Effect of Angiotensin II Type 2 Receptor Deletion in Hematopoietic Cells on Brain Ischemia-Reperfusion Injury

Jun Iwanami, Masaki Mogi, Kana Tsukuda, Li-Juan Min, Akiko Sakata, Fei Jing, Kousei Ohshima, Masatsugu Horiuchi

Abstract—The angiotensin II type 2 (AT2) receptor is expressed in bone marrow cells and may affect cell differentiation. We previously reported a beneficial role of the AT2 receptor in ischemic brain damage. Here, we investigated the effect of AT2 receptor stimulation in hematopoietic cells on ischemic brain injury using chimeric mice. Chimeric mice were generated by bone marrow transplantation into wild-type mice after irradiation. Bone marrow cells were prepared from wild-type (Agtr2+/+) or AT2 receptor-deficient mice (Agtr2−/−). Six weeks after bone marrow transplantation, these chimeric mice were subjected to ischemia/reperfusion injury. Both Agtr2+/+ and Agtr2−/− chimeric mice did not show a significant change in systolic and diastolic blood pressures, whereas body weight decreased in Agtr2−/− chimera. Twenty-four hours after ischemia/reperfusion injury, ischemic brain damage in Agtr2−/− chimera was exaggerated compared with that in Agtr2+/+ chimera. Moreover, cerebral blood flow in the peripheral region before and after ischemia/reperfusion injury was decreased in Agtr2−/− chimera. The inflammatory response in the ipsilateral hemisphere was not significantly different, whereas tumor necrosis factor-α and monocyte chemoattractant protein 1 expressions tended to increase in the Agtr2−/− chimeric brain. Expression of methylmethane sulfonate 2, which has a neuroprotective effect, was lower in the brain of Agtr2−/− chimera. These results indicate that deletion of AT2 receptor in blood cells has a harmful effect on ischemic brain injury. (Hypertension. 2011;58:404-409.) ● Online Data Supplement

Key Words: angiotensin II receptor ● chimera ● stroke ● cerebral blood flow

The renin-angiotensin system is well known to be involved in the pathogenesis in multiorgan damage involving the heart, kidney, blood vessels, and brain. Recent large clinical trials have also indicated that angiotensin receptor blockers are effective to prevent a first or recurrent stroke beyond their blood pressure–lowering effect.1–3 Angiotensin II, a major player in the renin-angiotensin system, binds to 2 major receptors, the angiotensin II type 1 (AT1) receptor and type 2 (AT2) receptor. Treatment with angiotensin receptor blocker has been expected to have protective effects for multiorgans via not only AT1 receptor blockade but also relative stimulation of the AT2 receptor. In the brain, we reported recently that activation of the AT2 receptor attenuated brain injury partly because of a reduction of oxidative stress in the ischemic brain and an increase in cerebral blood flow (CBF) in the penumbral region in mice subjected to middle cerebral artery (MCA) occlusion.4 Moreover, we also demonstrated that AT2 receptor signaling enhanced neural differentiation and the repair of damaged DNA by induction of a neural differentiating factor, methyl methanesulfonate-sensitive 2 (MMS2), which is one of the ubiquitin-conjugating enzyme variants.5,6 Stimulation of the AT2 receptor promoted cell differentiation and regeneration in neuronal tissue.7 Accumulating evidence indicates that AT2 receptor signaling could act as a crucial cerebroprotective factor.

It is known that hematopoietic cells, such as leukocytes, platelets, macrophages, and erythrocytes, accumulate in the ischemic territory and infiltrate the brain after stroke. These blood cells interact with the microvasculature after ischemia and reperfusion because of platelet activation, fibrin formation, and leukocyte adhesion.8–10 In addition, leukocyte-derived cytokines, such as matrix metalloproteinase 9, have been reported to participate in dysregulation of the blood-brain barrier after ischemic brain injury,11 indicating that hematopoietic cells may play an important role in stroke expansion.

Angiotensin II receptors are highly expressed in bone marrow cells12 and are reported to induce proliferation of hematopoietic and erythroid progenitors.13 Kato et al14 reported that renin-angiotensin system–activated transgenic mice show increased erythropoiesis via the AT1 receptor. On the other hand, inflammatory cytokines from hematopoietic cells are increased in the brain after stroke and enlarge
ischemic brain injury.\textsuperscript{15,16} Recently, it was reported that chimeric mice with AT\textsubscript{1} receptor–deficient bone marrow show pathological differences in leukocyte recruitment and attenuation of platelet-vessel wall adhesion.\textsuperscript{17} On the other hand, AT\textsubscript{2} receptors are reported to be involved in hematopoietic cell differentiation and activation. For example, Okamura et al.\textsuperscript{18} reported that AT\textsubscript{1} and AT\textsubscript{2} receptors were upregulated during differentiation to macrophages from monocytes. AT\textsubscript{2} receptor signaling induces differentiation of human monocytes to dendritic cells.\textsuperscript{19} In monocytes, the angiotensin II–induced inflammatory response was enhanced by treatment with an AT\textsubscript{2} receptor antagonist.\textsuperscript{20} These reports suggest important roles for AT\textsubscript{2} receptor stimulation of blood cells in pathological conditions.

Several reports have shown the effect of AT\textsubscript{1} receptor signaling in hematopoietic cells followed by stroke. AT\textsubscript{1} receptor stimulation in leukocytes and endothelial cells induces brain microvessel endothelial injury via an increase in adhesion molecules in stroke-prone spontaneously hypertensive rats.\textsuperscript{21} However, the role of the AT\textsubscript{2} receptor on hematopoietic cells in ischemic brain damage has never been investigated. These results led us to investigate the effect of lack of AT\textsubscript{2} receptor signaling in hematopoietic cells on ischemic brain damage, using bone marrow chimeric mice after ischemia/reperfusion (IR) injury.

Materials and Methods

Animals

Adult AT\textsubscript{2} receptor–deficient mice (Agtr\textsuperscript{2}\textsuperscript{−/−}; based on C57BL/6J strain) and wild-type mice (Agtr\textsuperscript{2}\textsuperscript{+/+}; C57BL/6J) at 10 to 12 weeks old for male mice and 6 to 7 weeks old for female mice were used in this study. This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. The experimental protocol was approved by the Ehime University Animal Studies Committee.

Generation of Chimeric Mice

Six- to 7-week–old female mice were given whole-body irradiation with a dose of 9 to 10 Gy. Bone marrow cells (BMCs) were isolated from 6 crushed bones (the bilateral tibias, femurs, and iliac bones) of adult 10- to 12-week–old male Agtr\textsuperscript{2}\textsuperscript{−/−} or Agtr\textsuperscript{2}\textsuperscript{+/+} mice. Bulk BMCs (1.0 × 10\textsuperscript{6} cells) were injected via the tail vein after dilution in PBS (200 μL) immediately after irradiation. After transplantation, irradiated mice were housed for 6 weeks. To determine the chimerism, blood samples were obtained from these chimeric mice, and PCR was performed for genotyping sex chromosome-linked genes, as described previously.\textsuperscript{22} Hemodynamic parameters, such as CBF and blood pressure, in each mouse did not change after BMC injection.

Measurement of Blood Pressure

Systolic blood pressure was monitored in conscious mice by the tail-cuff method (MK-1030, Muromachi Co, Tokyo, Japan), as described previously.\textsuperscript{24} For the more detailed blood pressure monitoring, we used surgical implantation of telemetry blood pressure monitoring systems (TA11PA-C10, Primetech Corporation, Tokyo, Japan).

Transient MCA Occlusion

Chimeric mice were subjected to transient MCA occlusion by the filament technique, as described previously.\textsuperscript{24} Mice were anesthetized by IP injection of 65 mg/kg of Nembutal in saline. A midline incision was made in the neck, and the left common and external carotid arteries were isolated. A silicon (PROVIL Novo Medium, Heraeus Kulzer, Hanau, Germany)-coated nylon (Ethilon, Ethicon, Norderstedt, Germany) filament was inserted into the common carotid artery, and the MCA was occluded. Three hours after MCA occlusion, the nylon filament was removed from the common carotid artery. A brain sample was obtained 24 hours after MCA occlusion.

Neurological Score

Neurological deficit was measured 24 hours after MCA occlusion using the neurological scores developed by Huang et al.\textsuperscript{23} Neurological score was defined as follows: 0 for no neurological deficit; 1, failure to extend left forelimb; 2, circling to the contralateral side; 3, falling to the contralateral side at rest; and 4, no spontaneous motor activity.

2,3,5-Triphenyltetrasodium Chloride Staining

To evaluate the ischemic volume in the brain, the extracted brain was sliced into 7 coronal sections with 1-mm thickness and stained with 2% 2,3,5-triphenyltetrasodium chloride. Ischemic volumes were determined as the percentage of 2,3,5-triphenyltetrasodium chloride–stained area in the total area.

Cerebral Blood Flow

CBF before and after MCA occlusion and reperfusion was measured with a 2D blood flow meter (Omegazone, laser speckle blood flow imager, Omegawave, Tokyo, Japan), as described previously.\textsuperscript{26} Mice were anesthetized with Nembutal in saline, and a midline incision was made in the scalp. The skull was exposed and wet with saline. A 780-nm laser semiconductor laser was used to illuminate the area of interest. CBF was measured in the whole of the skull surface and in the core and periphery of the MCA territory before and 24 hours after MCA occlusion.

Real-Time RT-PCR

Real-time quantitative RT-PCR was performed with a SYBR green 1 kit (MJ Research, Inc, Waltham, MA) using the whole cortex of the brain before MCA occlusion in MMS2 and 24 hours after MCA occlusion in all of the genes. We compared the gene expressions between brain samples obtained with or without the perfusion of PBS. However, there were no significant differences in mRNA expressions in these groups. Therefore, we prepared brain samples without PBS perfusion to avoid brain damage.

The PCR primers for tumor necrosis factor-α were 5′-CTCACATCATTTCTCAA-3′ (forward) and 5′-CAGACCATGACCTC-CAAA-3′ (reverse); those for monocyte chemoattractant protein 1 were 5′-GGCTCAGCAATGCGTAA-3′ (forward) and 5′-GTGAATGAGTAGCAGCAGGTGAGT-3′ (reverse); those for MMS2 were 5′-ATGGCAGTCTCCAGGAGT-3′ (forward) and 5′-GCCCAATATCATGGCAGTTGC-3′ (reverse).

Quantification of 8-Hydroxy-2′-Deoxyguanosine

Immunoactivity of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in DNA of the brain cortex 24 hours after MCA occlusion was detected by an ELISA. Briefly, genomic DNA was extracted using a DNA Extractor TIS kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and pretreated using 8-OHdG Assay Preparation Reagent Set (Wako Pure Chemical Industries, Ltd). The 8-OHdG level in 50 μg of DNA samples was measured by an 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging). The ratio of nanograms of 8-OHdG per milligram of DNA was calculated.

Statistical Analysis

All of the values were expressed as mean±SD for ischemic volume and mean±SEM for other results. 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

Systolic Blood Pressure and Body Weight

Chimeric mice were confirmed 6 weeks after BMC transplantation by assessing the change in DNA band pattern from the
recipient pattern (female; 1 PCR band at 300 bp) to the donor pattern (male; 2 PCR bands at 300 and 330 bp), indicative of successful reconstitution of BMC by transplantation (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). In chimeric mice, there was no significant change in blood pressure measured by tail-cuff method. Moreover, 24-hour blood pressure monitoring revealed that systolic and diastolic blood pressures did not differ over a 24-hour period in these mice (Figure S2A and S2B). In addition, there was no significant difference of blood pressure in these mice 24 hours after MCA occlusion. Body weight was significantly decreased in Agtr2⁻→Agtr2⁺ mice (Agtr2⁻ chimera; 18.6±0.2 g) compared with Agtr2⁺→Agtr2⁺ mice (Agtr2⁺ chimera; 19.1±0.2 g; P<0.05).

**Increased Brain Damage After I/R Injury by AT₂ Receptor Deletion in Hematopoietic Cells**

At 24 hours after I/R injury, ischemic size was assessed by 2,3,5-triphenyltetrasodium chloride staining. As shown in Figure 1, ischemic volume in Agtr2⁻ chimera was significantly increased compared with that in Agtr2⁺ chimera (Figure 1). Neurological deficit in Agtr2⁻ chimera 24 hours after I/R injury was also worse compared with that in Agtr2⁺ chimera (Figure 2). Survival ratio 24 hours after I/R injury determined by 2,3,5-triphenyltetrasodium chloride staining of brain sections obtained 24 hours after I/R injury. B, Quantitative analysis of ischemic volume at 24 hours after I/R injury determined by 2,3,5-triphenyltetrasodium chloride staining, n=7 to 10 for each group. *P<0.05 vs Agtr2⁺ chimera. Values are mean±SEM.

**Decrease in CBF After I/R Injury by Deletion of AT₂ Receptor in Hematopoietic Cells**

CBF in the whole brain region before I/R injury was markedly decreased in Agtr2⁻ chimera compared with Agtr2⁺ chimera (Figure 3A and 3B). Change of regional CBF after I/R injury in the core and peripheral regions of the MCA territory was also assessed. In the core region, CBF was not significantly different between the 2 groups before I/R injury, whereas it showed a tendency to decrease in Agtr2⁻ chimera 24 hours after I/R injury (Figure 3C). In the peripheral region, CBF was significantly reduced in Agtr2⁻ chimera before I/R injury (Figure 3C). Twenty-four hours after I/R injury, CBF in the peripheral region in Agtr2⁻ chimera was also significantly decreased compared with that in Agtr2⁺ chimera.

**Inflammatory Response After I/R Injury**

Expression of inflammatory cytokines, such as tumor necrosis factor-α and monocyte chemoattractant protein 1 tended to increase in Agtr2⁻ chimera compared with Agtr2⁺ chimera (Figure 5B). MMS2 expression in the ipsilateral hemisphere compared with that in the contralateral hemisphere (Figure 4). In the ipsilateral hemisphere, expression of tumor necrosis factor-α and monocyte chemoattractant protein 1 tended to increase in Agtr2⁻ chimera compared with Agtr2⁺ chimera; however, significant difference was not obtained between Agtr2⁺ and Agtr2⁻ chimera.

**Low Level in MMS2 Expression by Deletion of AT₂ Receptor in Hematopoietic Cells**

We assessed mRNA expression of the AT₂ receptor and MMS2, which is regulated by AT₂ receptor signaling, as described previously.⁶ AT₂ receptor expression tended to increase in the ipsilateral hemisphere compared with the contralateral hemisphere in both strains but did not show any significant difference between the 2 groups (Figure 5A). On the other hand, MMS2 expression in the brain before MCA occlusion was significantly lower in Agtr2⁻ chimera compared with Agtr2⁺ (Figure 5B). MMS2 expression in the contralateral and ipsilateral sides after MCA occlusion was significantly lower in Agtr2⁻ chimera compared with Agtr2⁺ chimera (Figure 5B).

MMS2 is a family of ubiquitin-conjugating enzyme variants and induces to repair damaged DNA.²⁷ Finally, we assessed the level of 8-OHdG, one of the DNA damage

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**Figure 1.** Stroke volume after ischemia-reperfusion (I/R) injury. A, Representative photos of 2,3,5-triphenyltetrasodium chloride staining of brain sections obtained 24 hours after I/R injury. B, Quantitative analysis of ischemic volume at 24 hours after I/R injury determined by 2,3,5-triphenyltetrasodium chloride staining, n=7 to 10 for each group. *P<0.05 vs Agtr2⁺ chimera. Values are mean±SEM.

**Figure 2.** Neurological score after ischemia-reperfusion (I/R) injury. n=10 to 15 for each group. *P<0.05 vs Agtr2⁺ chimera. Values are mean±SEM.

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makers 24 hours after MCA occlusion. 8-OHdG level tended to increase in the ipsilateral hemisphere of Agtr2-/- chimera compared with that of Agtr2+/+ chimera, but a significant change was not obtained (Figure 6).

**Discussion**

The present study demonstrated that deletion of the AT2 receptor in hematopoietic cells enhanced ischemic brain damage after I/R injury. Moreover, a remarkable decrease in CBF and lower level of MMS2 expression were observed in mice with deletion of the AT2 receptor in hematopoietic cells.

Recently, AT1 receptor signaling in bone marrow and hematopoietic cells has been highlighted using chimeric mice, especially in the pathogenesis of atherosclerosis.28,29 Tsubakimoto et al30 also reported that bone marrow AT1 receptor activation regulates differentiation of monocyte lineage progenitors from hematopoietic stem cells. Strawn and Ferrario31 also showed that AT1 receptor stimulation increases hematopoietic cell differentiation into monocytes expressing proinflammatory markers, such as CD11b. However, although a previous report showed the effect of the AT2 receptor on the differentiation of human and mouse myeloid dendritic cells,19 there are few articles describing the effect of AT2 receptor activation on differentiation of hematopoietic cells. Moreover, there are also few reports on the effects of angiotensin receptors in hematopoietic cells on ischemic brain injury. The present article focused on the role of the renin-angiotensin system in hematopoietic cells in stroke, using chimeric mice.

Recently, we reported that AT2 receptor signaling in bone marrow stromal cells plays an important role in bone marrow stromal cell–induced brain protection, with an antiinflammatory effect, but not with an increase in CBF after I/R injury in mice.32 Thus, we expected that the inflammatory response might be enhanced in Agtr2-/- chimera. However, no significant difference in mRNA expression of tumor necrosis factor (TNF)-α (A) and monocyte chemoattractant protein (MCP) 1 (B), in whole cortex of the brain after ischemia-reperfusion (I/R) injury. n=6 to 7 for each group. Values are mean±SEM.
factor-α and monocyte chemoattractant protein 1 was observed between Agtr2\(^{-}\) and Agtr2\(^{+}\) chimera. In contrast, Agtr2\(^{-}\) chimera exhibited a decrease in CBF compared with Agtr2\(^{+}\) chimera. Therefore, we speculated that deletion of the AT\(_2\) receptor in hematopoietic cells affects blood circulation but not inflammatory response. Vascular endothelium is a key player in hematopoietic-vessel interactions, such as leukocyte recruitment and neutrophil-endothelial interactions. After stroke, AT\(_1\) receptor activation in leukocytes and endothelial cells is reported to induce brain endothelial injury via an increase in adhesion molecules.\(^{33}\) It is possible that loss of counteraction of AT\(_1\) receptor signaling by lack of AT\(_2\) receptors may increase leukocyte adhesion to endothelial cells and result in a decrease in CBF.

AT\(_2\) receptor activation stimulates the release of NO/cGMP and may mediate vascular relaxation and increase blood flow indirectly by the modulation of bradykinin release.\(^{33}\) Moreover, Sales et al\(^{34}\) demonstrated that lack of AT\(_2\) receptor signaling elevated a positive area of macrophage in atherosclerotic lesions in apolipoprotein E–deficient mice. These results provide us with a hypothesis that Agtr2\(^{-}\) chimeric mice decreased CBF probably at least in part via a decrease in the NO/cGMP pathway and activation of inflammatory cells, such as macrophages, which regulate vascular plasticity. Further studies on AT\(_2\) receptor functions in hematopoietic cells are necessary to address the roles of the AT\(_2\) receptor more in detail.

Previously, we reported that AT\(_2\) receptor signaling regulates MMS2, which could act as an important factor for neuronal cell differentiation and an increase in survival, possibly through enhancement of DNA repair.\(^{5}\) After cerebral ischemia, oxidative DNA damage occurs in a larger area than that where cell death is recognized.\(^{35}\) In this study, we observed that brain MMS2 expression was markedly lower in Agtr2\(^{-}\) chimera compared with Agtr2\(^{+}\) chimera, suggesting that lack of AT\(_2\) receptor signaling in hematopoietic cells might enhance the induction of DNA damage, with enhancement of ischemic brain damage. Although we could not obtain a significant difference in 8-OHdG level, DNA damage tended to increase in the ipsilateral hemisphere of Agtr2\(^{+}\) chimera compared with that of Agtr2\(^{-}\) chimera. We have been investigating the detailed mechanism of the downregulation of MMS2 in the brain of Agtr2\(^{+}\) chimera, which would contribute to further understanding of the roles of AT\(_2\) receptor stimulation in hematopoietic cells to prevent ischemic brain damage. In conclusion, deletion of the AT\(_2\) receptor in hematopoietic cells has a harmful effect against cerebral ischemic damage.

**Perspectives**

Our findings indicate that presence of the AT\(_2\) receptor in hematopoietic cells has a protective effect against cerebral ischemic damage. Stimulation of the AT\(_2\) receptor on hematopoietic cells by such as angiotensin receptor blocker may be therapeutically useful for the prevention of ischemic brain damage. Clinical studies are anticipated in patients taking AT\(_1\) receptor blockers, focusing on the function of blood cells.

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**Disclosures**

None.
References


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Figure S1. Representative photos for detection of chimerism using PCR amplification of X and Y chromosome specific sequence in mice six weeks after bone marrow transplantation following irradiation.
Figure S2A. Radio telemetry recording of systolic blood pressure 6 weeks after bone marrow transplantation. n=4 for each group. Values are mean±SEM.
Figure S2B. Radio telemetry recording of diastolic blood pressure 6 weeks after bone marrow transplantation. n=4 for each group. Values are mean±SEM.