Angiotensin Receptor Blocker Prevented β-Amyloid–Induced Cognitive Impairment Associated With Recovery of Neurovascular Coupling

Shuko Takeda, Naoyuki Sato, Daisuke Takeuchi, Hitomi Kurinami, Mitsuru Shinohara, Kazue Niisato, Masanobu Kano, Toshio Ogihara, Hiromi Rakugi, Ryuichi Morishita

Abstract—Recent studies suggest that vascular risk factors play a considerable role in the development of Alzheimer disease. Furthermore, the use of antihypertensive drugs has been suggested to reduce the incidence of dementia, including Alzheimer disease. In this study, we examined the effects of an angiotensin receptor blocker, olmesartan, on β-amyloid–induced cerebrovascular dysfunction and cognitive impairment. Oral administration of a low dose of olmesartan attenuated cerebrovascular dysfunction in young Alzheimer disease–model transgenic mice (APP23 mouse), without a reduction in the brain β-amyloid level. Moreover, treatment of APP23 mice with olmesartan decreased oxidative stress in brain microvessels. Using an acute mouse model induced by ICV administration of β-amyloid 1-40, we assessed the effect of oral administration of olmesartan on spatial learning evaluated with the Morris water maze. Olmesartan significantly improved cognitive function independent of its blood pressure–lowering effect, whereas there was no improvement by other types of antihypertensive drugs (hydralazine and nifedipine). We found that pretreatment with a low dose of olmesartan completely prevented β-amyloid–induced vascular dysregulation and partially attenuated the impairment of hippocampal synaptic plasticity. These findings suggest the possibility that amelioration of cerebrovascular dysfunction with an angiotensin receptor blocker could be a novel therapeutic strategy for the early stage of Alzheimer disease. (Hypertension. 2009;54:1345-1352.)

Key Words: angiotensin receptor blocker ■ hypertension ■ renin-angiotensin system ■ dementia ■ Alzheimer disease

Alzheimer disease (AD) is the most common form of dementia. One of the major neuropathological hallmarks of AD is accumulation of β-amyloid peptide (Aβ) in the brain.1 Aβ is a 38- to 43-amino acid peptide produced from amyloid precursor protein (APP) by proteolytic processing. Abnormal accumulation of Aβ-peptide in the brain is associated with a cascade of pathological events, resulting in dementia.1 Recently, alterations in cerebrovascular regulation related to vascular oxidative stress have been implicated in the mechanisms of the early stage of AD.2–4 The strategy for AD treatment is shifting to earlier stage intervention or prevention of this disease. Amelioration of cerebrovascular dysregulation could be a novel therapeutic target.

Importantly, there is a growing body of evidence indicating an association between vascular risk factors and AD.5 Several epidemiological studies have shown that hypertension in midlife is related to the development of AD.5,6 Furthermore, some antihypertensive drugs reduce the incidence of dementia, including AD. For example, in the Systolic Hypertension in Europe Study, active therapy with a calcium channel blocker, nitrendipine, reduced the incidence of AD.7 The Cache County Study of Memory and Aging concluded that the use of antihypertensive medication is associated with a lower incidence of AD.8 On the other hand, previous studies have revealed contributory functions of the renin-angiotensin system (RAS) in the pathogenesis of AD. Savaskan et al9 reported increased immunoreactivity of perivascular angiotensin-converting enzyme (ACE) and angiotensin II surrounding cortical vessels in the AD brain, suggesting RAS activation in the cerebral vessels in AD. Activation of RAS in the AD brain has also been reported by other groups.10,11 These findings suggest that inhibition of brain RAS could be a new therapeutic strategy for AD.12 More recent studies have shown that ACE inhibitors and angiotensin receptor blockers (ARBs) have favorable effects on cognitive function.13–15 Importantly, these studies suggest the possibility that these medications may produce their effects on cognitive function independent of their antihypertensive actions, although the underlying mechanisms are still unknown.
In this study, we investigated the effect of an ARB, olmesartan, on cognitive dysfunction in an AD mouse model, focusing on its effect on cerebrovascular function. Our results suggest that oral administration of olmesartan at a relatively low dosage prevents Aβ-mediated cognitive decline, associated with recovery of neurovascular coupling.

Methods
Please see also the online Data Supplement (http://hyper.ahajournals.org).

Animals and Drug Treatment
All of the animal experiments were performed in compliance with the Osaka University School of Medicine Guidelines for the Care and Use of Laboratory Animals. Male ddY mice and transgenic APP23 mice were used for the study. In all of the experiments, mice were analyzed at the age of 12 to 15 weeks. Administration of antihypertensive drugs was started at 8 weeks of age and continued until the end of experiments.

Behavioral Analysis
Hippocampus-dependent learning and memory function of the mice were investigated with the Morris water maze task. To assess basal activity of animals, the open-field test was carried out.

Electrophysiological Tests
Electrophysiological assessment of hippocampal function in Aβ-injected mice was carried out 6 to 7 days after ICV administration. Field excitatory postsynaptic potential was studied in the CA1 region of hippocampal sections.

Measurement of Aβ
Fresh-frozen mouse brain was serially homogenized into detergent-soluble and guanidine HCl–soluble fractions. The amounts of Aβ X-40 and Aβ X-42 in each fraction were determined by BNT-77/BA-27 and BNT-77/BC-05 sandwich ELISAs (Wako Pure Chemical Industries), respectively, according to the manufacturer’s instructions.

Isolation of Brain Microvessels and Immunocytochemical Staining
Mouse brain microvessels were isolated and characterized as detailed in the Online Data Supplement.

Measurement of Reactive Oxygen Species
Reactive oxygen species (ROS) production in brain microvessels was measured by dihydroethidium (DHE) microfluorography (Please see the online Data Supplement for more details).

Cerebral Blood Flow Analysis
Assessment of cerebral blood flow (CBF) and cerebrovascular reactivity was carried out as described previously with some modifications. All of the parameters were simultaneously monitored with a computerized data acquisition system (Unique Acquisition software; Unique Medical).

Statistical Analysis
All of the data were expressed as mean±SEM. Comparison of 2 groups was performed by 2-tailed t test for dependent or independent samples, as appropriate. Comparison of means among ≥3 groups was performed by ANOVA or repeated-measure ANOVA followed by the Tukey-Kramer multiple-range test.

Results
Olmesartan Improved Neurovascular Dysfunction and Decreased ROS in Brain Microvessels in Young APP23 Mice
With the view of establishing a new therapeutic strategy to prevent AD, we initially used young APP23 transgenic mice, a well-validated animal model of AD. Although APP23 mice at the age of 12 weeks had no detectable amyloid plaque in the brain and did not exhibit apparent impairment of cognitive function, the amount of soluble Aβ was significantly elevated as compared with that in wild-type mice. Because neural activity is closely related to CBF, and its regulation during brain activity largely depends on reactivity of cerebral vessels, we focused on cerebrovascular regulation. It is reported that Aβ (especially Aβ1-40) disrupts cerebrovascular reactivity, which contributes to cognitive dysfunction. To evaluate cerebrovascular reactivity, we used an in vivo CBF monitoring system and examined functional hyperemia and CBF autoregulation.

There was no significant difference in body weight and resting CBF after 4 weeks of pretreatment with olmesartan (Figure S1B and S1C, available in the online Data Supplement). Arterial blood gases and pH were in the physiological range and did not differ among the 3 groups (Figure S1A). In this study, to minimize confounding effects of a possible difference in neuronal activation on functional hyperemia, we recorded somatosensory evoked potentials (SEPs) at the site of CBF recording and adjusted the magnitude of whisker stimulation (Figure 1A, left). The stimulation pulse was adjusted to evoke half of the maximum amplitude of SEPs. Functional hyperemia evoked by whisker stimulation was significantly attenuated in young APP23 mice (Figure 1A, right). Olmesartan partially, but significantly, ameliorated Aβ-induced impairment of functional hyperemia (Figure 1A and 1B). Because the neuronal activation evoked by whisker stimulation was comparable among the 3 groups (Figure S1D), altered neuronal activation might not contribute to the difference in functional hyperemia. Moreover, the magnitude of adjusted stimulation on the basis of SEP level and the amplitude of stimulation required for maximum SEP response were not different among the groups (Figure S1E and S1F), which indicates that the neuronal response to whisker stimulation in young APP23 mice was comparable to that in transgene-negative littermates. Moreover, APP23 mice showed profoundly impaired CBF autoregulation compared with wild-type mice, whereas olmesartan significantly improved the impairment in CBF during stepwise hypotension (Figure 1C).

Because Aβ-induced oxidative stress in brain blood vessels is thought to contribute to cerebrovascular dysfunction and cognitive impairment in APP transgenic mice, we next evaluated the effect of olmesartan on ROS production in brain microvessels of young APP23 mice. Using DHE microfluorography, ROS signals in blood vessels were significantly stronger in young APP23 mice than in wild-type mice (Figure 2A through 2C; P<0.01). Treatment with olmesartan significantly reduced ROS production in brain microvessels of APP23 mice. Especially in capillaries, ROS signals in APP23 mice treated with olmesartan were comparable with those in wild-type mice (Figure 2A and 2C).

To examine whether the decreased ROS production in brain microvessels and improvement of cerebrovascular reactivity were related to a reduction in Aβ load in the brain, we measured brain Aβ in mice with or without olmesartan treatment. Although the levels of Aβ40 and Aβ42 in the APP23 mouse brain were significantly elevated as compared...
Olmesartan Improved Cognitive Dysfunction in Aβ1-40–Injected Mice

Unfortunately, young APP23 mice did not exhibit apparent cognitive dysfunction. Given the favorable effects of olmesartan, we then examined the effects of olmesartan on cognitive dysfunction using an Aβ1-40–injected mouse model, because this model is one of the useful, well-validated animal models of AD. The validity of this model as an AD model was confirmed by our recent study. Treatment with olmesartan could not be attributed to reduction in brain Aβ.

Figure 1. Olmesartan improves neurovascular dysfunction in young APP23 mice. A and B, Functional hyperemia evoked by whisker stimulation. Representative waveforms of SEP (A, left). CBF and mean arterial blood pressure were simultaneously recorded (A, right). Stim, whisker stimulation. B, CBF change during whisker stimulation. *P<0.05, **P<0.01. C, Relationship between CBF and mean arterial blood pressure. Representative waveforms of SEP (A, left). CBF and mean arterial blood pressure were simultaneously recorded (A, right). Stim, whisker stimulation. B, CBF change during whisker stimulation. *P<0.05, **P<0.01, APP23 + olmesartan 1.0 mg/kg per day vs APP23 + vehicle; #P<0.05, APP23 + olmesartan 1.0 mg/kg per day vs wild-type + vehicle. n=4 to 5 per group.

with those in wild-type mice, no significant difference was observed between APP23 mice with or without olmesartan treatment (Figure S2A and S2B). In this early stage of disease, no obvious amyloid plaque was observed in the brains of both groups (Figure S2C). Therefore, the improved cerebrovascular reactivity in APP23 mice treated with olmesartan could not be attributed to reduction in brain Aβ.

Decreased CBF and MABP in APP23+Vehicle mice relative to wild-type (Figure 3A; P<0.05). However, administration of olmesartan at doses of 0.5 and 1.0 mg/kg per day significantly decreased the escape latency as compared with vehicle treatment (Figure 3A). In the visible platform test, there were no significant differences in escape latency among all of the groups, indicating that visual function was not different among them (Figure S3C). In the probe test, Aβ1-40 injection alone resulted in significantly less time in the target quadrant as compared with Aβ40-1 injection (P<0.05), whereas olmesartan significantly attenuated the impairment caused by Aβ1-40 (Figure 3B; P<0.01). These results clearly demonstrated that olmesartan significantly improved Aβ-induced cognitive dysfunction. In contrast, we found that higher doses of olmesartan (3.0 mg and 6.0 mg/kg per day) resulted in no favorable effect on cognition because of excessive hypotension (Figure S4A and S4B).

However, we found that the dose of olmesartan (1.0 mg/kg per day) slightly but significantly reduced blood pressure (Figure S3B), although olmesartan ameliorated cognitive dysfunction in Aβ1-40–injected mice. To exclude the possibility that this favorable effect on cognitive function might depend on its blood pressure–lowering effect, we also examined other antihypertensive drugs with non-RAS mechanisms, such as hydralazine (a nonspecific vasodilator) and nifedipine (a calcium channel blocker). Of importance, hydralazine (30 mg/kg per day) and nifedipine (10 mg/kg per day) did not improve cognitive dysfunction, although these
drugs reduced systolic arterial blood pressure to a similar degree compared with olmesartan (1.0 mg/kg per day; Figure S5A). In the hidden platform test (Figure 4A), administration of olmesartan at a dose of 1.0 mg/kg per day significantly ameliorated the impairment of spatial learning caused by Aβ1-40 injection (P<0.01), whereas the escape latency in the hydralazine and nifedipine groups was comparable with that in vehicle-treated mice with Aβ1-40 injection. In the probe test, the olmesartan-treated group showed a significant increase in the number of crossings of the trained platform (Figure S5C) and searched in the target quadrant for a significantly longer time than did the vehicle-treated group (Figures 4B and S5E). In contrast, the performances of hydralazine- and nifedipine-treated mice were comparable with that of vehicle-treated animals (Figures 4B and S5C). There were no significant differences in escape latency in the visible platform test (Figure S5B) and swim speed (Figure S5D) among these groups, indicating that the differences in performance in this test reflected neither an alteration of visual function or swimming ability. Furthermore, we evalu-

Figure 2. ROS production in brain microvessels assessed by DHE staining. A, Isolated cerebral microvessels were stained for CD31 (endothelial cell marker) and with DHE (ROS signal). Scale bar, 50 μm. B and C, The increased ROS signal in brain microvessels of young APP23 mice was attenuated by treatment with olmesartan (1.0 mg/kg per day), both in arterioles (B) and capillaries (C). *P<0.05, **P<0.01. n=4 to 5 per group.

Figure 3. Low doses of olmesartan improve cognitive function in Aβ-injected mice. Morris water maze test. Escape latencies in hidden-platform (A) and spatial preference pattern in a probe test (B) are shown. #P<0.05 vs Aβ1-40 + vehicle; *P<0.05 vs respective target quadrant. n=5 to 7 per group. T, target quadrant: the area where the platform was constantly located in the hidden-platform task; L, the quadrant left of the target; R, the quadrant right of the target; O, the quadrant opposite the target.
ated the basal activity of olmesartan-treated mice in the open-field test and confirmed that they showed exploratory activity comparable with that of vehicle-treated mice (Figure S6A and S6C). In this test, we also observed that olmesartan-treated mice showed more habituation (a decrease in locomotion and rearing score on examination days) than did vehicle-treated mice, suggesting that olmesartan might decrease anxiety or aggressiveness of Aβ1-40-injected mice (Figure S6B and S6D). Taken together, the present data suggest that improvement of Aβ-induced cognitive dysfunction by olmesartan is independent of its blood pressure-lowering effect.

Olmesartan Prevented Impairment of Synaptic Plasticity and Cerebrovascular Dysfunction Induced by Aβ1-40

To elucidate the cellular mechanisms of the improvement in cognitive dysfunction by olmesartan, we further examined hippocampal function as assessed by long-term potentiation (LTP) in Aβ-injected mice with or without olmesartan treatment. LTP recorded at CA1 was significantly suppressed in Aβ1-40-injected mice compared with that in control (Aβ40-1-injected) mice (Figure 5A and 5B). However, pretreatment with olmesartan significantly attenuated the suppression of LTP in Aβ1-40-injected mice (P<0.01).

Figure 4. Improvement of Aβ-induced cognitive dysfunction by olmesartan is independent of blood pressure-lowering effect. Morris water maze test. Escape latencies in hidden platform test (A) and spatial preference pattern (B) during the probe test are shown. *P<0.05, **P<0.01. n=9 to 15 per group.

Figure 5. Effect of olmesartan administration on LTP in CA1/Schaffer collaterals in Aβ-injected mice. A, Time course of LTP in 3 groups. Tetanic stimulation (100 Hz, 1 second) was delivered at 0 minutes. B, Summary of LTP experiments. Potentiation ratios were calculated by dividing the averaged excitatory postsynaptic potential slope values from 50 to 60 minutes by those from -30 to 0 minutes. **P<0.01. n=11 to 13 per group.
We then focused on cerebrovascular regulation. Although arterial blood gases and pH were not outside of the physiological range among all of the groups (Table S1), direct topical application of Aβ1-40 onto the mouse somatosensory cortex significantly attenuated the increase in CBF evoked by whisker stimulation (Figure 6A). Pretreatment with olmesartan significantly prevented Aβ-induced attenuation of functional hyperemia (Figure 6A).

We further assessed CBF autoregulation. Under topical application of Aβ1-40, the reduction of CBF in response to stepwise hypotension in the range of 60 to 90 mm Hg was larger than that in control (Aβ40-1+vehicle-treated) mice (Figure 6B), which indicates that cerebrovascular autoregulation was disrupted by Aβ1-40. On the other hand, animals treated with olmesartan showed recovery of impaired autoregulation in the presence of Aβ1-40. It is noteworthy that treatment with olmesartan ameliorated Aβ-induced cerebrovascular dysfunction.

Discussion
Recent studies have revealed significant contributory functions of RAS in the pathophysiology of AD. Activation of RAS in the AD brain was reported by some groups.9–11 These findings support the attractive hypothesis that inhibition of the brain RAS could be a new therapeutic strategy for AD.12

Indeed, a recent study showed that treatment with brain-penetrating ACE inhibitors slowed the rate of cognitive decline in AD patients in comparison with other antihypertensive drugs (hydralazine and nifedipine) did not, although the Systolic Hypertension in Europe Study showed that calcium channel blockers might depend on their blood pressure–lowering effect.13,25 Importantly, a recent study demonstrated that an ARB, valsartan, attenuated oligomerization of Aβ into toxic oligomers, and treatment of Tg2576 mice with valsartan reduced amyloid neuropathology.26 Another report also revealed that telmisartan prevented cognitive deficit in an Aβ injection mouse model through angiotensin receptor blockade and activation of peroxisome proliferator-activated receptor-γ.27 However, it is still an enigma regarding whether ARBs improve cognitive dysfunction. The present study demonstrated that oral administration of an ARB, olmesartan, prevented cognitive impairment in a mouse model of AD, associated with amelioration of Aβ-induced cerebrovascular dysfunction. Our findings suggest important novel mechanisms for the improvement of Aβ-mediated cognitive dysfunction by ARBs.

Our present study provides several important observations. First, low doses of olmesartan (≈1.0 mg/kg per day) improved Aβ-induced cognitive dysfunction. However, other antihypertensive drugs (hydralazine and nifedipine) did not, although the Systolic Hypertension in Europe Study showed that a calcium channel blocker significantly reduced the incidence of AD.2 The neuroprotective effect of calcium channel blockers might depend on their blood pressure–lowering effect. The present study strongly suggests that olmesartan ameliorated Aβ-mediated cognitive deficit through mechanisms independent of blood pressure lowering. Second, olmesartan improved neurovascular dysfunction and decreased ROS production in APP23 transgenic mice. Considerable evidence now indicates the importance of cerebrovascular dysfunction in the pathogenesis of AD.17,28,29 Interestingly, neurovascular coupling is reported to be compromised in AD27 by the enhanced generation of superoxide radicals.4 Thus, the beneficial effect of olmesartan on cognitive dysfunction could be attributed to recovery of cerebrovascular function associated with a significant reduction in oxidative stress. Third, olmesartan restored LTP in the
hippocampus of Aβ1-40-injected mice and ameliorated the impairment of cerebrovascular regulation induced by Aβ1-40. Brain activity critically depends on a continuous blood supply, and a focal increase in CBF in a functionally activated area (functional hyperemia) supports normal cognitive function. It has been reported that synaptic transmission partly depends on the brain microcirculation. Therefore, it is possible that improvement of the microcirculation by olmesartan might contribute to the attenuation of Aβ-mediated suppression of LTP. It is also possible that olmesartan may act directly on neurons in the brain and exert a neuroprotective effect. Angiotensin II induces inflammation and ROS production in the brain via the angiotensin II type 1 receptor, which caused neuronal dysfunction. Selective blockade of the angiotensin II type 1 receptor in the brain might abolish these unfavorable effects directly or through the protective effect of angiotensin II type 2 receptor stimulation.

Importantly, improvement in CBF regulation occurred without a reduction of brain Aβ level, implying that olmesartan protects brain vessels from Aβ-mediated oxidative damage without a reduction of the Aβ level. A recent study also showed that a peroxisome proliferator-activated receptor-γ agonist completely restored cerebrovascular function in transgenic APP mice, without a reduction in brain Aβ level. Another report demonstrated that aged Nox2-null APP mice showed preserved cerebrovascular function and reduced oxidative stress in cerebral vessels and, furthermore, preserved cognitive function without improvement of amyloid neuropathology.

Perspectives

Overall, the present study demonstrated that low doses of olmesartan (~1.0 mg/kg per day) significantly ameliorated Aβ-mediated cerebrovascular and cognitive dysfunction. It is noteworthy that the dose for cognitive improvement (0.5 to 1.0 mg/kg per day) in this study was approximately within the equivalent range of the recommended dosage of olmesartan (20 to 40 mg/d) for the treatment of hypertension in humans. This animal study demonstrated the potential therapeutic strategy using ARB, although further studies are necessary to investigate the clinical effects of olmesartan in AD patients.

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Disclosures

None.

References


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Short title: ARB prevents Aβ-Induced Cognitive Impairment

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Supplementary methods

Animals
Male ddY mice (7–8 weeks old) were obtained from CLEA (Japan). A transgenic APP23 mouse model that over-expressed human-type APP containing the Swedish double mutation (K670N/M671L) driven by the neuron-specific Thy-1 promoter was bred as previously described 1. Total hAPP expression is seven times the endogenous mouse APP level, although no Aβ deposition is observed in the brain until 6 months of age. Male APP23 mice and age-, sex-, and strain-matched wild-type mice were used for the study. Animals were maintained at room temperature (25 ± 2 °C) under a standard 12 / 12-h light-dark cycle with lights on at 7:00 A.M. Food and water were available ad libitum.

Assessment of Blood Pressure
Systolic blood pressure was measured in conscious mice by the tail-cuff method (BP-98A; Softron Co., Tokyo, Japan). Blood pressure for each mouse was calculated as the mean of at least nine individual measurements. Measurements were performed before drug treatment (day -30), just before and after Aβ administration (day -1 and 2), and at the end of the experiment (day 14).

Drug Treatment
In Experiment 1 (Figure 1), APP23 and wild-type mice were treated with olmesartan (donated by Daiichi-Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan) at a dose of 1.0 mg/kg/day or vehicle. Drugs were administered in drinking water, and solutions were freshly prepared twice a week. In Experiment 2 (Figure 3), ddY mice were treated with olmesartan at a dose of 0.5 mg or 1.0 mg/kg/day or vehicle once daily via oral gavage. Solution was administered at approximately the same time each day. In Experiments 3 and 4 (Figure 4-6), ddY mice were randomly assigned to the following antihypertensive drug treatment groups: olmesartan 1.0 mg/kg/day, hydralazine (Sigma-Aldrich) 30 mg/kg/day, nifedipine 10 mg/kg/day (WAKO, Japan), and vehicle treatment control group. Drugs were administered in drinking water, and solutions were freshly prepared twice a week. The doses of hydralazine and nifedipine were determined to reduce the systolic arterial blood pressure to the same degree as did olmesartan 1.0 mg/kg/day. Selection of drug dosages was based upon previously published studies 2, 3 and our preliminary study.

Preparation of Aβ Peptide
Aβ1-40 and Aβ40-1 were obtained from Peptide Institute (Osaka, Japan), and Aβ solution was prepared for each experiment as described previously 4. Briefly, 0.55 mg Aβ1-40 peptide was dissolved in 3250 µl PBS with 35 % acetonitrile and 0.1 % trifluoroacetic acid. To remove the remaining undissolved Aβ1-40, centrifugation was performed at 15,000 x g for 3 min before Aβ1-40 solution was aliquoted. The control peptide Aβ40-1 was also prepared in the same way as was Aβ1-40.

Intracerebroventricular Administration
Intracerebroventricular (i.c.v.) administration of Aβ was carried out in accordance with the procedure described previously 5, 6 with minor modifications. Briefly, mice were anesthetized with isoflurane gas, and Aβ1-40 or Aβ40-1 (200 pmol / 5 µl) solution was
injected slowly (1.0 µl/min) into the lateral ventricles bilaterally (0.5 mm posterior to bregma, 1.5 mm lateral to midline, 2.5 mm ventral to the brain surface) using an injection needle (27-gauge) connected by polyethylene tubing to a 50-µl Hamilton micro-syringe (Hamilton, Reno, NV). The needle was left in place for another 45 sec before it was slowly withdrawn. Mice with excess bleeding were excluded from the test (less than 5 % of all animals).

**Morris water maze**

1. **Water maze apparatus**

   The Morris water maze test was conducted in a circular pool filled with water at a temperature of 25.0 ± 1 °C as described previously with minor modifications. In the hidden platform test, a transparent platform was submerged 1 cm below the water level. The water tank was located in a test room in which there were many cues external to the maze. The position of the cues remained unchanged throughout the water-maze task. Swimming paths were tracked with a camera fixed on the ceiling of the room and stored in a computer.

2. **Procedure**

   A pre-training session was carried out on day 7 (6 days after 2nd Aβ administration, Figure S3a), in which animals were given 60-sec free swimming without the platform. In the hidden-platform test (from day 8 to day 10), the mice were given 2 trials (1 session) on the first day and 4 trials (2 sessions) per day on the 2nd and 3rd day. The inter-trial interval was about 30 min and the inter-session interval was 2 hours. During each trial, the mice were released from four pseudorandomly assigned starting points and allowed to swim for 60 s. After mounting the platform, the animals were allowed to remain there for 15 s, and were then placed in the home cage until the start of the next trial. If a mouse was unable to find the platform within 60 s, it was guided to the platform and allowed to rest on the platform for 15 s. Probe trials were performed on day 11. In the probe trial, the hidden platform was removed and the animal was released from the right quadrant and allowed to swim freely for 60 sec. The time spent in the target quadrant, where the platform had been located during training, and the time spent in the other quadrants were measured. In the visible-platform test (day 12), the platform was elevated above the water surface and placed in a different position. The mice were given four trials with an inter-trial interval of 10 min. All of the experiments were conducted at approximately the same time each day. Obvious floating rather than swimming mice (less than 5 % of the mice tested) were disqualified from statistical analysis.

**Open-field test**

A transparent cubic box without a ceiling (30 × 30 × 30 cm) was housed in a ventilated sound-attenuating chamber. An overhead incandescent bulb provided room lighting that measured approximately 110 lx inside the chamber. In addition, a fan attached in the upper part of the wall at one end of the chamber presented a masking noise of 45 dB. On each X and Y bank of the open-field area, two infrared-ray beams were attached 2 cm above the floor at a 10 cm interval, generating a flip-flop circuit between the two beams. The total number of successive interceptions of the two adjoining beams on each bank was counted as the locomotion behavior. On the X bank, another 12 infrared-ray
beams were attached 4.5 cm above the floor at 2.5 cm intervals. The total number of vertical beam interceptions was counted as the rearing behavior. Each mouse was allowed to explore freely in the open-field area for 10 min each day. Testing was carried out for three consecutive days.

**Electrophysiological Tests**

Transverse hippocampal slices (400-µm thickness) were prepared from mice, and placed in an interface-type holding chamber for at least 1 hour. A single slice was then transferred to the recording chamber and submerged beneath a continuously perfusing medium saturated with 95 % O₂ and 5 % CO₂. The composition of the medium was (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1.0 NaH₂PO₄, 26.2 NaHCO₃ and 11 glucose. Field EPSPs were recorded with a glass electrode (filled with 3 M NaCl) placed in the stratum radiatum. To evoke synaptic responses, a bipolar tungsten stimulating electrode was placed in the stratum radiatum, and Schaffer collateral/commissural fibers were stimulated at 0.1 Hz. An Axopatch 1D amplifier (Axon Instruments, CA, USA) was used, and the signal was filtered at 1 kHz, digitized at 10 kHz and stored on a personal computer. The stimulus strength was adjusted so that it gave rise to AMPA receptor-mediated EPSPs with a slope between 0.10 and 0.15 mV/ms. For analysis of EPSPs, their early rising phase was measured, to avoid contamination of voltage-dependent components as much as possible. All experiments were performed at 25 °C. The majority of electrophysiological experiments were performed in a blind manner, and the results were essentially identical to those of the non-blind experiments, and thus all the data were pooled.

**Measurement of Aβ**

First, the cerebral hemispheres were homogenized in 5 volumes (w/v) of 1 % Triton X-100 in Tris-buffered saline (TBS) (25 mM Tris and 137 mM NaCl, pH 7.6) containing protease inhibitors (protease inhibitor cocktail, 1 tablet in 50 ml solution; Roche), with a Teflon-glass homogenizer. The homogenate was centrifuged at 100,000 g for 60 min at 4 °C, and the supernatant was saved as the Triton X-100-soluble fraction. The resulting pellet was solubilized by sonication in 5 M guanidine HCl in 50 mM Tris (pH 8.0) with the protease inhibitor mixture, incubated for 2 h at 25 °C, and spun at 13,000 g for 20 min at 4 °C. The supernatant was diluted 10 fold to reduce the concentration of guanidine HCl and used as the guanidine HCl-soluble fraction.

**Isolation of Brain Microvessels**

Mouse brain microvessels were isolated and characterized as described previously with some modification. The hemispheres of the mice used for CBF studies were removed after saline perfusion through the heart. Then, they were rinsed in cold isotonic sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4) and homogenized in 1.5 ml sucrose buffer with a Teflon-glass homogenizer, followed by centrifugation at 4500 g for 10 min at 4 °C. The precipitates were resuspended in sucrose buffer and centrifuged at 4500 g for 10 min at 4 °C. The final pellets were resuspended in 2 ml sucrose buffer, and the solution was passed through a 40-µm nylon mesh. Brain microvessels retained on this mesh were washed, eluted from the mesh, and collected by centrifugation. The purification of the microvessels was assessed by microscopic observation (dried smear stained with toluidine blue and immunolabeling for
endothelial and smooth muscle cell markers as described below). This protocol allows isolation of a range of vessels ranging from capillaries (positive for only endothelial marker without smooth muscle cell component, < 10 µm diameter) to arterioles/venules (positive for both endothelial and smooth muscle cell markers, > 10 µm diameter).

**Immunocytochemical staining**

Immunocytochemical characterization of brain microvessels was performed as described earlier\(^9,10\). Brain microvessels were isolated and fixed in acetone (100 %) for 5 min at 4 °C. Samples were blocked with 0.5 % goat serum in PBS for 10 min at room temperature and then incubated with primary antibody in PBS at 4 °C overnight. A rat anti-CD31 (PECAM-1) monoclonal antibody (1:50, BD Pharmigen) and a mouse anti-α-smooth muscle actin (αSMA) monoclonal antibody conjugated to Cy3 (1:100, Sigma-Aldrich) were used to visualize endothelial cells and vascular smooth muscle cells, respectively. After two 5-min washes in PBS, they were incubated with Alexa Fluor 488 goat anti-rat fluorescent secondary antibody (1:200; Molecular Probes) for 1 h at room temperature. Slides were washed three times in PBS, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images of brain microvessels were captured with a fluorescence microscope (BZ-9000, KEYENCE, Japan). The specificity of the labeling was established by omitting the primary antibody. Brain amyloid plaques were immunodetected with an antibody against Aβ. The contralateral hemisphere of APP23 mice used for CBF analysis was post-fixed in 4 % PFA. Then, 15-µm sections of paraffin-embedded brain tissue were probed with anti-Aβ (6E10) antibodies, detected with diaminobenzidine (DAB) and counterstained with hematoxylin.

**Measurement of Reactive Oxygen Species in Brain Microvessels**

Reactive oxygen species was measured by dihydroethidium (DHE) microfluorography, as described previously\(^9,11\). Isolated mouse brain microvessels were immediately incubated with DHE (10 mol/L; Molecular Probes Inc.) in PBS for 30 min at 37 °C in a light-protected humidified chamber. DHE is cell permeable and is oxidized on reaction with superoxide to ethidium, which binds to double-stranded DNA in the nucleus and fluoresces red. To identify DHE-stained cells, samples were processed for immunocytochemical staining for the endothelial cell marker CD31 (1:50, BD Pharmigen). They were washed and incubated with Alexa Fluor 488 goat anti-rat fluorescent secondary antibody (1:200; Molecular Probes). Nuclei were counterstained with DAPI. Fluorescent images of brain microvessels were captured with a fluorescence microscope (BZ-9000, KEYENCE, Japan) equipped with a digital camera, using the same fluorescence settings in all cases. The pixel intensities of the fluorescent signal were analyzed and quantified using National Institutes of Health Image software. Briefly, imaged blood vessels were classified into capillaries and arterioles according to diameter (capillary < 10 µm; arteriole > 10 µm). Pixel intensities of the DHE fluorescent signal were quantified and adjusted for the number of nuclei (identified according to their DAPI signal) included in the region of interest. They were expressed as relative fluorescence units (RFU). Usually, 20–30 vessel fragments were analyzed for each animal. The specificity of the DHE fluorescent signal was tested by replacing the DHE solution with PBS.
Cerebral Blood Flow Analysis

1. Surgical preparation
Mice were anesthetized with isoflurane (2 % induction, 1 % maintenance). One of the femoral arteries was cannulated for recording mean arterial blood pressure (MABP) (UB-103; Unique Medical, Tokyo, Japan) and collection of blood samples. After surgery, anesthesia was switched to ketamine (50 mg/kg intraperitoneally, repeated every 30 to 45 minutes), and mice were placed on a stereotaxic device. The skull was exposed, and small holes (2 × 2 mm) were drilled at the site where recording probes were to be positioned (somatosensory cortex), under saline cooling. The dura was removed with care, and the site was superfused with artificial CSF (the same perfusion medium used for electrophysiological studies). Core body temperature was monitored and maintained at 37.0 °C using a heating pad with a thermprobe (ATC-101B; Unique Medical, Tokyo, Japan) placed in the rectum of the mouse. The adequacy of anesthesia was regularly checked by confirming the absence of an arterial blood pressure response and a motor response to pinching the tail. To minimize confounding effects of anesthesia on cerebrovascular reactivity, the time interval between starting anesthesia and the measurement of CBF was kept consistent among the groups.

2. Measurement of cerebral blood flow
Arterial blood gases and pH were measured just before CBF measurement (ABL505, RADIOMETER; COPENHAGEN), and confirmed to be in the physiological range (Figure S1a, Table S1). CBF was analyzed with a laser-Doppler probe (0.8 mm, model TBF-LN1; Unique Medical, Tokyo, Japan) 12. CBF analysis was started after confirming a stable flow wave for at least 10 min. CBF values were expressed as the percent increase relative to baseline.

3. Experimental protocol for CBF analysis
In experiment 1, cerebrovascular reactivity was tested in AD model transgenic mice (APP23) with or without olmesartan treatment. After four weeks of pretreatment, functional hyperemia and cerebrovascular autoregulation were assessed under normal artificial CSF superfusion. For assessment of functional hyperemia, changes in CBF before, during, and after whisker stimulation (30 sec at 5 Hz) were recorded, with three to five recordings acquired every 60 to 90 sec and averaged for each animal. For stimulation of the somatosensory cortex, a small bipolar electrode was attached to the contralateral whisker area, and rectangular pulses of 0.3-ms duration were applied. No motor movement or change in cardiac activity (confirmed by stable arterial blood pressure and regular heart beat) was observed during stimulation. Cerebrovascular autoregulation 13, 14 was investigated by elevating MABP by intravenous infusion of phenylephrine (15 to 40 µg/kg) or inducing hypotension by controlled, stepwise withdrawal of 100 µL arterial blood. The range of MABP studied was 30–150 mmHg. CBF values were recorded 2 min after MABP was changed. Transgene-negative littermates served as controls. In this test, to minimize confounding effects of a possible difference in neuronal activation on cerebrovascular reactivity, somatosensory evoked potentials (SEPs) were recorded 15. SEPs were recorded with a small electrode (Unique Medical) placed stereotaxically over the somatosensory cortex at the same position as CBF monitoring. SEPs were averaged using data acquisition software (Unique Acquisition; Unique Medical). For the measurement of peak-to-peak amplitude,
averages of 100 consecutive SEPs were taken. The current of whisker-stimulation pulses was adjusted to evoke half the maximum amplitude of SEP. In experiment 4, the effect of olmesartan on Aβ-induced impairment of functional hyperemia and cerebrovascular autoregulation was evaluated by topical application of Aβ peptide to the somatosensory cortex of pre-treated ddY mice. After testing CBF responses during artificial CSF superfusion, 10 µM Aβ1-40 or Aβ40-1 was topically applied to the somatosensory cortex. Aβ peptide was solubilized in artificial CSF and freshly prepared for each experiment. The concentration of Aβ1-40 was selected based upon previously published studies 16, 17, in which it was found to produce a significant cerebrovascular effect. We also confirmed this effect in our experimental setting. Functional hyperemia and cerebrovascular autoregulation were tested 20–30 min after Aβ application.

**Experimental Design**

**Experiment 1**
The effect of olmesartan on cerebrovascular reactivity was tested using APP23 transgenic mice. Animals were pretreated with olmesartan (1.0 mg/kg/day) or vehicle for 4 weeks starting at 8 weeks of age. At the end of the experiment, the brain was removed and processed for Aβ quantitation and ROS assessment of isolated microvessels.

**Experiment 2**
To examine the effect of oral administration of ARB on cognitive function in Aβ1-40 injection model mice, treatment with the ARB olmesartan (0.5, 1.0 mg/kg/day) was started 4 weeks before (day -28) Aβ administration. Control mice received no medication. On day 0 and day 1, Aβ1-40 or 40-1 (control peptide) was injected into the brain. Behavioral analysis (Morris water maze test) was carried out on day 7 to 12.

**Experiment 3**
The aim of Experiment 3 was to compare the effect of an ARB and other antihypertensive medication with a non-RAS-dependent blood pressure-lowering effect on Aβ-induced cognitive dysfunction. Treatment with olmesartan (1.0 mg/kg/day), hydralazine (30 mg/kg/day), nifedipine (10 mg/kg/day), or no medication was started 4 weeks before (day -28) Aβ injection. Aβ injection and behavioral analysis were carried out with the same schedule as that in Experiment 2. A set of animals was subjected to open field test on day 4 to 6.

**Experiment 4**
To examine the mechanism underlying the effect of olmesartan on cognitive function, electrophysiological assessment of hippocampal function was conducted and cerebrovascular reactivity was examined in mice with or without olmesartan (1.0 mg/kg/day; 4 weeks) treatment.

**Supplementary references**
2. Tsukuda K, Mogi M, Li JM, Iwanami J, Min LJ, Sakata A, Fujita T, Iwai M,


**a**

### Resting Physiological Parameters

<table>
<thead>
<tr>
<th></th>
<th>Wild type + vehicle</th>
<th>APP23 + vehicle</th>
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<td>11.2±0.2</td>
<td>P = 0.98</td>
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</tbody>
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**b**

- **Wild**
- **APP23**

**c**

- Resting CBF

**d**

- Sensory evoked potential

**e**

- Stimulation

**f**

- Stimulation for MAX response

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**Figure S1**

**Resting physiological parameters in APP23 mice and amplitude of whisker stimulation.** (a) Resting physiological parameters. Arterial blood was analyzed just before CBF measurement. (b) Body weight of animals at the age of 12 weeks (after 4 weeks of treatment). (c) Resting CBF at start of the experiment. (d) SEP evoked by whisker stimulation. (e) Level of whisker stimulation required to evoke half of the maximum SEP response. (f) Level of whisker stimulation required to produce maximum SEP response. n = 4–5 / group.
Figure S2
Effects of olmesartan on brain Aβ burden. Brain levels of Aβ40 and Aβ42 in APP23 mice with or without olmesartan treatment were examined. Both Triton-X 100 soluble (a) and insoluble (guanidine extract) (b) Aβ were measured. Treatment did not affect brain Aβ load in APP23 mice (P > 0.05; n = 4–5 / group). (c) Aβ immunostaining (6E10) of the brain of APP23 mice. There was no detectable amyloid plaque in this early age (12 weeks) in mice with (right) and without (left) olmesartan treatment. Scale bar, 300 μm.
Figure S3
(a) Experimental schedule. BP, blood pressure measurement. (b) Blood pressure in Aβ-injected mice treated with various doses of olmesartan. **P < 0.01 versus vehicle treatment group (Aβ40-1 + vehicle and Aβ1-40 + vehicle). (c) Morris water maze test. Escape latencies in visible-platform test. n = 5–7 / group.
Dose-dependency of the effects of olmesartan on blood pressure and cognitive function. ddY mice were treated with olmesartan at a dose of 0.5 mg, 1.0 mg, 3.0 mg, 6.0 mg/kg/day or vehicle once daily via oral gavage. Experimental design was the same as Experiment 2 (see Supplementary methods). (a) Systolic blood pressure after four weeks treatment (just before Aβ administration). (b) Cognitive function of treated mice was evaluated with Morris water maze test. Performance in probe test (%time in target quadrant) is shown. Olmesartan at doses of 0.5 and 1.0 mg/kg/day significantly attenuated the memory impairment caused by Aβ1-40, however, this favorable effect was not observed at higher doses (3.0 and 6.0 mg/kg/day). *P < 0.05, n = 5-7 / group.
Figure S5
(a) Blood pressure in A ؚ -injected mice treated with olmesartan (1.0 mg/kg/day), hydralazine (30 mg/kg/day), nifedipine (10 mg/kg/day), and vehicle. **P < 0.01 versus vehicle treatment group (A ؚ 40-1 + vehicle and A ؚ 1-40 + vehicle). (b–e) Morris water maze test. Escape latencies in visible platform test (b), mean number of annuls crossings (c), swim speed (d), representative swim paths (e) during the probe test are shown. **P < 0.01. n = 9–15 / group.
Figure S6

**Locomotion and rearing in open field test.** Mean locomotion scores (a) and rearing scores (c) on each experimental day (Day 1–3) are shown. One-way ANOVA and subsequent Tukey-Kramer multiple range test indicated that there was no significant group effect on the locomotion score (Day 1: $F(2, 33) = 0.35, P = 0.71$; Day 2: $F(2, 33) = 0.34, P = 0.72$; Day 3: $F(2, 33) = 0.79, P = 0.46$) and the rearing score (Day 1: $F(2, 33) = 0.78, P = 0.47$; Day 2: $F(2, 33) = 0.33, P = 0.72$; Day 3: $F(2, 33) = 0.23, P = 0.80$). NS, not significant. The change in locomotion score during the experimental days is shown in (b), in which values were expressed as the percentage relative to baseline (locomotion score on Day 1). The locomotion score in control mice (Aβ40-1 injection + vehicle treatment) significantly decreased on test days (**$P < 0.01$ compared with Day 1; paired $t$ test), whereas that in the Aβ1-40 injection + vehicle-treated group did not change. The lack of habituation (no reduction of locomotion score) represents a higher level of anxiety or aggressiveness of the animal. These kinds of behavior are often observed in AD patients. A significant reduction in locomotion score was also observed in olmesartan (1.0 mg/kg/day)-treated mice on Day 3 (*$P < 0.05$ versus Day 1; paired $t$ test), suggesting that treatment with olmesartan decreased anxiety or stabilized the mood of Aβ1-40-injected mice. The same tendency was observed in the change in rearing score (d) on test days (*$P < 0.05$, **$P < 0.01$ versus Day 1; paired $t$ test).
### Resting Physiological Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Aβ 40-1 +vehicle</th>
<th>Aβ 1-40 +vehicle</th>
<th>Aβ 1-40 +Olme 1.0 mg</th>
<th>P value</th>
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<td>Arterial blood pH</td>
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<td>P = 0.74</td>
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</tbody>
</table>

**Table S1**

Resting physiological parameters. Aβ was applied topically onto the somatosensory cortex. Arterial blood was analyzed just before CBF measurement.