Angiotensin II Peptide Vaccine Protects Ischemic Brain Through Reducing Oxidative Stress

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Background and Purpose—Medication nonadherence is one of major risk factors for the poor outcome in ischemic stroke. Vaccination is expected to solve such a problem because of its long-lasting effects, but its effect on ischemic brain damage is still unknown. Here, we focused on vaccination for renin–angiotensin system and examined the effects of angiotensin II (Ang II) peptide vaccine in permanent middle cerebral artery occlusion model in rats.

Methods—Male Wistar rats were exposed to permanent middle cerebral artery occlusion after 3× injections of Ang II peptide vaccine, and the serum or brain level of anti–Ang II antibody was examined. The effects of the vaccine were evaluated by differences in infarction volume, brain renin–angiotensin system components, and markers for neurodegeneration and oxidative stress.

Results—Ang II vaccination successfully produced anti–Ang II antibodies in serum without concomitant change in blood pressure. Sufficient production of serum anti–Ang II antibody led to reduction of infarct volume and induced the penetration of anti–Ang II antibody in ischemic hemisphere, with suppressed expression of Ang II type 1 receptor mRNA. Vaccinated rats with sufficient antibody production showed the reduction of Fluoro-Jade B–positive cells, spectrin fragmentation, 4-hydroxynonenal-positive cells, and Nox 2 mRNA expression.

Conclusions—Our findings indicate that Ang II vaccination exerts neuroprotective and antioxidative effects in cerebral ischemia, with renin–angiotensin system blockade by penetration of anti–Ang II antibodies into ischemic brain lesion. Ang II peptide vaccination could be a promising approach to treat ischemic stroke. (Stroke. 2017;48:1362-1368. DOI: 10.1161/STROKEAHA.116.016269.)

Key Words: angiotensin II ▪ blood pressure ▪ oxidative stress ▪ stroke ▪ vaccine

Inhibition of renin–angiotensin system (RAS) and control of blood pressure by angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin II (Ang II) receptor blockers (ARBs) are important for in the primary and secondary prevention of ischemic stroke. The protective effect of ACE-Is and ARBs has been attributed to their inhibitory effect on the expression of proinflammatory cytokines and oxidative stress in postischemic brain or their direct neuroprotective effects. However, recent studies have revealed inconsistent drug intake in >60% patients 1 year after hospitalization. Nonadherence to secondary prevention medication is a major risk factor for the recurrence of ischemic stroke. In addition, economic burden is a concern pertaining to long-term treatment. To alleviate the compliance concerns and improve preventive outcomes, we assessed the effects of the recently developed Ang II peptide vaccine in preventing ischemic stroke.

Although vaccines have traditionally been used to prevent infectious diseases, their therapeutic use has been recently expanded against the adult common diseases, such as hypertension, Alzheimer’s disease, and so on, by targeting self-antigens. Immunization is a cost-effective intervention compared with conventional therapy owing to its long-last- ing effects and does not require daily intake of medication. However, there are only few reports regarding the development of a vaccine to treat ischemic stroke.

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A vaccine targeting NR1 subunit of N-methyl-D-aspartate receptor in rats with ischemic stroke has been reported earlier; however, clinical trials for the same have not been conducted. The association of auto-antibodies to N-methyl-D-aspartate receptor NR1 subunit with neuropsychiatric systemic lupus erythematosus and anti-N-methyl-D-aspartate receptor encephalitis is noteworthy. Selection of appropriate target molecules is a key challenge for therapeutic vaccination in patients with ischemic stroke owing to safety concerns.

Therefore, here we focused on developing a vaccine against RAS because the inhibition of RAS by ACE-Is and ARBs has been widely accepted as a safe and effective treatment for the primary and secondary prevention of ischemic stroke. Because previous reports demonstrated that Ang II peptide vaccine or Ang II DNA vaccine was free from anti-Ang II autoimmune response and exerted a long-lasting antihypertensive effect in animal experiments and human clinical trials, Ang II peptide vaccine is a promising therapeutic modality for the treatment of hypertension. However, it is still unclear whether the protective effect of Ang II peptide vaccine against ischemic damages is over and above its antihypertensive effects. To clarify this important question, we examined its efficacy in preventing ischemic damage using a permanent focal ischemia model of normotenive rats.

Methods

Animals
Male Sprague–Dawley rats (3 weeks old) obtained from CLEA Japan, Inc (Tokyo, Japan) were housed under a 12-hour light/12-hour dark cycle with free access to food and water under temperature- and humidity-controlled conditions. Female rats were not used to avoid any influences of sex steroids. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Tokyo.

Peptide Syntheses
To induce sufficient immune response, keyhole limpet hemocyanin (KLH; Wako Pure Chemical Industries, Osaka, Japan) carrier protein was conjugated to the N-terminus of Ang II using glutaraldehyde (Peptide Institute Inc, Osaka, Japan) as previously described.

Ang II Peptide Vaccine Immunization
Immunization was performed at the age of 4, 6, and 7 weeks. Detailed procedure is described in the online-only Data Supplement.

Study Design
Detailed information is described in the online-only Data Supplement.

Surgical Procedure
Permanent middle cerebral artery occlusion (pMCAo; n=92) or sham (n=16) surgery was performed at 1 week or 15 days after the third vaccination or saline injection. Detailed information is described in the online-only Data Supplement.

Measurement of BP and Sample Collection
Systolic BP was recorded using the tail-cuff system (BP-98A; SOFTRON Co., Tokyo, Japan) at 0, 14, 21, and 28 days after the first vaccination and at 24 hours after pMCAo. Detailed procedures are described in the online-only Data Supplement.

Histological Analysis
To identify the infarct area, cresyl violet staining was performed and infarction volume was calculated. Neurodegeneration and oxidative stress in the brain was evaluated using Fluoro-Jade B staining and immunohistochemistry for Ang II type 1 receptor (AT1R) and 4-hydroxynonenal (HNE). NeuroTrace 530/615 Red Fluorescent Nissl Stain was used to label neurons. Detailed procedure is described in the online-only Data Supplement.

Western Blot
Protein expression of spectrin alpha chain or 4-HNE was evaluated using Western blot. Detailed procedure is described in the online-only Data Supplement.

Quantification of Serum Anti–Ang II Antibody Titer by Enzyme-Linked Immunosorbent Assay
Serum anti–Ang II antibody titer was quantified using enzyme-linked immunosorbent assay. The anti-Ang II specific antibody titer was defined as the serum dilution that exhibited half-maximal optical density (OD) at 450 nm (OD50%). Detailed procedure is described in the online-only Data Supplement.

Anti–Ang II Antibody Penetration Into Brain Parenchyma
Anti–Ang II antibody penetration into brain parenchyma was measured by enzyme-linked immunosorbent assay using the brain homogenate. Detailed procedure is described in the online-only Data Supplement.

Quantification of Ang II Protein Expression in Plasma and Brain
Detailed procedure for sample processing is described in the online-only Data Supplement. Ang II concentration in brain homogenate or plasma samples was measured using standard radioimmunoassay by an external laboratory (SRL, Tokyo, Japan).

Real-Time Polymerase Chain Reaction
The mRNA levels of AT1R, angiotensinogen, NADPH oxidase 2, and 18s ribosomal RNA (endogenous control) were measured by real-time polymerase chain reaction. For more detail, please see online-only Data Supplement.

Statistical Analysis
Data are expressed as mean±standard deviation. Data were analyzed using Statview for windows version 5 (SAS Institute Inc, Tokyo, Japan). Detailed information is described in the online-only Data Supplement.

Results

Anti–Ang II Antibody in Immunized Rats
Normal rats were immunized using 3 doses of Ang II peptide; the antibody titers against Ang II and the concentration of Ang II in plasma and brain homogenates were measured at 28 days after first immunization (Figure 1A). Consistent with a previous report stating that BP was not altered in normotenive Ang II peptide vaccinated mice, no differences in BP were observed between the groups (Figure I in the online-only Data Supplement). Although anti–Ang II antibody was not observed in saline-injected rats (controls), serum titer of anti–Ang II antibody was significantly increased in the vaccinated rats (Figure 1B). The concentration of Ang II in plasma was
significantly increased in the vaccinated rats (Figure IIA in the online-only Data Supplement). A significant positive correlation between serum anti–Ang II antibody titer and plasma Ang II level was observed in the vaccinated rats (Figure IIB in the online-only Data Supplement; \( r = 0.669, P = 0.0153 \)). To investigate whether vaccination affected the brain Ang II concentration, the concentration of Ang II in the brain was examined. However, no significant differences were observed between the groups (Figure 1C). To examine whether anti–Ang II antibodies penetrate from systemic circulation to nonischemic brain tissue, anti–Ang II antibody level was quantified by enzyme-linked immunosorbent assay using brain homogenate. There was no significant change in anti–Ang II antibody levels in the intact brain tissue between vaccinated and control rats (Figure 1D).

**Effect of Ang II Peptide Vaccine in the pMCAo Rats**

Next, we examined whether Ang II peptide vaccine affected ischemic brain damage. Ang II–vaccinated rats and control rats were subjected to pMCAo at 1 week after the third vaccination (Figure 2A). Rats with high titer of serum anti–Ang II antibody showed smaller infarct volume (Figure 2B; \( r = -0.759, P = 0.006 \)) at 24 hours of pMCAo. Based on the result, we divided the rats into 2 groups: low titer group (OD50% < 6000, \( V_L \) group) and high titer group (OD50% ≥ 6000, \( V_H \) group). Representative images of the cresyl violet–stained sections from each group showed a reduced infarct volume in \( V_H \) rats (Figure III in the online-only Data Supplement). On quantitative analysis, \( V_H \) rats showed a significant reduction in the infarct volume compared with the \( V_L \) and control rats (Figure 2C). These results indicate that high titers of serum anti–Ang II antibody may be necessary to demonstrate the protective effects against ischemic brain. Next, we examined whether the protective effects of Ang II vaccine could last 15 days after the vaccination. As expected, infarct volume was less in \( V_H \) rats when the rats were exposed to pMCAo at 15 days after third vaccination (Figure IV in the online-only Data Supplement). Because no differences were observed in BP between \( V_H \) and control rats (Figure V in the online-only Data Supplement), the protective effects of Ang II vaccine were not because of its actions on BP.

**Penetration of Anti–Ang II Antibody Into Brain Parenchyma**

To clarify the mechanism of preventive effects of Ang II peptide vaccine on cerebral infarction, we first measured brain anti–Ang II antibody level at 24 hours after pMCAo in the brain homogenate samples. Anti–Ang II antibody in the brain tissue of \( V_H \) rats was significantly increased by 56-fold compared with that in the brain tissue of control rats, whereas brain anti–Ang II antibody was also significantly increased in \( V_H \) rats compared with that in the \( V_L \) rats (Figure 3A). These findings indicate that anti–Ang II antibody penetrated into ischemic lesion. There was a strong positive correlation between serum anti–Ang II antibody titer and brain parenchymal anti–Ang II antibody change (Figure VI in the online-only Data Supplement; \( r = 0.747, P = 0.0003 \)). Temporal profile of brain anti–Ang II antibody showed the penetration of anti–Ang II antibody into ischemic lesion as early as 12 hours after cerebral ischemia (Figure 3B).
Subsequently, we examined the effects of Ang II peptide vaccine on the brain RAS components. Because $V_H$ rats showed preventive effects in terms of the infarct volume and penetration of anti–Ang II antibody into the parenchyma, we compared the expression of RAS components between $V_H$ and control rats at each time point. In sham-operated rats, the expression of AT1R or angiotensinogen mRNA was not altered by Ang II peptide vaccination (Figure 4A and 4B). In comparison with sham-operated rats, AT1R mRNA was significantly increased at 24 hours after pMCAo in ischemic hemisphere of nonvaccinated rats, whereas the increased expression was significantly inhibited in $V_H$ rats (Figure 4A). Although angiotensinogen mRNA was decreased in the ischemic hemisphere at 24 hours after pMCAo in nonvaccinated control rats, its expression was attenuated in $V_H$ rats (Figure 4B).

Figure 2. Effect of angiotensin II (Ang II) peptide vaccine on cerebral infarct volume. A, Cerebral infarct volume was evaluated at 24 hours after permanent middle cerebral artery occlusion (pMCAo) surgery. B, A significant negative correlation was observed between the infarct volume and the serum anti–Ang II antibody titer ($r=−0.759, P=0.008$). C, Infarct volume was reduced in $V_H$ rats whose serum anti–Ang II antibody titer was OD50% $>6000$ compared with that in $V_L$ rats whose serum anti–Ang II antibody titer was OD50% $<6000$ and saline-treated (S) rats. *$P<0.05$ vs S, **$P<0.05$ vs $V_H$. Each group included n=5 (sham), n=5 ($V_L$), n=6 ($V_H$), or n=8 (S) in C.

Figure 3. Anti-angiotensin (Ang II) antibody penetration into ischemic lesion in vaccinated rats. A, Rats with high titers of serum anti–Ang II antibody ($V_H$, OD50% $>6000$) showed a significant increase in anti–Ang II antibody in ischemic brain tissue as compared with that in rats with low titers of serum anti–Ang II antibody ($V_L$, OD50% $<6000$) and saline-treated (S) rats. B, Anti-Ang II antibody in ischemic brain had increased as early as 12 hours after pMCAo in $V_H$ rats as compared with that in sham-operated $V_L$ rats. ***$P<0.01$ vs $V_L$, ###$P<0.01$ vs S, 1P<$P<0.05$ vs $V_H$ (sham). Each group included n=7 (S), n=7 ($V_L$), n=10 ($V_H$) in A and n=5 ($V_H$ [sham]), n=7 ($V_H$ [6 h]), n=6 ($V_H$ [12 h]) in B.

Figure 4. Effect of angiotensin (Ang II) peptide vaccination on gene expression of brain renin–angiotensin system in ischemic lesion. Ang II type 1 receptor (AT1R) and angiotensinogen mRNA expressions were not affected by vaccination in sham-operated rats. A, Reduced AT1R mRNA expression was observed in ischemic hemisphere of serum anti–Ang II high titer ($V_H$) rats at 24 hours after pMCAo as compared with that in saline-treated (S) rats. B, S rats had significantly decreased expression of angiotensinogen mRNA in ischemic hemisphere as compared with that in saline-treated sham rats at 24 hours after pMCAo. **$P<0.01$ vs saline-treated sham, ###$P<0.01$ vs S (24 h), 1P<$P<0.05$ vs saline-treated sham. Each group included n=6 except for n=7 (6 h [$V_H$]), n=5 (12 h [S]) in A and n=8 except for n=7 (12 h [$V_H$]), n=5 (12 h [S]) in B.

Effect of Ang II Peptide Vaccine on Neurodegeneration and Oxidative Stress

Finally, we examined the molecular mechanisms of the protective effects of Ang II peptide vaccine against ischemic brain damage. The number of Fluoro-Jade B–positive cells, which are degenerated neurons in the early phase of cerebral ischemia,21 was significantly decreased in $V_H$ rats compared with control rats (Figure 5A). In addition, Western blotting of spectrin breakdown products, reliable markers for neurodegeneration,22 revealed a significant reduction in 150/145 kDa fragments of spectrin (Figure 5B). These results suggest that the reduction in the infarct volume by Ang II peptide vaccine is because of the prevention of neurodegeneration after pMCAo.

Because the pharmacological inhibition of RAS using ACE-Is or ARBs has been reported to exert its neuroprotective effects via its antioxidative property,23 we further examined whether the neuroprotective effect of Ang II peptide vaccine may be related to the antioxidative mechanism in neurons. First, we checked whether neurons expressed AT1R and 4-HNE, oxidative stress marker in the ischemic brain. Immunohistochemistry showed that the AT1R-positive cells were increased mainly in neurons in ischemic core and peri-infarct region (Figure VIIIIB in the online-only Data Supplement). Also, most 4-HNE-positive cells showed increased expression of AT1R in ischemic hemisphere (Figure VIIIC in the online-only Data Supplement) and 4-HNE-positive cells were predominantly composed of neurons (Figure VIIID in the online-only Data Supplement). These results indicate that increased expression of AT1R was associated with increased oxidative stress in neurons in the ischemic hemisphere. In the vaccinated rats with Ang II peptide vaccine, the expression of 4-HNE was markedly decreased (Figure 5C), suggesting that Ang II vaccine suppressed oxidative stress through AT1R signalings in neurons. Western blot assay also showed that 4-HNE formation was significantly increased in the ischemic hemisphere of nonimmunized rats.
whereas its expression was significantly decreased by Ang II vaccination. Additionally, the expression of NADPH oxidase 2 mRNA, which is the most critical NADPH oxidase in the ischemic brain, was significantly decreased in vaccinated rats (Figure VIIB in the online-only Data Supplement). Thus, Ang II vaccine inhibited oxidative stress in the ischemic brain.

**Discussion**

In the present study, immunization with Ang II peptide vaccine significantly prevented the exacerbation of ischemic brain damage by the inhibition of neurodegeneration and antioxidative effects. Although anti–Ang II antibody does not seem to penetrate into the intact brain, our data revealed that anti–Ang II antibody may penetrate the brain once the blood–brain barrier has been broken down as a result of ischemic damage. This observation was confirmed by the significant positive correlation in the amount of anti–Ang II antibody between serum and brain. Circulating high titer of anti–Ang II antibody may cross blood–brain barrier and increase its expression in the ischemic brain tissue and prevent further ischemic damage. Increased expression of plasma Ang II in the vaccinated rat might be explained by diminished suppression of feedback on renin secretion via blockade of systemic RAS as previously reported. Independent of systemic RAS, the brain has been the center of interest as an important site for the production of Ang II from tissue angiotensinogen by renin and ACE. Similar to the previous reports that showed increased AT1R mRNA expression at 24 hours after middle cerebral artery occlusion, this change was observed in nonimmunized rats. Inhibition of the upregulation of AT1R expression by vaccination may prevent the activation of brain RAS. In contrast, angiotensinogen mRNA expression in ischemic brain tissue was shown to increase rapidly at 1 to 2 hours followed by return to baseline level at 6 hours after middle cerebral artery occlusion. In the present study, angiotensinogen mRNA expression was decreased in the ischemic hemisphere in the control group after 24 hours of PmCAo, whereas Ang II vaccination attenuated this decrease in the present study. Decreased expression of angiotensinogen mRNA in nonimmunized rats is probably because of negative regulation by the activation of AT1R signaling pathway induced by Ang II in astrocytes.

Ang II regulates reactive oxygen species production and oxidative stress in ischemic brain. Because the expression of 4-HNE, which was expressed in AT1R-positive neurons, and AT1R mRNA was reduced in the vaccinated rats, suppression of oxidative stress in damaged neurons through inhibition of AT1R signaling might be one of the mechanisms for the amelioration of ischemic injury. Considering that NADPH oxidase 2 is involved in the AT1R-reactive oxygen species axis in neurons and its expression was less in the vaccinated rats, the antioxidative stress effects might be through the inhibition of NADPH oxidase 2. These might be supported by the previous reports showing the neuroprotective and antioxidative stress effects of ARBs in the cultured neurons. Another possible mechanism of protective effects of vaccination is the inhibitory effect on calpain and caspase activities because the fragmentation of spectrins, cleaved forms from full spectrin (240 kDa) by caspase-3 and calpain, was significantly reduced. Ang II increased the intracellular Ca and Ang II may have directly regulated the calpain activity in the

**Figure 5. Effect of angiotensin (Ang) II peptide vaccine on neurodegeneration and oxidative stress in ischemic lesion.**

**A**, Schematic illustration of a coronal brain section. The shaded area represents the ischemic lesion, and the boxed area was the observed ischemic core region. **B**, Twenty-four hours after PmCAo, Fluoro-Jade B (FJB)–positive cells (arrows) were significantly decreased in serum anti–Ang II high titer (VH) rats as compared with that in saline-treated control (S) rats. **C**, More 4-hydroxynonenal (4-HNE)–positive cells (arrows) were observed in lesions of S rats as compared with that in VH rats. Densitometric analysis of Western blot showed significantly decreased 4-HNE expression in VH rats as compared with that in S rats. Bar=20 μm, ##P=0.0067 vs S, **P<0.01 vs sham, #P<0.05 vs S. Each group included n=5 in A and n=8 (sham), n=6 (S), n=6 (VH) in B.
brain, as reported in other types of tissues, such as aorta, kidney, and cardiomyocyte.

In the present study, the cerebroprotective effects of Ang II vaccine lasted 15 days after the vaccination. Although we could not examine whether the effects were preserved >15 days because of the limitation of the stroke model, the preventive effects of Ang II peptide vaccine may have lasted up to several months because we observed the continuing existence of anti-Ang II antibody in serum at least ≤3 months after immunization. In addition, the previous clinical study using Ang II vaccine demonstrated the reversible antibody response against Ang II, with a half-life of ≈4 months after immunization. In clinical settings, this possible long-lasting efficacy would be promising compared with the standard inhibitor of the RAS because poor adherence to secondary preventive medication owing to poststroke dementia or other neurological deficits is a major risk of the recurrence of ischemic stroke. One may assume that excessive lowering of BP by Ang II peptide vaccine may be a risk factor for ischemic stroke. However, it is unlikely because no effects on BP were observed in the present study. This finding is compatible with the previous reports showing no BP lowering effects of Ang II peptide vaccine in normotensive mice, despite the reduction of BP in other reports showing no BP lowering effects of Ang II peptide in the present study. This finding is compatible with the previous studies showing no BP lowering effects of Ang II peptide vaccine in normotensive mice, but they are not necessarily incompatible with the findings of the present study, which demonstrated the reversible antibody response against Ang II, with a half-life of ≈4 months after immunization. In clinical settings, this possible long-lasting efficacy would be promising compared with the standard inhibitor of the RAS because poor adherence to secondary preventive medication owing to poststroke dementia or other neurological deficits is a major risk of the recurrence of ischemic stroke. One may assume that excessive lowering of BP by Ang II peptide vaccine may be a risk factor for ischemic stroke. However, it is unlikely because no effects on BP were observed in the present study. This finding is compatible with the previous reports showing no BP lowering effects of Ang II peptide vaccine in normotensive mice, but they are not necessarily incompatible with the findings of the present study, which demonstrated the reversible antibody response against Ang II, with a half-life of ≈4 months after immunization. In clinical settings, this possible long-lasting efficacy would be promising compared with the standard inhibitor of the RAS because poor adherence to secondary preventive medication owing to poststroke dementia or other neurological deficits is a major risk of the recurrence of ischemic stroke.

Because KLH itself is an immune activator and affects immune response under pathological conditions, KLH might have some influences on the production of anti-Ang II antibodies or the ischemic injury. However, in our previous study, we demonstrated that KLH itself did not produce the antibodies for Ang II. Also, in the present study, low Ang II titer level did not affect cerebral infarct size in the vaccinated rats. This indicated that nonspecific immune reaction by KLH did not affect the ischemic damages. In the clinical application, one of the concerns is the high variability of antibody production. Although further study is necessary, we speculate that higher dose of the vaccine, improvement of the timing of vaccination, and optimization of the carrier protein or adjuvant could increase the antibody titer and reduce the variability. Alternatively, DNA vaccination might be another option because we recently found that DNA vaccine encoding Ang II was more effective than Ang II peptide vaccine in lowering BP in SHR rats.

To summarize, prior immunization by Ang II peptide vaccine significantly ameliorated the neurodegenerative changes in cerebral infarction through the suppression of activated brain RAS and oxidative stress. The present study demonstrated the usefulness of Ang II peptide vaccination for the treatment of ischemic stroke.

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Angiotensin II Peptide Vaccine Protects Ischemic Brain through Reducing Oxidative Stress

Cover title: Angiotensin II Vaccine for Ischemic Brain

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Methods

Study Design

Rats were divided into Ang II peptide vaccinated (n = 77) or saline-treated (n = 55) rats. Saline-treated rats were injected with an equal volume of normal saline at the same time points. The rats were divided into non-surgical (n = 24) and surgical treatment (n = 108) rats. Non-surgical rats [Ang II vaccinated (n = 12), saline-treated (n = 12)] were used to investigate the influence of vaccination in non-ischemic healthy rats, while surgical treated rats [Ang II vaccinated (n = 65), saline-treated (n = 43)] were used to investigate the protective effects of vaccination in cerebral infarction. Power analysis was not performed in this study because the effect size could not be estimated due to lack of previous studies showing the effects of any vaccines in pMCAo models in rats. Exclusion criteria were a missing neurological deficit after cerebral ischemia, massive bleeding or death from surgery-related causes during 24 hours of cerebral ischemia. All procedures and analyses were conducted by examiners blinded to experimental conditions.

Surgical Procedure

Permanent middle cerebral artery occlusion (pMCAo) (n = 92) or sham (n = 16) surgery was performed under anesthesia with chloral hydrate (300 mg/kg, intraperitoneally) at 1 week or 15 days after the third vaccination or saline injection. In pMCAo rats, the right MCA was occluded by placement of poly-L-lysine-coated 4-0 nylon, as previously described\(^1\). In sham-operated rats, the same procedure was performed except for pMCAo. Nine rats (12%) were excluded from this study because of quick recovery in neurological deficit (Bederson score = 0) at 1 hours after pMCAo. Two rats died within 24 hours after pMCAo; one in vaccinated rat (1.1%) and one in saline treated group (1.1%).

Angiotensin II peptide vaccine immunization

Single dose of angiotensin (Ang) II peptide vaccine was prepared as the emulsified mixture of 200 μl peptide solution containing 10 μg Ang II-KLH with equal volume of Freund’s adjuvant. The antigen dose corresponded to the dose of Ang II in Ang II-KLH. Immunization was performed at the age of 4, 6, and 7 weeks. Priming was performed using complete Freund’s adjuvant and the boosts were performed using incomplete Freund’s adjuvant. The Ang II peptide vaccine was injected subcutaneously under anesthesia with chloral hydrate (300 mg/kg, intraperitoneally).

Measurement of systemic blood pressure and sample collection

Systolic blood pressure (BP) was recorded using the tail-cuff system (BP-98A, SOFTRON Co., Tokyo, Japan) at 0, 14, 21, and 28 days after the first vaccination or saline injection to investigate whether Ang II vaccination affected BP in the healthy rats. To examine whether Ang II vaccination affects BP after cerebral ischemia, BP was measured at 24 hours after pMCAo, which was performed at 15 days after the third vaccination.

Sample collection from animal was performed under anesthesia with chloral hydrate (300 mg/kg, intraperitoneally). Blood samples were collected via the femoral vein at 1 week after the third vaccination or saline injection and subjected to centrifugation under 8000 rpm
during 10 minutes to separate plasma and serum from whole blood. Brains were extracted from the skull after perfusion with ice-cold normal saline for complete blood removal and decapitation. In the non-surgical rats, brain samples were collected at 1 week after the last vaccination. Whereas, in the surgical rats, permanent middle cerebral artery occlusion (pMCAo) surgery or sham surgery was performed at 1 week or 15 days after third vaccination, and brain samples were collected at 6, 12, and 24 hours after pMCAo surgery or 24 hours after sham surgery. In Western blotting and ELISA, samples were frozen in liquid nitrogen and stored at −80°C until the assay. Histopathological samples were prepared from the tissues which were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 24 hours after pMCAo.

**Quantification of infarct volume**

After perfusion fixation with 4% PFA, the brain was removed from the skull and post-fixed overnight in 4% PFA at 4°C followed by cryoprotection using 30% sucrose in PBS. Serial coronal sections of 7.0 μm thickness were prepared on a freezing microtome (Leica, Wetzlar, Germany) at 1.0 mm intervals (+3.2 mm to −7.8 mm from the bregma). To identify the infarct area, cresyl violet staining was performed with minor modifications of procedures described elsewhere. The slides were stained for 10 minutes in filtered cresyl violet solution, briefly rinsed in distilled water, followed by dehydration in 70%, 95%, and 100% ethanol. Finally, the slides were cleared in xylene and mounted with a coverslip. Images were digitized with Leica DFC450 C on a Leica DM2500 LED microscope (Leica). All acquired images of serial sections were imported into Adobe Photoshop CS3 (Adobe System, CA, USA), and the infarct area quantified in square millimeters. The infarct volume was calculated from the infarct areas in nine sections as follows: Infarct volume = Σ (Infarct area) (mm³).

**Western blot assay**

Brains were sliced into 1.0 mm coronal sections using a brain slicer and the slices at the level of striatum-caudate putamen (CPu) were separated into two cerebral hemispheres. The right hemispheres on the brain slices were homogenized in ice-cold RIPA buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) that contained Complete ultra mini protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenized tissues were centrifuged at 4°C for 20 minutes at 15000 rpm, and supernatants transferred to new tubes. Protein concentration was determined using Pierce BCA Protein Assay kit (Thermo Scientific, MA, USA), according to the manufacturer’s instructions. Ten micrograms of protein extracts loaded on tris–glycine gel (8%, 12%) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Then, the membrane was preincubated with PVDF Blocking Reagent for Can Get Signal (Toyobo, Osaka, Japan) for 1 hour followed by a 3-time wash using tris-buffer saline-0.05% Tween-20 (TBS-T) and reacted overnight at 4°C in Can get Signal Solution-1 (Toyobo) with the primary antibodies of mouse antispectrin alpha chain (1:1000; Millipore) or mouse 4- hydroxynonal (HNE) (1:200; JaICA, Shizuoka, Japan). Horseradish peroxidase (HRP)-conjugated anti-ß actin antibody (1:10000; Sigma-Aldrich, MO, USA) diluted in TBS-T was incubated overnight at 4°C as the internal
control. After washing, the membrane was incubated in Can get Signal Solution-2 (Toyobo) with HRP-conjugated anti-mouse antibody (1:5000; Cell Signaling Technology, MA, USA) for 1 hour at room temperature to label the primary antibody. Finally, the signals were visualized with ECL prime detection reagent (GE healthcare, Little Chalfont, UK) using LAS-3000 image station (Fuji Photo Film, Tokyo, Japan). Densitometric analysis was performed using ImageJ (National Institute of Health, MD, USA).

**Quantification of serum anti-Ang II antibody titer by enzyme-linked immunosorbent assay**

Serum anti-Ang II antibody titer was quantified using enzyme-linked immunosorbent assay (ELISA) by a modification of the protocol described elsewhere. Ang II-BSA conjugate was coated at 10 μg/ml concentration diluted in 50 mM carbonate buffer overnight at 4°C on ELISA plates. After blocking with 5% skimmed milk in PBS, the sera were serially diluted from 1:10 to 1:1000000 in skimmed milk and plated on each well followed by overnight incubation at 4°C. After washing the plates with 0.05% PBS-Tween-20 (PBS-T), these were incubated with anti-rat specific horseradish peroxidase HRP-conjugated antibodies (1:1000; GE Health care) for 3 hours at room temperature. After washing the plates, the color was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) solution (Sigma-Aldrich), followed by reaction stop using 0.5 N sulfuric acid. The absorbance was read using iMark microplate absorbance reader (Bio-Rad, CA, USA). The anti-Ang II specific antibody titer was defined as the serum dilution that exhibited half maximal binding.

**Anti-Ang II antibody penetration into brain parenchyma**

Intact brain hemispheres and ischemic brain hemispheres taken from the brain slices (1.0 mm thick) at the level of CPu of non-surgical rats or surgical rats (both pMCAo and sham-operated rats) were homogenized in ice-cold PBS followed by centrifugation at 4°C for 20 minutes at 15000 rpm; the supernatants were transferred to new tubes. Each 50μl supernatant diluted in 5% skimmed milk containing 60 μg of total protein was incubated overnight at 4°C in Ang II-BSA conjugate-coated ELISA plates. The subsequent procedure to record anti-Ang II antibody in brain parenchyma was performed in a manner similar to the ELISA procedure to quantify serum anti-Ang II antibody titer. The absorbance at 450 nm in brain homogenate from right hemisphere of non-surgical vaccinated or saline-treated control rats was measured to assess whether anti-Ang II antibody penetration into brain tissue occurred in healthy rats. Although the absorbance at 450 nm in brain homogenates from ischemic hemisphere of vaccinated or saline treated control pMCAo rats was recorded in a manner similar to that of non-surgical rats, fold increase compared to the average of absorbance at 450 nm in non-surgical rats was calculated by the following formula considered to reflect anti-Ang II antibody penetration into the ischemic brain lesion.

\[
\text{(Fold increase of anti - Ang II antibody) = } \frac{\text{(Absorbance at 450 nm of the brain homogenate from vaccinated MCAo rats)}}{\text{(Average of absorbance at 450 nm of the brain homogenate from vaccinated healthy rats)}}
\]
Quantification of Ang II protein expression in brain and plasma

The right hemispheres of non-surgical rats containing cortex, CPu and thalamus were homogenized in ice-cold PBS followed by centrifugation at 4°C for 20 minutes at 15000 rpm. Total protein concentration of supernatants was determined using Pierce BCA Protein Assay kit (Thermo Scientific) and all samples were adjusted to a final total protein concentration of 2.5 mg/ml after dilution in PBS. The brain homogenate and plasma samples were stored at −80°C until assay. Measurement of Ang II concentration in brain homogenate or plasma samples was performed using standard radioimmunoassay by an external laboratory (SRL, Tokyo, Japan).

Real-time polymerase chain reaction

Total RNA extraction from the stored brain hemisphere was processed using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), as per the manufacturer’s instructions. The cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA), according to the manufacturer’s instructions. Real-time PCR was performed in a StepOne real time PCR system (Life technologies, CA, USA). The following Taqman® Gene Expression Assays were used for expression analysis: Ang II receptor type 1a (AT1R): Rn02758772_s1, angiotensinogen: Rn00593114_m1, cytochrome b-245, beta polypeptide (Cybb, the gene encoding Nox2): Rn00576710_m1, (Applied Biosystems). Quantitative data was calculated using comparative Ct (ΔΔCT) method. The expressions of target genes were normalized against the expression level of 18s ribosomal RNA with control primer of eukaryotic 18s rRNA endogenous control (4319413E, Applied Biosystems).

Fluoro-Jade B staining

Fluoro-Jade B (FJB) is an anionic dye that specifically stains the soma and neurites of degenerating neurons. Coronal frozen brain sections (7.0 μm thick) were cut at the level of CPu on a freezing microtome. The sections were dried and placed in 80% ethanol/1% NaOH for 5 minutes and 70% ethanol for 2 minutes followed by wash with distilled water for 2 minutes. Subsequently, sections were soaked in a solution of 0.06% KMnO₄ for 10 minutes and washed in distilled water for 2 minutes and stained using 0.0004% FJB (Millipore) in 0.1% acetic acid for 20 minutes. Slides were washed three times in distilled water for 1 minute each and dried on heat block at 50°C for 10 minutes. Finally, slides were cleared in xylene and coverslips were mounted using DPX mountant for histology (Sigma-Aldrich). Digital images were acquired using a DFC450 C on a DM2500 LED microscope (Leica).

Immunohistochemical analysis

Brain sections were prepared using the procedure described for FJB staining. Sections were washed three times with PBS-T and blocked with 5% normal goat serum (NGS) in PBS for 1 hour. Thereafter, sections were incubated overnight with the primary monoclonal antibody of 4-HNE (1:100; JaICA) or rabbit polyclonal AT1R (1:10; SantaCruz)
diluted in 5% NGS at 4°C. After washing three times with PBS-T, sections were incubated for 1 hour at room temperature with Alexa-488 or Alexa-594 conjugated to goat anti-mouse secondary antibody (1:500; Thermo Scientific) to detect 4-HNE primary antibody and Alexa-488 conjugated to goat anti-rabbit secondary antibody (1:500; Thermo Scientific) to detect AT1R primary antibody. The sections were mounted with coverslips using Vectashield mounting medium with DAPI (Vector Laboratories, CA, USA). Digital images were acquired using a DFC450 C on a DM2500 LED microscope (Leica).

**NeuroTrace Fluorescent Nissl stain**

NeuroTrace Nissl stain was performed to label neurons in combination with immunohistochemistry. After immunohistochemical staining for AT1R or 4-HNE, the sections were washed for 10 minutes in PBS with 0.1% Triton X-100 to permeabilize the tissues. Then the sections were washed in PBS two times and incubated with NeuroTrace® 530/615 Red Fluorescent Nissl stain (Thermo Scientific) diluted in PBS (1:50) for 20 minutes followed by 10 minutes’ wash in PBS with 0.1% Triton X-100. The sections were washed and mounted with coverslips using Vectashield mounting medium with DAPI. Digital images were acquired using a DFC450 C on a DM2500 LED microscope (Leica).

**Statistical Analysis**

Data are expressed as mean ± standard deviation (SD). Data were analyzed using Statview for windows version 5 (SAS Institute Inc, Tokyo, Japan). Between-group differences were assessed by unpaired t-test (Figure 1B-D, 5A, Supplemental Figure IIA, IV and V). Comparison between multiple groups was performed using ANOVA followed by Tukey-Kramer’s post-hoc comparison (Figure 3A-B, 4A-B, 5B, Supplemental Figure I and VIIB-B). Correlation analyses were performed by Pearson correlation (Figure 2B, Supplemental Figure IIB and VI). $P < 0.05$ was considered as statistically significant.
Supplemental Figure I

Effect of angiotensin (Ang) II peptide vaccine on systemic blood pressure.

No significant between-group difference in systolic blood pressure was observed. Each group included n = 10, V: Ang II vaccinated rats, S: saline treated rats.
Supplemental Figure II

**Plasma Ang II concentration in vaccinated rats**

(A) Plasma Ang II concentration was significantly increased in Ang II peptide vaccine immunized (V) rats as compared to that in saline-treated control (S) rats. (B) A significant positive correlation was observed between plasma Ang II concentration and serum anti-Ang II antibody titer ($r = 0.669$, $p = 0.0153$). ## $p = 0.003$ vs. S, n = 8 in each group in A.
Supplemental Figure III

Relationship between the serum anti-Ang II antibody titer and the size of cerebral infarction.

Representative images of cresyl violet-stained brain slices from vaccinated rats whose serum Ang II titer was ≥ 6000 (V_H), serum Ang II titer was < 6000 (V_L) and saline-treated control (S) rats 24 hours after permanent middle cerebral artery occlusion (pMCAo).
**Supplemental Figure IV**

**Infarct volume in rats exposed to pMCAo at 15 days after the third vaccination**

Rats were subjected to pMCAo at 15 days after the third vaccination or saline injection and the infarct volume at 24 hours of cerebral ischemia was compared between the groups. Infarct volume was less in $V_H$ rats, whose serum Ang II titer was OD50% ≥ 6000, than that in saline-treated rats (S). **$p < 0.001$ vs. S**, each group included n = 5.

### Supplemental Table IV

<table>
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<tr>
<th>Age (week)</th>
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<td>2$^{\text{nd}}$</td>
<td>3$^{\text{rd}}$</td>
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<td>Days after 3rd Vaccination</td>
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<td>7</td>
<td>15</td>
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Brain extraction

Titer test

pMCAo 24 h

24h, MCAo

Infarct Volume (mm$^3$)

\begin{center}
\begin{tabular}{c c}
S & $V_H$ \\
\hline
250 & **
\end{tabular}
\end{center}
Supplemental Figure V

Systolic blood pressure (BP) in rats at 24 hours after pMCAo.

pMCAo surgery was performed 15 days after third vaccination or saline injection and BP was recorded at 24 hours after cerebral ischemia. There was no significant difference in BP between $V_H$ rats, whose serum Ang II titer was OD50% $\geq$ 6000, and saline-treated rats (S) at 24 hours after pMCAo. $n = 5$ in each group.
Supplemental Figure VI
Serum anti-Ang II antibody titer and change of the brain parenchymal anti-Ang II antibody level in the ischemic hemisphere of vaccinated rats.

A strong positive correlation was observed between serum anti-Ang II antibody titer and fold increase of anti-Ang II antibody in ischemic brain tissue at 24 hours after pMCAo in vaccinated rats ($r = 0.747, p = 0.0003$).
Supplemental Figure VII

Attenuation of cleavage of spectrin and Nox2 mRNA expression in vaccinated rats.

(A) Densitometric analysis demonstrated accelerated cleavage of full spectrin (240 kDa) into 150/145 kDa fragments in ischemic lesion of the saline-treated control (S) rats as compared with that in ischemic lesion of sham rats at 24 hours after pMCAo. Reduced cleavage of the full spectrin into 150/145 kDa fragments was observed in rats with high titers of serum anti-Ang II antibody (V_H, OD 50% ≥ 6000) compared with S rats. (B) Quantitative analysis of Nox2 mRNA expression by real-time polymerase chain reaction. Twenty-four hours after pMCAo, Nox2 mRNA expression in ischemic hemisphere was significantly lower in V_H rats as compared to that in S rats. **p < 0.01 vs. sham, ‡‡ p < 0.01 vs. S, # p < 0.05 vs. S. Each group included n = 5 in A and n = 8 (sham), n = 6 (S), n = 6 (V_H) in B.
Supplemental Figure VIII

Expression of AT1R or 4-HNE in neurons

(A) Schematic illustration of a coronal brain section. The shaded area represents the ischemic lesion and the boxed areas were observed regions. (B) Double labeling images for AT1R (green) and neuron (NeuroTrace, red) revealed that the expression of AT1R was increased in neurons in the ischemic core and peri-infarct tissue (arrows). (C) Formation of 4-HNE (red) was observed only in the ischemic hemisphere and most of 4-HNE immunopositive cells showed the increased expression of AT1R (green) in the ischemic hemisphere (thick arrows). (D) Double labeling images for 4-HNE (green) and neuron (NeuroTrace, red) demonstrated that 4-HNE positive cells were observed only in the ischemic hemisphere.
hemisphere and most of them were neurons (arrowheads) although other cells also expressed 4-HNE (arrows). IC = Ischemic core, Bar = 20μm.
Supplemental references


**Stroke Online Supplement**

**Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation**

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| **Experimental groups and study timeline** | ☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
☑ An overall study timeline is provided. |
| **Inclusion and exclusion criteria** | ☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. |
| **Randomization** | ☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
☑ Type and methods of randomization have been described.  
☑ Methods used for allocation concealment have been reported. |
| **Blinding** | ☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment. |
| **Sample size and power calculations** | ☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided. |
| **Data reporting and statistical methods** | ☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups.  
☑ Baseline data on assessed outcome(s) for all experimental groups have been reported.  
☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
☑ Statistical methods used have been reported.  
☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures. |
| **Experimental details, ethics, and funding statements** | ☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described.  
☑ Different sex animals have been used. If not, the reason/justification is provided.  
☑ Statements on approval by ethics boards and ethical conduct of studies have been provided.  
☑ Statements on funding and conflicts of interests have been provided. |