Continuous Activation of Renin-Angiotensin System Impairs Cognitive Function in Renin/Angiotensinogen Transgenic Mice

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Abstract—We examined the possibility that continuous activation of the human brain renin-angiotensin system causes cognitive impairment, using human renin (hRN) and human angiotensinogen (hANG) gene chimeric transgenic (Tg) mice. Cognitive function was evaluated by the shuttle avoidance test once a week from 10 to 20 weeks of age. The avoidance rate in wild-type mice gradually increased. In contrast, the avoidance rate in chimeric hRN/hANG-Tg mice also increased; however, no further increase in avoidance rate was observed from 14 weeks of age, and it decreased thereafter. Cerebral surface blood flow was markedly reduced in 20-week-old hRN/hANG-Tg mice. Superoxide anion production in the brain was already higher in 10-week-old hRN/hANG-Tg mice and further increased thereafter with an increase in NADPH oxidase activity. Moreover, expression of p47phox and Nox4 in the brain of hRN/hANG-Tg mice also increased. Administration of an angiotensin II type 1 receptor blocker, olmesartan (5.0 mg/kg per day), attenuated the increase in blood pressure and ameliorated cognitive decline with enhancement of cerebral surface blood flow and a reduction of oxidative stress in hRN/hANG-Tg mice. On the other hand, hydralazine (0.5 mg/kg per day) did not improve the decrease in avoidance rate, and did not influence cerebral surface blood flow or oxidative stress in hRN/hANG-Tg mice, in spite of a similar reduction of blood pressure to that by olmesartan. Moreover, we observed that treatment with Tempol improved impaired cognitive function in hRN/hANG-Tg mice. These results suggest that continuous activation of the brain renin-angiotensin system impairs cognitive function via stimulation of the angiotensin II type 1 receptor with a decrease in cerebral surface blood flow and an increase in oxidative stress. (Hypertension. 2009;53[part 2]:356-362.)

Key Words: angiotensin II receptors ▪ cognitive function ▪ blood flow ▪ oxidative stress ▪ transgenic mice

Dementia is a common serious health problem that impairs quality of life. A continuous decline in cognitive function occurs as the natural aging course in both humans and animal models. Hypertension is a major risk factor for cerebrovascular disease, including stroke, and contributes to the development of vascular dementia.1 Several clinical studies, such as Perindopril Protection Against Recurrent Stroke Study, Systolic Hypertension in Europe, and Study on Cognition and Prognosis in the Elderly, have shown that antihypertensive drug treatment is associated with reduced cognitive decline.2-4 However, it is not still clear which classes of antihypertensive drugs provide greater benefits than others.

Activation of the renin-angiotensin system (RAS) plays major roles in elevated blood pressure and the development of cerebrovascular disorders. All of the components of the classic RAS have been identified in the brain.5,6 Recent clinical trials, such as the Losartan Intervention for Endpoint Reduction in Hypertensive Study, Morbidity and Mortality After Stroke, Eprosartan Compared With Nitrendipine for Secondary Prevention, and the Jikei Heart Study, demonstrated that blockade of RAS with angiotensin (Ang) II receptor blockers (ARBs) is effective to prevent the onset of stroke, irrespective of their blood pressure–lowering effect.7-9 Moreover, we have reported previously that blockade of RAS with ARBs attenuates ischemic brain damage.10,11 However, the roles of Ang II in cognitive function are not well defined. In addition, the relationship between continuous activation of the brain RAS and cognitive function is not understood. Therefore, in the present study, we tested the hypothesis that continuous activation of the brain RAS is involved in impairment of cognitive function.

For this purpose, we used chimeric double transgenic (Tg) mice of the human renin (hRN) and human angiotensinogen (hANG) genes. The chimeric (hRN/hANG-Tg) mice were produced by mating of hRN-Tg and hANG-Tg mice.
chimeric mice have been developed as a mouse model of human hypertension induced by activation of the human RAS.\textsuperscript{12–14} Our recent article showed that Ang II content is significantly increased in the brain, as well as in plasma, in hRN/hANG-Tg mice, and that activation of the human RAS in the brain is involved in exaggeration of ischemic brain damage attributed partly to enhancement of oxidative stress.\textsuperscript{15} Ang II is known to increase oxidative stress, and several studies have demonstrated that impairment of cognitive function is associated with an increase in oxidative stress.\textsuperscript{16–18} Therefore, we examined the roles of continuous activation of the human RAS in the brain, focusing on oxidative stress, as well as cerebral blood flow.

**Methods**

**Animals and Treatment**

Adult male mice aged 10 weeks were used in this study. Transgenic mice carrying both the human renin and angiotensinogen genes (hRN/hANG-Tg) were generated by mating of human renin-transgenic (hRN-Tg; C57BL/6J background) mice with human angiotensinogen-transgenic (hANG-Tg; C57BL/6J background) mice purchased from Riken Bioresource Center (Tsukuba, Japan).\textsuperscript{12} C57BL/6J mice were used as a genetic background–matched wild-type control. The animals were housed in a room with a 12-hour light/dark cycle with a temperature of 25±1°C. They were given standard laboratory chow (MF, Oriental Yeast Co, Ltd) and water ad libitum. Olmesartan, a selective Ang II type 1 (AT1) receptor blocker (5.0 mg/kg per day in laboratory chow, provided by Sankyo Co. Ltd), hydralazine (0.5 mg/kg per day in laboratory chow, Sigma-Aldrich Japan K.K.) were administered orally from 4 weeks of age through the experiment (until a maximum of 20 weeks of age). An electric shock was delivered by a shock generator to the stainless-steel rods on the floor of the cage. Each mouse was placed in 1 side of the cage and given 20 trials. Each trial consisted of a 5-second buzzer followed by an electric shock (0.3 mA, 3-second duration, 2-second interval) on the side of the mouse. The side where the mouse was present was detected by a sensor. When the mouse escaped the electric shock by moving from one side to the other during the buzzer, it was counted as avoidance. Avoidance rate was calculated as the ratio of avoidance in 20 trials. The test was performed once a week between 10 AM and 12 PM.

**Measurement of Cerebral Blood Flow**

Cerebral surface blood flow (CBF) was determined under anesthesia by laser speckle flowmetry (Omnigrow laser speckle blood flow imager, Omegawave, Inc), as described previously.\textsuperscript{21} Mean CBF was measured in a region of the same size (250×300 pixels), where the bregma was positioned at the center of the measured region. Images were analyzed by the color image program incorporated in the flowmetry system to obtain the average value of blood flow.

**Detection of Superoxide Anion Production in Brain Sections**

Histological in situ detection of superoxide anion was performed using freshly frozen sections stained with dihydroethidium (10 μmol/L in PBS for 30 minutes at 37°C, as described previously.\textsuperscript{11} 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 using computer-imaging software (Densiograph, ATTO Corporation).

**Measurement of NADPH Oxidase Activity**

Protein samples were obtained by homogenizing the brain with ice-cold Tris-sucrose buffer (10 mmol/L of Tris [pH 7.0], 340 mmol/L of sucrose, and 1 mmol/L of EDTA). NADPH oxidase activity was measured by cytochrome method and quantified from the difference in absorbance with and without superoxide dismutase, as described previously.\textsuperscript{22}

**Real-Time RT-PCR**

Total RNA was extracted from the brain at 20 weeks of age. Expression of mRNA was determined by quantitative real-time RT-PCR. The level of target gene expression was normalized against...
the GAPDH expression in each sample. PCR primers for p47phox were 5'-H11032-GTCCCTGCATCCTATCTGGA-3' (forward) and 5'-H11032-GGGACATCTCGTCCTCTTCA-3' (reverse); for Nox4 they were 5'-H11032-GAGTCACTCCATTTGCATCG-3' (forward) and 5'-H11032-TCCCATCTGTTTGACTGAGG-3' (reverse); for the AT1 receptor they were 5'-H11032-AGTCGCACTCAAGCCTGTCT-3' (forward) and 5'-H11032-ACTGGTCCTTGTGTCGTGAG-3' (reverse); for the Ang II type 2 (AT2) receptor they were 5'-H11032-CCTGCATGAGTGTCGATAGGT-3' (forward) and 5'-H11032-CCAAGCAGACCACTGAGCATA-3' (reverse); and for GAPDH were 5'-H11032-TGCGACTTCAACAGCAACTC-3' (forward) and 5'-H11032-ATGTAGGCCATGAGGTCCAC-3' (reverse).

Statistical Analysis
Values are expressed as means±SEMs in the text and figures. Data were analyzed by 1-way ANOVA. If a statistically significant effect was found, posthoc analysis was performed by Scheffe’s test to detect the difference between the groups. A value of $P<0.05$ was considered to indicate statistical significance.

Results

Systolic Blood Pressure in hRN/hANG-Tg Mice
Systolic blood pressure (SBP) in hRN/hANG-Tg mice was higher than that in wild-type (WT) mice even at 10 weeks of age and gradually increased thereafter, as reported previously. On the other hand, SBP in WT mice did not significantly change during the experimental period (Figure 1).

Decrease in Cognitive Function in hRN/hANG-Tg Mice
Cognitive function was evaluated by the shuttle avoidance test. The avoidance rate was measured once a week from 10 to 20 weeks. The avoidance rate in WT mice showed a gradual increase. In contrast, the avoidance rate in hRN/hANG-Tg mice also gradually increased; however, no further increase in avoidance rate was observed from 14 weeks of age, and it decreased thereafter (Figure 2).

Change in CBF in hRN/hANG-Tg Mice
CBF at the age of 10 weeks did not show a significant difference between WT and hRN/hANG-Tg mice. However, CBF in hRN/hANG-Tg mice markedly decreased, whereas that in WT mice did not significantly change (Figure 3). At 20 weeks of age, CBF in hRN/hANG-Tg mice was $\sim 80\%$ of that in WT mice.
Increase in Oxidative Stress in Brain of hRN/hANG-Tg Mice
Superoxide anion production in the cerebral cortex detected by dihydroethidium staining was already higher in hRN/hANG-Tg mice than in WT mice even at 10 weeks of age and increased thereafter (Figure 4A and 4B). We also observed that NADPH oxidase activity in the brain was also enhanced in hRN/hANG-Tg mice compared with that in WT mice (Figure 4C). We next examined expression of NADPH oxidase subunits p47phox and Nox4 mRNA in the brain at 20 weeks of age. Expression of p47phox (left) and Nox4 (right) mRNA was significantly higher in hRN/hANG-Tg mice than in WT mice (Figure 5).

Effect of AT1 Receptor Blockade on Cognitive Function in hRN/hANG-Tg Mice
Treatment with an AT1 receptor blocker, olmesartan, from 4 weeks of age inhibited the increase in SBP to a similar level compared with that in WT mice (Figure 1). Olmesartan prevented impairment of cognitive function and reduction of CBF in hRN/hANG-Tg mice throughout the experiment (Figures 2 and 3). Moreover, olmesartan inhibited overproduction of superoxide anion and enhancement of NADPH oxidase activity, together with suppression of expression of NADPH oxidase subunits p47phox and Nox4 mRNA in the brain (Figures 4 and 5). To examine the effect of elevated blood pressure on cognitive function in hRN/hANG-Tg mice, we next investigated the effect of treatment with hydralazine. Treatment with hydralazine from 4 weeks of age reduced SBP to a similar level to that caused by olmesartan but did not improve the decrease in avoidance rate, CBF, or increase in oxidative stress in hRN/hANG-Tg mice (Figures 1 to 5).

Treatment With Olmesartan Increased Expression of AT2 Receptor mRNA in hRN/hANG-Tg Mice
There was no significant difference in the brain AT1 receptor mRNA level in each group (Figure 6). AT2 receptor mRNA level in the brain did not differ between WT and hRN/hANG-Tg mice. Administration of olmesartan significantly increased the AT2 receptor mRNA level, whereas treatment with hydralazine did not influence it.

Effect of Antioxidant on Cognitive Decline in hRN/hANG-Tg Mice
Treatment with an antioxidant, Tempol, from 4 weeks of age attenuated the impairment of cognitive function in hRN/hANG-Tg mice (Figure 7A). In addition, administration of Tempol improved the decrease in CBF (Figure 7B). SBP after treatment with Tempol was slightly lower than that without Tempol in hRN/hANG-Tg mice at the age of 20 weeks, although there was no significant difference (119.8±6.5 mm Hg in hRN/hANG-Tg mice with Tempol).

Discussion
Previous clinical studies have suggested that blockade of the RAS could prevent cognitive impairment associated with hypertension.2,4 Our results demonstrated that continuous exaggeration of the human RAS in transgenic mice used in...
this experiment prevented the development of normal learning ability and, thereafter, impaired cognitive function. Moreover, administration of an ARB, olmesartan, ameliorated cognitive decline, with a reduction of blood pressure. In contrast, treatment with hydralazine did not improve cognitive decline, even with a similar decrease in blood pressure compared with that by olmesartan. These results strongly suggest that continuous exaggeration of RAS is involved in impaired development of cognitive function. It is also reported that administration of Ang II or renin into the central nervous system attenuates the retention of a passive avoidance task after learning and avoidance learning. The shuttle avoidance test in our study is similar to a passive avoidance test with electric shock to examine learning in those previous articles. However, electric shock may cause anxiety, which is difficult to be analyzed separately from learning. Wilson et al reported that transgenic (mRen2)27 rats that overexpressed mouse renin with the increase in Ang II showed an anxiatic profile, suggesting a possible involvement of Ang II in anxiety. Therefore, further examination is needed to elucidate possible involvement of Ang II–induced anxiety.

We demonstrated that continuous exaggeration of the human RAS decreased CBF at 20 weeks of age. Overproduction of Ang II plays major roles in the pathogenesis of cardiovascular and cerebrovascular diseases. Ang II induces cerebrovascular remodeling, promotes vascular inflammation and oxidative stress, and thereby impairs the regulation of CBF. Previous studies showed that endothelial function in cerebral vessels was impaired in a genetic model of Ang II–dependent hypertension. It is known that CBF decreases with aging. Hypertension is a risk factor for cognitive decline and dementia. Accumulating evidence suggests that Alzheimer disease and other types of dementia, including vascular dementia in humans, are associated with reduced CBF. Moreover, it has been reported that hypertensive patients had decreased CBF, although their cognitive function was normal. These results indicate that reduction of CBF could be involved in the development of neurodegenerative disorders. In the present study, we demonstrated that the decrease of CBF in hRN/hANG-Tg mice was improved by the administration of olmesartan, but not hydralazine, although similar reductions of blood pressure were observed with both drugs, supporting the notion that exaggeration of AT1 receptor activation really plays a role in the impairment of cognitive function. The reason that hydralazine did not recover the decrease in CBF is not yet totally clear. However, a possible explanation may be that the CBF in hRN/hANG-Tg mice is mainly regulated by activated RAS in the brain in this mouse model. In fact, as we have reported previously, superoxide anion production was also markedly increased in middle cerebral arteries of hRN/hANG-Tg mice, which was inhibited by an ARB but not by hydralazine. Accordingly, some studies demonstrated that treatment with an ARB exerted a protective effect on the autoregulation of CBF in the spontaneously hypertensive rat via a reduction of inflammation and superoxide generation, and restoration of the vascular structure, independent of blood pressure. Moreover, we have reported previously that capillary density in the brain was increased by treatment with an ARB. These results suggest that these beneficial effects on cerebral vessels by blockade of the AT1 receptor could contribute to suppression of the decrease in CBF.

In the present study, we demonstrated that superoxide anion production, NADPH oxidase activity, and expression of NADPH oxidase subunits, such as p47phox and Nox4, were enhanced in the brain of hRN/hANG-Tg mice. It was reported previously that the GAPDH mRNA level was decreased by stimulation with Ang II in cultured vascular smooth muscle cells. However, in the measurement of mRNA level, we applied the same amount of total RNA, and the mRNA level for GAPDH was not significantly different in all of the experimental groups. Overproduction of Ang II induces excessive release of superoxide radical by stimulation of reduced nicotinamide-adenine dinucleotide/NADPH oxidase via AT1 receptor activation. Yamamoto et al reported previously that reactive oxygen species produced by Ang II–activated NADPH oxidase increased cerebral neuronal apoptosis and inflammation in stroke-prone spontaneously hypertensive rats. Moreover, Wei et al reported that NADPH oxidase contributed to vascular inflammation, endothelial dysfunction, and remodeling in transgenic (mRen2)27 rats, which exhibit elevated tissue Ang II, similar to our experimental model, hRN/hANG-Tg mice. It has been recognized that oxidative stress is implicated in age-related cognitive impairment. In addition, there are reports indi-
ating that oxidative stress is increased in the brain of patients with Alzheimer disease and other neurodegenerative disorders. Experiments in *Drosophila* revealed that oxidative stress was a causal factor in r-induced neurodegeneration. In the present study, administration of an antioxidant, Tempol, prevented the cognitive decline and the decrease in CBF in hRN/hANG-Tg mice at 20 weeks of age. These results suggest that the decline of cognitive function by continuous activation of the brain RAS in hRN/hANG-Tg mice was closely associated with enhanced oxidative stress because of excessive stimulation of the AT1 receptor. There was no significant difference in CBF at 10 weeks of age when the oxidative stress was already elevated. This may be because of a time difference between the elevation of oxidative stress and its consequent effect on CBF. Therefore, we propose that the sustained decrease in oxidative stress by blockade of the AT1 receptor could contribute to neural protection and an increase in CBF, resulting in the prevention of consequent cognitive decline in hRN/hANG-Tg mice. Because it is reported that Tempol reduces activity of the sympathetic nervous system, there is a possibility that modulation of sympathetic nerve activity is also involved in the actions of Tempol and olmesartan.

Relative stimulation of AT2 signaling during ARB treatment has been highlighted in terms of protection against brain damage. We observed that treatment with olmesartan increased AT2 receptor expression in the brain. Therefore, we can postulate that the effect of treatment with olmesartan to prevent cognitive decline in hRN/hANG-Tg mice could possibly be because of blockade of the AT1 receptor with relative activation of the AT2 receptor. We have reported previously that AT2 receptor signaling contributes to neuronal differentiation and also that AT2 receptor signaling attenuates DNA damage and vascular senescence. Therefore, the inhibitory effect of treatment with olmesartan on cognitive decline might be partly because of activation of the AT2 receptor in the brain of hRN/hANG-Tg mice. However, the detailed mechanisms of the increase in expression of the AT2 receptor by olmesartan in the brain and its pathophysiological relevance to prevention of cognitive decline need to be addressed in more detail to further understand the roles of exaggeration of RAS in cognition.

**Perspectives**

Our results suggest that continuous exaggeration of Ang II production could result in impairment of normal development of cognitive function and, consequently, cognitive decline, probably independent of blood pressure increase. The decrease in CBF and continuous exaggeration of oxidative stress induced by overproduction of Ang II could be involved in cognitive decline. Taken together, our results provide new insights into understanding how continuous long-term stimulation of the AT1 receptor could mediate cognitive impairment, and earlier treatment of hypertensive patients with an ARB could have beneficial effects to prevent cognitive decline.

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**Disclosures**

None.

**References**


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