Original Article

Inhibition of Matrix Metalloproteinase-9 Activity by Trandolapril after Middle Cerebral Artery Occlusion in Rats

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We investigated whether an angiotensin-converting enzyme (ACE) inhibitor could inhibit matrix metalloproteinase (MMP) activities in cerebral infarct lesions after middle cerebral artery occlusion (MCAO) in rats. After placebo or trandolapril (5 mg/kg per day) was administered orally for 7 days, we permanently occluded the right middle cerebral artery. ACE activity in extracts from the infarct side of placebo-treated rats was significantly higher than that in extracts from the non-infarct side from 5 days after MCAO, though they did not differ at 1 day. ACE activities in extracts from both hemispheric segments in the trandolapril-treated group were significantly decreased compared with those in the placebo-treated group before MCAO, and this significant reduction persisted even at 7 days after MCAO. In the placebo-treated group, MMP-9 and MMP-2 activities in the infarct side were significantly increased at 12 h and at 1 day after MCAO, respectively. Trandolapril treatment significantly reduced MMP-9 and MMP-2 activities to 68.5% and 53.2%, respectively. Seven days after MCAO, the ratios of infarct areas to the hemispheric sectional areas in placebo- and trandolapril-treated rats were 55.4±2.1% and 30.9±2.9%, respectively, and this difference was significant. Neurological severity scores were significantly improved from 1 to 7 days after MCAO in trandolapril-treated rats. Cumulative survival in trandolapril-treated rats was significantly increased compared with that in placebo-treated rats. Thus, the inhibition of MMP-9 by trandolapril might be part of the mechanism that prevents cerebral damage after cerebral ischemia. (*Hypertens Res* 2007; 30: 469–475)

***Key Words*: angiotensin converting enzyme, cerebral infarction, macrophage, inhibitor**

Introduction

Angiotensin-converting enzyme (ACE) is a Zn^{2+}-dependent endopeptidase expressed in endothelial cells, macrophages and smooth muscle cells that converts angiotensin I to angiotensin II (*1*). Angiotensin II plays a crucial role in tissue remodeling *via* increases in growth factors, cytokines, chemokines and extracellular matrix, as well as in the regulation of blood pressure (*2*, *3*). In cerebral infarction after middle cerebral artery occlusion (MCAO), macrophages accumulate at the border between infarct and non-infarct areas and might induce the expansion of cerebral infarction (*4*, *5*). We reported that brain ACE activity is significantly increased in the infarct area after MCAO in rats (*6*). In that study, trandolapril significantly reduced not only brain ACE activity but also the ratio of the infarct to the total area 7 days after MCA occlusion. However, whether trandolapril has functional merit such as reducing neurological severity scores or increasing survival rates remains obscure.

Matrix metalloproteinases (MMPs) belong to a family of Zn^{2+}-dependent endopeptidases. Extracellular matrix mole-

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cules such as type IV collagen, laminin and fibronectin, which support the structure of the vascular basement membrane, are degraded by MMP-2 and MMP-9 (*7*). Their enzymatic functions play an important role in the progression of inflammation and atherosclerosis (*8*, *9*). The vascular basement membrane in the brain plays a crucial role in maintaining the blood-brain barrier (BBB) (*10*), which protects the central nervous system. However, focal stroke damages the BBB, resulting in neuronal cell death (*11*). Other studies have shown that MMP-2 and MMP-9, but not MMP-1 and MMP-3, are increased in brain infarcts after MCAO in rats (*12*). After cerebral ischemia and reperfusion, levels of type IV collagen, laminin and fibronectin decrease (*13*). These reports suggest that MMP-2 and MMP-9 are involved in the cerebral damage after focal ischemia. In fact, MMP-9 inhibition results in a decrease of the infarct area after rat MCAO (*12*).

The activities of MMP-2 and MMP-9 are inhibited by ACE inhibitors in extracts of rat renal and human cardiac tissues (*14*, *15*). However, whether ACE inhibitors affect MMP-2 and MMP-9 activities after focal ischemia remains unknown. Here, we examined the effects of ACE inhibitors on MMP-2 and MMP-9 activities after MCAO in rats.

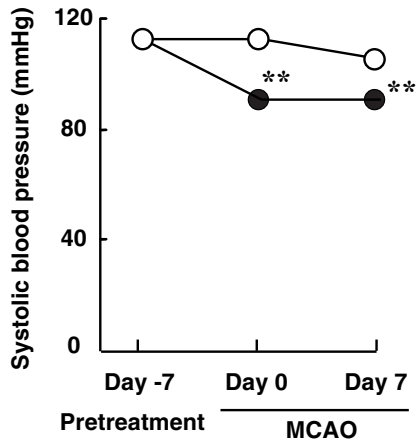

Fig. 1. *Systolic blood pressure 7 days before (day −7), immediately before (day 0) and 7 days (day 7) after MCAO in placebo- and trandolapril-treated rats (each group, n = 6). Open circles represent placebo-treated rats. Closed circles represent trandolapril-treated rats. **p < 0.01 vs. placebo-treated rats.*

Methods

Animals

Male Wistar Kyoto (WKY) rats weighing 230–260 g were purchased from Japan SLC (Shizuoka, Japan). All rats were housed individually in metabolic cages for measurement of urinary albumin at room temperature (23–26°C) under a 12-h light-dark cycle and had free access to standard food (F-2; Funahashi Co., Tokyo, Japan) and water. The rats were divided into 2 groups, the vehicle group and the trandolapril (5 mg/kg per day)-treated group. The experimental protocols involving the animals proceeded in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College, Takatsuki, Japan).

MCAO Model

Focal cerebral ischemia was accomplished by using the intraluminal filament model (4-0 nylon monofilament suture) of proximal MCAO as previously described (*6*, *16*). Rats were anesthetized initially with 3.5% halothan and maintained with 1.0% to 2.0% halothan in 70% N_2O and 30% O_2 using a face mask. The right common carotid artery was exposed through a lateral incision, separated from the vagus nerve, and ligated. For the permanent MCA occlusion, a 4-0 nylon monofilament, with a tip that was rounded by heating, was introduced from the bifurcation of the internal carotid artery and advanced until resistance was felt. Using this procedure, after the MCAO was completed, each rat had an infarct area of similar size.

Blood Pressure

Systolic blood pressure was monitored by tail-cuff plethysmography (BP-98; Muromachi Co., Kyoto, Japan).

Neurological Severity Score

The rats underwent behavioral tests before and 1, 3 and 7 days after MCAO as described previously (*16*). A modified neurological severity score (mNSS) (*16*) was used to grade the aspects of neurological function. Each test in mNSS has been used to grade various aspects of neurological function. mNSS is a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. A higher score reflects more severe injury.

Tissue Extract

Brain extracts for measurement of ACE and MMP activities were prepared as described previously (*17*). First, the forebrain, separated from the cerebellum, was cut in half into left and right hemispheric segments, and then minced and homogenized in 5 volumes (w/v) of 20 mmol/L Tris-HCl buffer, pH 8.3, containing 5 mmol/L $Mg(CH_3COO)_2$, 30 mmol/L KCl, 250 mmol/L sucrose, 0.5 U/mL aprotinin and 0.5% NP-40. The homogenate was centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was used for the measurement of ACE activity and protein concentration.

ACE Activity

The ACE activity in the brain extract was measured using a

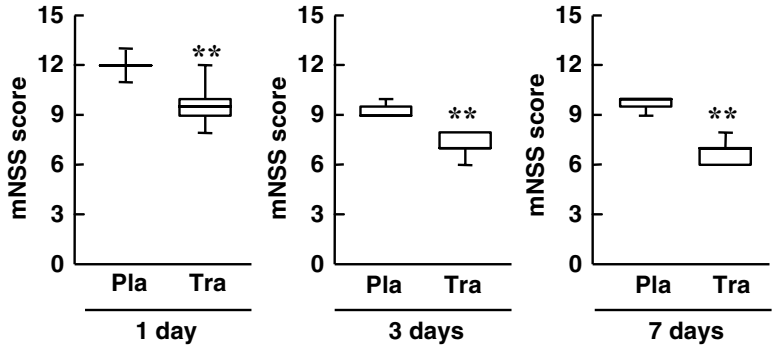

Fig. 2. *Neuronal function of placebo (Pla)- and trandolapril (Tra)-treated rats at 1, 3 and 7 days after MCAO as determined by mNSS (each group, n = 12). **p < 0.01 vs. placebo-treated rats.*

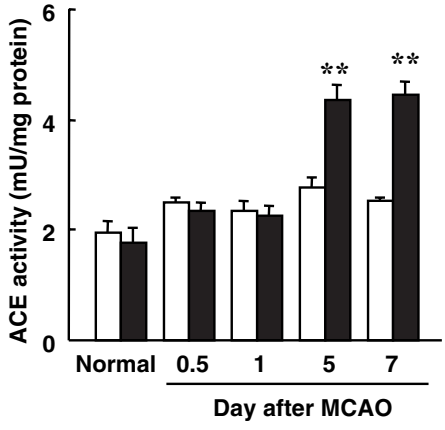

Fig. 3. *Changes in ACE activities of extracts from the non-infarct and infarct sides of the brain before and 0.5, 1, 5 and 7 days after MCAO in placebo-treated rats (each point, n = 6). Open bars represent the non-infarct side. Closed bars represent the infarct side. **p < 0.01 vs. before MCAO.*

synthetic substrate, hippuryl-His-Leu (HHL), specifically designed for ACE (Peptide Institute, Inc., Osaka, Japan). Fifty microliters of tissue extract were incubated for 30 min at 37°C with 5 mmol/L HHL in 250 μL of 10 mmol/L phosphate buffer, pH 8.3, containing 0.3 mol/L NaCl. The reaction was terminated by addition of 750 μL of 3% metaphosphoric acid, and then the mixture was centrifuged at 20,000 × *g* for 5 min at 4°C. The supernatant was applied to a reversed-phase column (4 mm i.d. × 250 mm; IRICA Instrument, Kyoto, Japan), which had been equilibrated with 10 mmol/L KH_2PO_4 and CH_3OH (1:1, pH 3.0), and eluted with the same solution at a rate of 0.3 mL/min. Hippuric acid was detected by ultraviolet absorbance at 228 nm. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 μmol of hippuric acid/min.

Gelatin Zymography

Gelatinolytic activity in the brain extract was assayed on gelatin zymography. Each sample was purified and concentrated using gelatin Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) as described previously (*18*). The eluted samples from gelatin Sepharose 4B were loaded onto 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels containing 1 mg/mL of gelatin. After electrophoresis, the gel was renatured in 50 mmol/L Tris-HCl, pH 7.5, containing 100 mmol/L NaCl and 2.5% Triton X-100 for 1.5 h to remove SDS, and the gel was then incubated with 50 mmol/L Tris-HCl (pH 7.5) containing 10 mmol/L $CaCl_2$. Subsequently, the gel was stained with Coomassie Brilliant Blue, and gelatinolytic activity, which appeared as clear bands against a blue-stained background, was quantified with NIH-Image 1.61 software (US National Institutes of Health, Bethesda, USA).

Histological Study

The infarct area was measured as described previously (*16*). Seven days after the MCA occlusion, rats were deeply anesthetized with halothan, and then the hearts were perfused with saline. The brain was immediately removed and sectioned into 7 equally spaced (2 mm) coronal blocks using a rodent brain matrix. These sections were stained with 2% 2-3-5-triphenylterazolium (TTC) in normal saline for 30 min at 37°C. The area left unstained by TTC staining was regarded as the infarct area. The volume of the lesion is presented as a percentage of the volume of the contralateral hemisphere.

Statistical Analysis

All data indicated in the text are expressed as the means±SEM. The statistical significance of differences between the mean values of two groups was determined using Student's *t*-test. Significant differences among the mean values of multiple groups were evaluated by one-way ANOVA followed by a post-hoc analysis (Bonferroni's test). Compar-

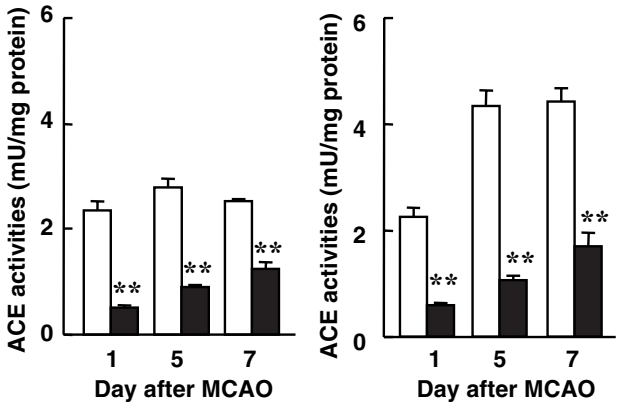

Fig. 4. *ACE activities of extracts from the non-infarct (left) and infarct (right) sides of the brain 1, 5 and 7 days after MCAO in placebo- and trandolapril-treated rats (each group, n = 4–7). Open bars represent placebo-treated rats. Closed bars represent trandolapril-treated rats. **p < 0.01 vs. placebo-treated rats.*

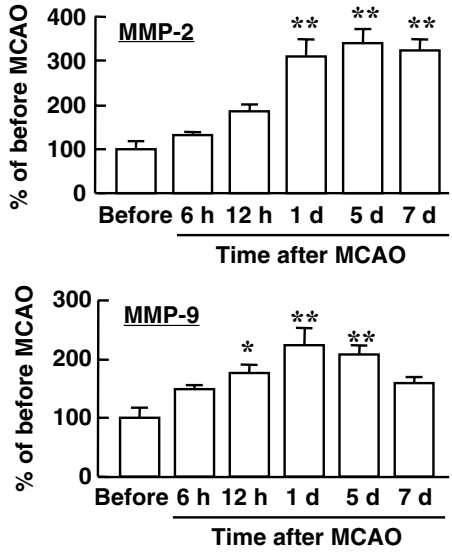

Fig. 5. *Changes in MMP-2 and MMP-9 activities of extracts from the infarct side of the brain before and 6 h, 12 h, 1 day (1 d), 5 days (5 d) and 7 days (7 d) after MCAO (each point, n = 5). *p < 0.05 and **p < 0.01 vs. before MCAO.*

isons of the mNSS between two groups were done by Mann-Whitney nonparametric test. Differences in cumulative survival between among the groups were analyzed according to Kaplan-Meier followed by a log-rank test. Differences were considered significant at $p<0.05$.

Results

Systolic Blood Pressure

Systolic blood pressure was significantly lower in the trandolapril-treated group than in the placebo-treated group before MCAO, and this persisted for 7 days (Fig. 1).

Neurological Function Test

Even at 1 day after MCAO, the mNSS scores of trandolapril-treated rats were significantly lower than those in the placebo-treated rats, and this persisted for 7 days (Fig. 2).

ACE Activity

ACE activities between left (non-infarct) and right (infarct) brain extracts did not significantly differ before MCAO in placebo-treated rats (Fig. 3). In addition, ACE activities did not significantly differ at 1 day after MCAO, whereas those in the infarct side were significantly higher than those in the non-infarct side at 5 and 7 days (Fig. 3).

In trandolapril-treated rats, ACE activities of both the left and right brain segments were significantly reduced before MCAO (Fig. 4). The ACE activities in the infarct side at 1 day after MCAO were 2.24±0.19 and 0.61±0.04 mU/mg protein in placebo- and trandolapril-treated rats, respectively, and this difference was significant (Fig. 4). In the contralateral side, ACE activities were also significantly lower in the trandolapril-treated group than in the placebo-treated group (Fig. 4). Even at 7 days after MCAO, ACE activities remained significantly reduced in both sides in the trandolapril-treated rats (Fig. 4).

MMP-2 and MMP-9 Activities

Before MCAO in the placebo-treated group, the level of MMP-9 activity was only slight, whereas the level of MMP-2 activity was more pronounced. After MCAO, MMP-9 activity was immediately increased from 12 h, peaked at 1 day, and then gradually decreased to the levels before MCAO at 7 days (Fig. 5). On the other hand, MMP-2 activity was significantly increased from 1 day after MCAO compared with the level before MCAO and the peak of the increase was observed at 5 days (Fig. 5).

Figure 6A shows photographs of typical MMP-2 and MMP-9 bands 1 day after MCAO. When the MMP-2 and MMP-9 activities in the placebo-treated rats were both regarded as 100%, trandolapril significantly reduced them to 53.2% and 68.5%, respectively, at 1 day after MCAO (Fig. 6B).

Infarct Area after MCAO

The ratios of the infarct areas to the hemispheric sectional areas in the placebo-treated rats 7 days after MCAO were 55.4±2.1%, whereas each of these ratios was significantly reduced to 30.9±2.9% in trandolapril-treated rats (Fig. 7).

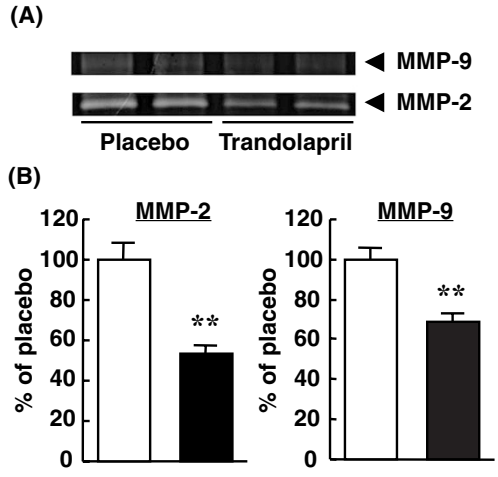

Fig. 6. *A: Typical photographs of gelatin zymograms of MMP-2 and MMP-9 from the infarct side of the brain 1 day after MCAO in placebo- and trandolapril-treated rats. B: MMP-2 and MMP-9 activities of extracts from the infarct side of the brain 1 day after MCAO in the placebo- and trandolapril-treated rats (each group, n = 5) (B). Open bars represent placebo-treated rats. Closed bars represent trandolapril-treated rats. **p < 0.01 vs. placebo-treated rats.*

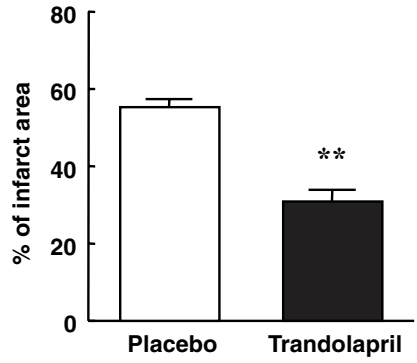

Fig. 7. *Ratios of the infarct areas to the hemispheric sectional areas 7 days after MCAO in the placebo- and trandolapril-treated rats (each group, n = 5). **p < 0.01 vs. placebo-treated group.*

Survival

Figure 8 shows survival curves of the placebo- and trandolapril-treated rats at 7 days after MCAO. The 7-day survival rates in the placebo- and trandolapril-treated rats were 77.1% and 94.3%, respectively, and the values differed significantly.

Discussion

We discovered that the ACE inhibitor trandolapril significantly reduced MMP-2 and MMP-9 activities in infarct regions after MCAO in rats. A previous report has shown that MMP-9 activity increases from 12 h after MCAO (*12*). On the other hand, MMP-2 activity also increases from 1 day after MCAO (*12*). The present study also found significant increases in MMP-9 and MMP-2 activities from 12 h and 1 day after MCAO, respectively, and trandolapril significantly reduced these increases. Previous reports have demonstrated that ACE inhibitors inhibit both MMP-2 and MMP-9 activities in extracts from rat and human tissues (*14*, *15*). However, whether or not ACE inhibitors can inhibit MMP-2 and MMP-9 activities *in vivo* has remained unclear. Angiotensin II induces MMP-2 and MMP-9 gene expression (*19*, *20*). We found here that ACE activity from brain extracts in the infarct side was significantly higher than that in extracts from the non-infarct side from 5 days after MCAO, whereas the ACE activities did not differ significantly between the two sides at 1 day after MCAO. Therefore, the production of angiotensin II by ACE in the infarct side might not be changed at 1 day after MCAO. However, trandolapril significantly suppressed MMP-2 and MMP-9 activities at 1 day after MCAO. These results indicate that the trandolapril-induced reduction of MMP-2 and MMP-9 activities at 1 day after MCAO in trandolapril-treated rats does not involve the suppression of angiotensin II production by ACE. These findings suggest that trandolapril directly inhibits MMP-2 and MMP-9 activities at 1 day after MCAO in rats.

We reconfirmed that trandolapril significantly reduces the ratio of the infarct area after MCAO (*6*) and found that neuronal function significantly improved after MCAO. Furthermore, cumulative survival was also significantly better in trandolapril-treated rats than in placebo-treated rats. To the best of our knowledge, the ability of angiotensin II–related drugs, such as ACE inhibitors and angiotensin II receptor blockers (ARBs), to increase cumulative survival has not been previously recognized. We found here that trandolapril not only reduced the infarct area but also improved neuronal function and increased survival rates after MCAO.

Administration of trandolapril (5 mg/kg per day, p.o.) for 7 days significantly suppressed ACE activity 1 day after MCAO, and this persisted in both the infarct and non-infarct sides for 7 days after trandolapril was withdrawn. We previously reported that the repeated administration of highly lipophilic ACE inhibitors such as trandolapril significantly suppresses brain ACE activity and continues to do so for 7 days after trandolapril withdrawal (*21*). The results of the present study reconfirmed these findings. On the other hand, other investigators and we have reported that hydrophilic ACE inhibitors such as enalapril do not inhibit brain ACE despite ACE inhibition in plasma and peripheral tissues (*22*, *23*). Repeated trandolapril administration has antinociceptive effects along with the suppression of brain ACE activity, but enalapril induces neither of these effects, although ACE activities are suppressed in plasma and peripheral tissues (*24*). In the present study, the lipophilic ACE inhibitor,

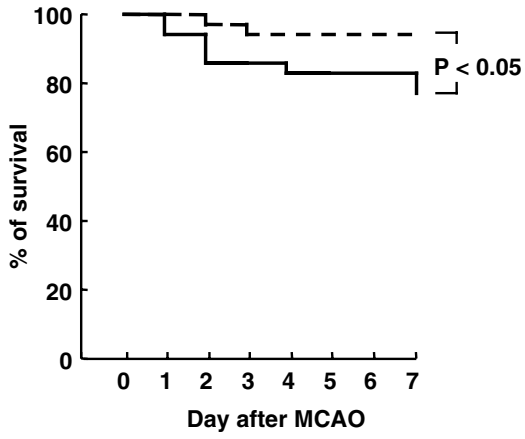

Fig. 8. *Cumulative survival rates of rats administered placebo (n = 35) or trandolapril (n = 35). The solid line represents placebo-treated rats. The broken line represents trandolapril-treated rats.*

trandolapril, which can penetrate the blood-brain barrier, not only reduced brain ACE activity and the size of the infarct area but also increased neurological severity scores and survival rates. On the other hand, the less lipophilic ACE inhibitor, enalapril, does not suppress either neurological severity scores or infarct areas after MCA occlusion in rats (*25*). Therefore, ACE inhibitors that are highly lipophilic might have an advantage for cerebral protection compared with those that are not lipophilic.

ARBs improve neuronal function and reduce infarct areas after MCAO in rats and mice (*25–27*). After MCAO in rats, levels of angiotensin II type 2 (AT_2) and AT_1 receptors in the brain significantly increase and decrease, respectively (*28*). Angiotensin II might further stimulate AT_2 receptors in the presence of administered ARBs. Iwai *et al*. (*29*) demonstrated significantly larger infarct areas in AT_2 receptor–deficient mice than in wild type mice. They also reported that increases of superoxide production and NADPH oxidase activity were significantly reduced by an ARB, but to a lesser extent in AT_2 receptor–deficient mice than in wild type mice (*29*). Although AT_1 receptors increase superoxide production and NADPH oxidase, AT_2 receptor stimulation partly contributes to the cerebral protection conferred by ARB. Both the ARB candesartan and the ACE inhibitor ramipril reduce infarct areas and improve neuronal function after MCAO in spontaneously hypertensive rats, but the AT_2 receptor blocker PD123319 almost completely attenuates the cerebral protection induced by candesartan (*27*). At the very least, it is clear that AT_2 receptor stimulation does not play a role in the mechanism by which ACE inhibitors protect the ischemic brain after MCAO, since angiotensin II formation is reduced by ACE inhibitors. Therefore, the mechanisms of ARB and ACE inhibitors might be quite different.

In the present study, trandolapril significantly suppressed both MMP-2 and MMP-9 activities 1 day after MCAO. The decrease of MMP-9 after MCAO might play an important role in protecting against cerebral damage, since an MMP-9–neutralizing monoclonal antibody and an MMP-9 inhibitor have been shown to improve neuronal function and reduce the infarct area after MCAO in rats (*12*, *30*). In MMP-9–deficient mice, the neuronal damage after MCAO was significantly attenuated (*30*). Minocycline, which inhibits MMP-9, inhibited ischemia-provoked pro–MMP-9 induction in wild-type mice, but was not protective in MMP-9–deficient mice (*31*). On the other hand, the role of MMP-2 after MCAO has been obscure. Therefore, the mechanism through which trandolapril protects against cerebral damage after MCAO might be predominantly related to a reduction in MMP-9 activity rather than MMP-2 activity.

In conclusion, we demonstrated for the first time that a lipophilic ACE inhibitor, trandolapril, could suppress an increase of MMP-9 activities at the acute phase after MCAO. The inhibition of MMP-9 activities by trandolapril might be involved in cerebral protection after MCAO.

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