done therapeutically to reverse the symptoms. Although the management of blood pressure is effective to prevent cognitive decline, a major clinical study (SCOPE) and a clinical double-blind study have proved that ARBs have a further therapeutic effect on impaired cognitive function beyond their antihypertensive effects compared with other antihypertensive drugs.

AT2 receptors are also reported to be expressed in areas related to learning and control of motor activity and in fetal tissues but are present at low levels in adult tissues and are re-expressed in certain pathological conditions, such as neuronal injury and vascular injury, suggesting that activation of the AT2 receptor may play a pivotal role in the repair and regeneration of injured tissues. Recent studies have demonstrated the possibility that stimulation of AT2 receptors may promote cell differentiation and regeneration in neuronal tissue. Li et al reported that AT2 receptor stimulation supported neuronal survival and neurite outgrowth in response to ischemia-induced neuronal injury. Moreover, Gendron et al demonstrated that Ang II induces neural differentiation and neurite outgrowth via mitogen-activated protein kinase or NO/through AT2 receptor activation and is involved in cerebellum development. Therefore, after focal brain injury, AT2 receptor stimulation could prevent damage of neurons or activate neural repair systems. However, the earliest events associated with activation of AT2 receptor and the contribution of AT2 receptor signaling to cognitive decline remains unclear.

Methyl methanesulfonate sensitive 2 (MMS2) belongs to a family of ubiquitin-conjugating enzyme variants that are highly similar to ubiquitin-conjugating enzymes E2 (Ubc) and forms a complex with Ubc-13. The MMS2/Ubc-13 complex has been reported to play an important role in DNA repair through a ubiquitin-proteasome system (UPS). DNA damage occurs with central nervous system (CNS) injury, and defects in repair mechanisms are associated with neurodegenerative diseases. Abnormalities of the UPS in the brain contribute to Parkinson disease, Alzheimer disease, prion disease, amyotrophic lateral sclerosis, and polyglutamine disease. Recently, MMS2 was reported to be highly expressed in the rat brain in late embryonic development and then to fall markedly during maturation of the CNS, suggesting that MMS2 plays a pivotal role in neuronal development and differentiation. However, the association of MMS2 with CNS injury has never been studied.

Here, we found that Ang II induced MMS2 mRNA expression in neurons through AT2 receptor stimulation. Focusing on the AT2 receptor–MMS2 signaling cascade, we demonstrated potential roles of AT2 receptor stimulation in neural protection and preservation of cognitive function after focal ischemia.

Materials and Methods
An expanded Materials and Methods section is available online at http://hyper.ahajournals.org.

Cell Culture
To assess the effects of Ang II on neural differentiation, we used in vitro fetal mouse neurospheres prepared as described previously.

AT2 receptor-deficient mouse (Agtr2−/−; based on C57BL/6J strain bred in our laboratory) and wild-type mouse (Agtr2+ + ) fetal brain tissue was dissected into cells. Counting neurospheres was performed in the view at ×20 magnification with an Axioskop microscope (Carl Zeiss).

Immunoblot Analysis
Total protein was prepared from neurospheres, and immunoblotting was performed as described previously using anti-MAP2 (Chemicon International Inc, Temecula, Calif), anti-βIII tubulin, anti-glial fibrillary acidic protein (Promega Corporation, Madison, Wis), and anti-MMS2 (Santa Cruz Inc, Santa Cruz, Calif) antibodies. Densitometric analysis was performed using an image scanner (EPSON GT-8000, Ricoh System Kaizatsu Company Ltd) and National Institutes of Health imaging software.

Immunofluorescence
Neurospheres were fixed with an ethanol and methanol solution (1:1 mixed) before incubation with rabbit polyclonal anti-mouse AT2, AT2 receptor antibody (Santa Cruz Inc), and mouse monoclonal anti-MMS2 antibody (Zymed Laboratories Inc, South San Francisco, Calif). The secondary antibody was goat anti-rabbit or anti-mouse Cy3-conjugated antibody (Jackson ImmunoResearch, West Grove, Pa). Cells were counterstained with 4',6-diamidino-2-phenylindole. Images were viewed at ×40 magnification with an Axioskop microscope (Carl Zeiss) using image analysis software (AxioVision, Carl Zeiss).

MMS2 Small Interfering RNA Assay
For small interfering RNA (siRNA) assay, neural stem cells isolated from the mouse fetal cortex and neurospheres were transiently transfected with negative control siRNA or MMS2-specific siRNA, a mixture of 3 siRNAs designed by B-Bridge using Lipofectamine PLUS (Invitrogen). Thirty-six hours after transfection, cells were observed and treated. Knockdown of MMS2 gene was evaluated by immunoblot using anti-MMS2 antibody (Zymed Laboratories Inc). The mixtures of siRNAs targeting to MMS2 are shown in Supplemental Table II (available online at http://hyper.ahajournals.org).

Immunohistochemical Study
Frozen, enzymatically intact, 10-μm–thick sections were prepared from mouse brain 24 hours after MCA occlusion and fixed with 10% formaldehyde solution before incubation with mouse polyclonal anti-MMS2 antibody (Zymed Laboratories Inc). After incubation, sections were stained by diaminobenzidine staining using an ABC staining kit (DakoCytomation).

Animals and Treatment
This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the Animal Studies Committee of Ehime University. An ARB, valsartan (provided by Novartis Pharma AG), was administered at a dose of 3 mg/kg per day IP via an osmotic minipump (Alzet model 1002, Durect Corporation) implanted IP 10 days before MCA occlusion. Blood pressure was measured by the tail-cuff method (MK-1030, Muromachi Co, Ltd).

MCA Occlusion
Focal cerebral ischemia was induced by occlusion of the MCA in 10-week–old mice by means of an intraluminal filament technique according to a method described previously. Brain samples were obtained 24 hours after MCA occlusion, and mRNA was isolated using Sepasol-RNAI (Nacalai Tesuque Inc). Neurological deficit was evaluated 24 hours after MCA occlusion using the neurological scores developed by Hara et al.

Procedure of Passive Avoidance Test
A shuttle avoidance cage (32×12.5×15 cm; Melquest) and an isolation cabinet (48×42×37 cm; Melquest) were used. Mice were
individually placed in a chamber and given 20 inescapable electric shocks (0.3 mA) of 10 seconds duration at intervals of 2 seconds. The number of escape failures was recorded. Escape failure was defined as a noncrossing response during shock delivery.

**Real-Time RT-PCR Method**

Real-time quantitative RT-PCR was performed with a SYBR green I kit (MJ Research, Inc). PCR primers for the AT₁, AT₂, and angiotensin II type-4 receptors (insulin regulated aminopeptidase [IRAP]) and MMS2 are shown in Supplemental Table I.

**In Vivo MMS2–siRNA Transfer**

We performed in vivo MMS2–siRNA transfer according to the method described previously. Mice were implanted with a stainless steel cannula in a unilateral cerebral ventricle. The stereotaxic coordinates were 0.5 mm posterior and 1.0 mm lateral to the bregma. ICV administration of 1 µL of naked siRNA (50 µg) was performed with the cannula. Animals were allowed 2 days of recovery before MCA occlusion. Transfection efficiency was evaluated by observation of stained cells using Cy3-labeled siRNA injection.

**Statistical Analysis**

Values are expressed as mean±SE in the text and figure. 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**

**Effects of AT₂ Receptor Stimulation on Neural Differentiation**

To assess the effect of Ang II stimulation on neural differentiation, we prepared neurospheres from mouse fetal brain cortex. As reported previously, immunofluorescent staining clearly showed that both AT₁ and AT₂ receptors were expressed in mouse neurospheres (Figure 1A). Usually, neurospheres keep floating in basic fibroblast growth factor–containing neural stem cell–specific medium, but after

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** AT₂ receptor stimulation increased neural differentiation of neurospheres from mouse fetal cortex. A, Both AT₁ and AT₂ receptors were expressed in neurospheres. Representative photographs of 4',6-diamidino-2-phenylindole, AT₁-Cy3, AT₂-Cy3, and merged images of 4 separate experiments are shown. B, AT₂ receptor stimulation enhanced attachment of mouse neurospheres to culture dishes. Neurospheres were cultured in noncoated 6-well dishes with basic fibroblast growth factor–containing neural growth medium. Administration of Ang II (10⁻⁷ mol/L) for 3 days increased the number of attached spheres on the bottom of dishes and induced their differentiation into neuron-like cells. Valsartan (10⁻⁵ mol/L) also enhanced their expression, but an AT₂ receptor antagonist, PD123319 (10⁻⁵ mol/L), inhibited the Ang II–induced effects. *P*<0.05 vs no treatment. †*P*<0.05 vs Ang II (n=3). C, Increase in expression of mature neural cell markers, βIII-tubulin, and MAP2 by Ang II stimulation. Valsartan also enhanced their expression, but PD123319 inhibited their increase. *P*<0.05 vs no treatment. †*P*<0.05 vs Ang II (n=3). D, Glial fibrillary acidic protein expression was not changed by each treatment (n=3). E, Ang II stimulation prevented attachment of mouse neurospheres generated from AT₂ receptor–deficient mice. Comparison of attached neurospheres between age-matched wild-type and AT₂ receptor–deficient mice after stimulation with Ang II and valsartan. Agtr₂⁺, wild-type mice; Agtr₂⁻, AT₂ receptor–deficient mice. *P*<0.01 vs Agtr₂⁺ (n=3).
attaching to culture dishes, differentiate into neurons, astrocytes, or oligodendrocytes. Administration of Ang II (10^{-7} mol/L) for 3 days significantly increased not only a total number of neurospheres but also attachment of neurospheres (Figure 1B), even in basic fibroblast growth factor–containing neural stem cell–specific medium, and induced their differentiation into neuron-like cells. Similarly, as shown in Figure 1C, Ang II stimulation increased the number of cells expressing mature neural cell markers, such as βIII-tubulin and MAP2 by immunoblot analysis. Interestingly, no significant change was found in the expression of a mature glial cell marker, glial fibrillary acidic protein (Figure 1D). These effects of Ang II were further enhanced by treatment with an ARB, valsartan (10^{-5} mol/L), and were markedly inhibited by an AT2 receptor–specific blocker, PD123319 (10^{-5} mol/L; Figure 1B through 1D). Moreover, administration of Ang II and valsartan

Figure 2. AT2 receptor stimulation increased MMS2 mRNA expression in mouse neurospheres determined by real-time RT-PCR. Treatment with Ang II (10^{-7} mol/L) increased mRNA expression. Valsartan (10^{-5} mol/L) enhanced such an increase. PD123319 (10^{-5} mol/L) inhibited Ang II–induced MMS2 expression.

*P<0.05 vs no treatment. †P<0.05 vs Ang II (n=3).

Figure 3. MMS2 played a pivotal role in maintaining neurospheres. A, Mouse neurospheres expressed MMS2 determined by immunofluorescence. Representative photos of 3 separate experiments on 4',6-diamidino-2-phenylindole and MMS2-Cy3, their merged images, and a phase contrast image are shown. B, Representative photos of neurospheres treated with control–siRNA and MMS2–siRNA. MMS2–siRNA–treated neurospheres exhibited loose sphere formation, resulting in collapse. C, Comparison of number of neurospheres between control–siRNA and MMS2–siRNA. *P<0.01 vs control–siRNA (n=3). D, Comparison of number of “formed” neurospheres between transfection of control–siRNA and MMS2–siRNA to mouse single cells immediately after isolation. *P<0.01 vs control–siRNA (n=3). E, AT2 receptor stimulation failed to induce attachment of mouse neurospheres treated with MMS2–siRNA. Comparison of attached neurospheres between control–siRNA and MMS2–siRNA after stimulation with Ang II and valsartan. *P<0.01 vs control–siRNA treatment (n=3). F, Comparison of mRNA expressions of Ang II receptors between control–siRNA and MMS2–siRNA. No significant difference was observed between 2 groups (n=3).
to neurospheres generated from Agrt2− mice failed to increase the number of attached spheres (Figure 1E), indicating that Ang II–induced neural differentiation is mediated via AT2 receptor stimulation.

Association of AT2 Receptor Stimulation and MMS2 Induction in Neural Differentiation

To further examine the mechanism of neural differentiation induced by AT2 receptor stimulation, we focused on MMS2 expression in neurons. In cultured rat neuronal cells, we observed that an increase in mRNA expression of MMS2 reached a peak just before or after birth, and a similar expression pattern was observed for the AT1 receptor.32 Real-time RT-PCR analysis showed that Ang II increased MMS2 mRNA expression in mouse neospheres, and this increase was enhanced by valsartan but inhibited by PD123319 (Figure 2A). Similar results were obtained in the protein expression of MMS2 by immunoblot (Figure 2B), indicating that Ang II–induced MMS2 expression was via AT2 receptor activation.

MMS2 is reported to be expressed in rat fetal brain and to affect brain development;34 however, the expression and effect of MMS2 on neospheres have never been studied. Immunofluorescent staining clearly showed that MMS2 was expressed in mouse neospheres (Figure 3A). Therefore, we assessed the function of MMS2 in neospheres using siRNA for gene knockdown of MMS2. Treatment of neospheres with MMS2–siRNA caused failure to “maintain” tight sphere formation as shown in Figure 3B and caused their collapse and a reduction in sphere number (Figure 3C). Next, we performed MMS2–siRNA treatment in neural stem cells that were just isolated from mouse fetal cortex. Treatment of neural stem cells with MMS2–siRNA caused failure to “generate” neospheres, resulting in a reduction of sphere number (Figure 3D). The Ang II–induced increase in attached sphere number induced by valsartan was significantly inhibited in neospheres treated with MMS2–siRNA, as shown in Figure 3E. MMS2–siRNA treatment in neospheres had no effect on mRNA expressions of AT1 and AT2 receptors (Figure 3F). These results indicate that Ang II–induced neural differentiation was via stimulation of the AT2 receptor and involved the induction of MMS2 expression.

mRNA Expression of Angiotensin Receptors After Focal Ischemia

Next, to assess AT2 receptor–induced neural differentiation in vivo, mice were subjected to MCA occlusion to the effect of the AT2 receptor after brain injury. The left MCA was permanently occluded by insertion of a nylon monofilament coated with silicon resin. Evaluation of the expression of angiotensin receptors in the ischemic and nonischemic areas of the brain after MCA occlusion showed that the AT2 receptor mRNA level was significantly increased in the ischemic area compared with the nonischemic area 24 hours after MCA occlusion, whereas AT1 receptor mRNA expression was not significantly changed (Figure 4). Angiotensin IV, which is also a ligand for IRAP, is reported to be involved in long-term potentiation of neurons and associative and spatial learning.33 However, IRAP mRNA expression was significantly decreased not only in the ischemic area but also in the nonischemic area after MCA occlusion.

Figure 4. Expression of AT2 receptor mRNA was markedly increased in the ischemic side of the brain. mRNA expression of AT1, AT2, and IRAP (angiotensin II type-4 receptor) after MCA occlusion in ischemic and nonischemic side detected by real-time PCR method. *p<0.05 vs Sham (n=5 to 7).

Figure 5. Effects of AT2 Receptor Signaling on Cognitive Function After Focal Ischemia

In previous studies, we demonstrated the possible inhibition of stroke size via AT2 receptor stimulation.35 Here, we examined cognitive function after stroke to evaluate neuronal loss in the injured brain. To assess the effects of MCA occlusion on subsequent learning and memory, Agrt2+ and Agrt2− mice were subjected to 20 trials of a shuttle avoidance test just before and 3 days after MCA occlusion. Because Agrt2− mice display a lower neurological score compared with Agrt2+ mice, as described previously,6 mice with similar neurological scores were chosen to exclude any difference in locomotor activity in these experiments. Focal brain injury by MCA occlusion in Agrt2+ mice resulted in a lower avoidance rate and a lower total response rate, which were evaluated by the avoidance rate and escape rate. Interestingly, we demonstrated that the avoidance rate in Agrt2− mice was significantly lower before MCA occlusion and was more markedly impaired than in age-matched Agrt2+ litter mates (Figure 5A). Moreover, IP administration of a nonhypotensive dose of valsartan via an osmotic minipump at a dose of 3 mg/kg per day for 10 days before MCA occlusion significantly improved the avoidance rate and also improved the total response rate (Figure 5B). Interestingly, treatment with valsartan enhanced cognitive function before MCA occlusion, indicating that the AT2 receptor plays a pivotal role in cognition even before focal cerebral ischemia.

Effect of MMS2 on Cognitive Function After Focal Ischemia

Finally, we analyzed the effect of MMS2 on cognitive function after focal ischemia. Brain samples were obtained 24 hours after MCA occlusion. MMS2 mRNA expression was increased in the ischemic brain region in Agrt2+ mice but not in Agrt2− mice (Figure 6A). Valsartan significantly increased MMS2 mRNA expression in mouse neospheres, and this increase was enhanced by valsartan but inhibited by PD123319 (Figure 2A). Similar results were obtained in the protein expression of MMS2 by immunoblot (Figure 2B), indicating that Ang II–induced MMS2 expression was via AT2 receptor activation. MMS2 is reported to be expressed in rat fetal brain and to affect brain development; however, the expression and effect of MMS2 on neospheres have never been studied. Immunofluorescent staining clearly showed that MMS2 was expressed in mouse neospheres (Figure 3A). Therefore, we assessed the function of MMS2 in neospheres using siRNA for gene knockdown of MMS2. Treatment of neospheres with MMS2–siRNA caused failure to “maintain” tight sphere formation as shown in Figure 3B and caused their collapse and a reduction in sphere number (Figure 3C). Next, we performed MMS2–siRNA treatment in neural stem cells that were just isolated from mouse fetal cortex. Treatment of neural stem cells with MMS2–siRNA caused failure to “generate” neospheres, resulting in a reduction of sphere number (Figure 3D). The Ang II–induced increase in attached sphere number induced by valsartan was significantly inhibited in neospheres treated with MMS2–siRNA, as shown in Figure 3E. MMS2–siRNA treatment in neospheres had no effect on mRNA expressions of AT1 and AT2 receptors (Figure 3F). These results indicate that Ang II–induced neural differentiation was via stimulation of the AT2 receptor and involved the induction of MMS2 expression.

Effect of MMS2 on Cognitive Function After Focal Ischemia

Finally, we analyzed the effect of MMS2 on cognitive function after focal ischemia. Brain samples were obtained 24 hours after MCA occlusion. MMS2 mRNA expression was increased in the ischemic brain region in Agrt2+ mice but not in Agrt2− mice (Figure 6A). Valsartan significantly increased MMS2 mRNA expression in mouse neospheres, and this increase was enhanced by valsartan but inhibited by PD123319 (Figure 2A). Similar results were obtained in the protein expression of MMS2 by immunoblot (Figure 2B), indicating that Ang II–induced MMS2 expression was via AT2 receptor activation. MMS2 is reported to be expressed in rat fetal brain and to affect brain development; however, the expression and effect of MMS2 on neospheres have never been studied. Immunofluorescent staining clearly showed that MMS2 was expressed in mouse neospheres (Figure 3A). Therefore, we assessed the function of MMS2 in neospheres using siRNA for gene knockdown of MMS2. Treatment of neospheres with MMS2–siRNA caused failure to “maintain” tight sphere formation as shown in Figure 3B and caused their collapse and a reduction in sphere number (Figure 3C). Next, we performed MMS2–siRNA treatment in neural stem cells that were just isolated from mouse fetal cortex. Treatment of neural stem cells with MMS2–siRNA caused failure to “generate” neospheres, resulting in a reduction of sphere number (Figure 3D). The Ang II–induced increase in attached sphere number induced by valsartan was significantly inhibited in neospheres treated with MMS2–siRNA, as shown in Figure 3E. MMS2–siRNA treatment in neospheres had no effect on mRNA expressions of AT1 and AT2 receptors (Figure 3F). These results indicate that Ang II–induced neural differentiation was via stimulation of the AT2 receptor and involved the induction of MMS2 expression.

mRNA Expression of Angiotensin Receptors After Focal Ischemia

Next, to assess AT2 receptor–induced neural differentiation in vivo, mice were subjected to MCA occlusion to the effect of the AT2 receptor after brain injury. The left MCA was permanently occluded by insertion of a nylon monofilament coated with silicon resin. Evaluation of the expression of angiotensin receptors in the ischemic and nonischemic areas of the brain after MCA occlusion showed that the AT2 receptor mRNA level was significantly increased in the ischemic area compared with the nonischemic area 24 hours after MCA occlusion, whereas AT1 receptor mRNA expression was not significantly changed (Figure 4). Angiotensin IV, which is also a ligand for IRAP, is reported to be involved in long-term potentiation of neurons and associative and spatial learning.33 However, IRAP mRNA expression was significantly decreased not only in the ischemic area but also in the nonischemic area after MCA occlusion.
expression in the ischemic area in Agtr2+ mice. Immunohistochemical staining of MMS2 also showed an increase of MMS2 expression in the ischemic brain of Agtr2+/− mice (Figure 6B). These findings suggest that AT2 receptor signaling could contribute to neural repair via MMS2 expression after brain injury. To further investigate the effect of MMS2 on cognitive function after focal ischemia, we performed the ICV administration of MMS2–siRNA. Treatment with MMS2–siRNA showed more impaired avoidance rate after MCA occlusion compared with that in negative control siRNA–transfected mice (Figure 6C), suggesting that inhibiting the increase in MMS2 expression by knocking down the MMS2 gene in vivo enhanced the decline in cognitive function after stroke.

**Discussion**

In this study, we demonstrated that the direct effects of AT2 receptor stimulation contribute to neural differentiation and could prevent the cognitive decline after brain injury. First, in cultured neurons, AT2 receptor stimulation enhanced the differentiation of neurospheres and induced MMS2 expression, which affects neural differentiation or protection, demonstrated by a gene knockdown assay of MMS2. Second, we showed that Agtr2− mice exhibited greater impairment of cognitive function after focal cerebral ischemia. Third, MMS2 induction after focal ischemia was found in wild-type mice but not in Agtr2− mice. Therefore, these experimental results and our previous report support the clinical evidence for cerebroprotection by ARB and indicate multiple mechanisms of prevention of neural damage by AT2 receptor stimulation.

This study demonstrated the association of AT2 receptor stimulation with MMS2 induction. Although the signaling cascade from AT2 receptor stimulation to MMS2 induction remains unclear, downstream targets of the AT2 receptor may be involved in such a cascade. The AT2 receptor belongs to the superfamily of G protein–coupled receptors and negatively regulates cell proliferation via signaling pathways that mainly involve activation of tyrosine phosphatases, such as Src homology 2 domain–containing protein-tyrosine phosphatase 1 (SHP-1), and inhibition of protein kinases. SHP-1 is reported to be a critical regulator of developmental signals leading to terminal differentiation and myelin sheath formation by oligodendrocytes, suggesting that SHP-1 may be involved in AT2 receptor–induced neural differentiation.
Moreover, we have recently cloned AT2 receptor–interacting protein, which interacts with the C-terminal tail of the AT2 receptor, and it has been indicated recently to cooperate with the AT2 receptor to trans-inactivate receptor tyrosine kinases.36 Our recent experimental results indicate that interaction of SHP-1 and AT2 receptor–interacting protein may be involved in MMS2 upregulation by AT2 receptor stimulation.32 Further study will be needed to confirm the steps of the signaling cascade from AT2 receptor stimulation to MMS2 transactivation.

Here, we showed that lack of the MMS2 gene caused failure to maintain sphere formation of neurospheres and generation of neurospheres from neural stem cells. It can be speculated that MMS2 seems to act as a control factor of neural cell differentiation, survival, and fate. The MMS2/Ubc-13 complex has been reported to play an important role in DNA repair through a UPS,21 and MMS2 is also necessary for error-free lesion bypass in the RAD6 pathway with cell cycle regulation.37 After cerebral ischemia, oxidative DNA damage occurs in a more extensive area than that where cell death is recognized.38 Because severe DNA damage triggers neuronal cell apoptosis, such extended DNA damage could result in more prolonged cell dysfunction and eventual neuronal loss.39,39 To prevent neuronal loss, a complex DNA repair system network is activated after cerebral ischemia.22 Therefore, MMS2 may play an important role in neural protection after stroke through the DNA repair system.

The detail mechanism of the decrease in mRNA expression of IRAP after MCA occlusion is unclear. However, focal cerebral ischemia induced an imbalance of blood flow in both hemispheres through secondary remote effects40 or diaschisis.41 To prevent neural loss and avoid the development of a necrosis, neurons induce preconditioning and decrease genes involved in metabolic pathways.42 IRAP regulates glucose metabolism through glucose uptake by regulating trafficking of GLUT4. Therefore, focal ischemia may reduce IRAP expression to reduce glucose metabolism for the inhibition of neuronal loss.

Ang II inhibited the induction of long-term potentiation in rat hippocampal granule cells, an ARB blocked the inhibition of long-term potentiation,43 and an angiotensin-converting enzyme inhibitor attenuated the age-related impairment of learning and memory,44 suggesting that blockade of the RAS also inhibits the cognitive decline not only through its hypotensive effect but also by other mechanisms. Agtr2− mice have been reported to exhibit attenuation of exploratory behavior and anxiety-like behavior45 and abnormality in water intake.30 In our experiments on behavioral tasks, Agtr2− mice showed a less social approach to a strange mouse and exhibited anxiety behavior (Supplemental Figure I, available at http://hyper.ahajournals.org), indicating that Agtr2− mice may have a neuropsychiatric disorder by nature. Agtr2− mice are reported to have an increased number of cells in the brain46 because of abnormal regulation of apoptosis. Indeed, we also observed an increased cell number around the hypothalamus of Agtr2− mice (32±3.1 in wild-type mice, 56.3±2.4 in Agtr2− mice at ×20 magnification detected by hematoxylin/eosin staining). These results suggest that lack of the AT2 receptor gene may generate an abnormality of brain development. Indeed, in humans, mutations of the AT2 receptor located on the X chromosome were found in a female patient with mental retardation,47 indicating that the AT2 receptor gene may be involved in brain development and neural maturation.

**Perspectives**

Our present findings demonstrate the possibility that the AT2 receptor is an important molecular determinant of neural differentiation, and AT2 receptor activation prevents the cognitive decline after focal brain injury, involving a new neuroprotective factor, MMS2. Therefore, we expect that relative stimulation of AT2 receptor signaling by ARB treatment could have a therapeutic advantage to prevent neurological disorders after stroke.

**Sources of Funding**

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan; the Takeda Science Foundation; and the Novartis Foundation for Gerontological Research (to M.H.) and the Mitsubishi Pharma Research Foundation (to M.M.).

**Disclosures**

None.

**References**

Angiotensin II Type-2 Receptor Stimulation Prevents Neural Damage by Transcriptional Activation of Methyl Methanesulfonate Sensitive 2

Masaki Mogi, Jian-Mei Li, Jun Iwanami, Li-Juan Min, Kana Tsukuda, Masaru Iwai and Masatsugu Horiuchi

Hypertension. 2006;48:141-148; originally published online June 12, 2006; doi: 10.1161/01.HYP.0000229648.67883.f9

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/48/1/141

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2006/06/26/01.HYP.0000229648.67883.f9.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/