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ORIGINAL ARTICLE

Postischemic administration of angiotensin II type 1 receptor blocker reduces cerebral infarction size in hypertensive rats

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Lowering the blood pressure (BP) during the acute period following ischemic stroke is still a controversial treatment. In this study, we investigated the effect of postischemic treatment using the angiotensin II type 1 receptor blocker, candesartan, on brain damage in focal cerebral ischemia. Spontaneously hypertensive rats underwent transient occlusion of the middle cerebral artery for 1 h. Candesartan (0.1, 1 and 10 mg kg^{-1}) or vehicle was administered orally 3 and 24 h after ischemia. Blood pressure and neurological function were monitored, and infarct volume was evaluated 48 h after occlusion. Cerebral blood flow was measured using laser Doppler flowmetry before and after treatment with candesartan. Activation of Rho-kinase in cerebral microvessels was evaluated by immunohistochemistry. Systolic blood pressure was markedly lowered with both moderate and high doses, but it did not fall with a low dose of candesartan. The infarct volume was reduced in rats treated with the low dose of candesartan but not in those treated with the moderate or high doses. Cerebral blood flow decreased in parallel with the reduction in BP 3 h after treatment using the moderate dose, but it did not change after treatment with the low dose of candesartan, compared with vehicle. Rho-kinase was activated in the brain vessels of the ischemic cortex, but treatment with candesartan suppressed it. Our results show that oral administration of candesartan after transient focal ischemia reduced infarct volume at doses that showed little effect on BP. The neurovascular protective effects of candesartan may be caused by the inhibition of Rho-kinase in brain microvessels.
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Keywords: angiotensin II type 1 receptor antagonist; cerebral blood flow; focal cerebral ischemia; Rho-kinase

INTRODUCTION

Hypertension is one of the major risk factors of stroke. In the acute phase of stroke, blood pressure (BP) is often elevated, so patients with hypertension have a higher risk of early death, cerebral edema, and stroke recurrence. However, treatment by lowering the BP in the acute phase of ischemic stroke has been controversial.[1]

In experimental animal stroke models, recent studies have shown that postischemic administration of an angiotensin II type 1 receptor (AT_1) blocker (ARB) after transient middle cerebral artery (MCA) occlusion improves neurological outcomes and reduces infarct size.[2–5] However, it remains unclear whether the beneficial effects of ARBs are dose dependent. Several studies have suggested that high doses of ARBs are not beneficial because they cause hypotension.[5,6]

Molecular mechanisms underlying the protective effects of ARBs on the postischemic brain could include the suppression of inflammation,[3,7–9] oxidative stress,[10] and apoptosis.[11] Recently, Rho-kinase has attracted attention because it is a potential target for the attenuation of ischemic brain injury.[12,13] We have demonstrated earlier that endothelial Rho-kinase was activated in the ischemic cortex after MCA occlusion.[14] Although intracellular signaling through the AT_1 receptor is closely associated with the activation of Rho-kinase in the field of vascular biology,[15] the effect of ARBs on Rho-kinase activity has not been examined in the ischemic brain.

In this study, we evaluated the dose-dependent effects of orally administered candesartan after focal cerebral ischemia in spontaneously hypertensive rats (SHR) on infarct size in relation to changes in BP and cerebral blood flow. We also examined the effect of candesartan on Rho-kinase activity in brain microvessels.

METHODS

Adult male, spontaneously hypertensive rats (Charles River Inc., Yokohama, Japan) weighing 250–350 g (10–15 weeks old) were used in this study. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. The rats were fed standard laboratory chow and had free access to water before and after all procedures.

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Experiment 1

Surgical procedure. Each animal (n=48 in total, n=12 per group) was anesthetized with halothane, and occlusion of the left MCA was accomplished according to the procedure used by Koizumi *et al.*[16] and Longa *et al.*[17] (Figure 1). Briefly, the left common carotid artery was exposed by a midline incision, and the internal carotid artery was isolated and carefully separated. A 4-0 nylon monofilament, whose tip was rounded by heating, was introduced from the bifurcation of the internal carotid artery and advanced until resistance was felt. At 60 min after MCA occlusion, the filament was withdrawn to allow reperfusion. The animals recovered rapidly from anesthesia and were observed postoperatively for 48 h. Rectal temperature was monitored routinely during the surgical procedure to ensure that it was maintained at 37.0 ± 0.5 °C.

Administration of drugs and measurement of blood pressure. Vehicle (n=12) or candesartan (n=12 per group) was given orally using gavage twice (3 and 24 h after MCA occlusion), at three different doses (0.1, 1, and 10 mg kg^{-1}). We measured arterial BP in conscious rats by the tail-cuff method before ischemia and at 3, 6, 24, and 48 h after ischemia.

Determination of neurological deficits. Evaluation of neurological deficits was carried out before ischemia and at 3, 6, 24, and 48 h after ischemia. Neurological findings were scored on a 5-point scale:[17] 0 indicating no neurologic deficit; 1, failure to extend the left and/or right forepaw fully; 2, circling to the right; 3, falling to the right; and 4, inability to walk and a depressed level of consciousness.

Measurement of infarct volume. At 48 h after MCA occlusion, rats were killed with an overdose of halothane. The brain was rapidly and carefully removed, cooled in ice-cold saline for 5 min, and then dissected into coronal 2-mm sections by using a rat brain matrix (Muromachi Kikai Co. Ltd, Tokyo, Japan). For the delineation of infarct area, the brain slices were incubated in saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min and stored in 10% neutral buffered formalin.[18] The ischemic lesions with no reaction towards TTC were measured on the posterior surface of each slice. The volume of ischemic lesions was measured by using an MCID image analysis system (Imaging Research Inc., St Catharines, Ontario, Canada). The area with ischemic lesions and the area of both hemispheres (mm^2) were calculated on TTC-stained coronal sections by tracing these areas on the computer screen. The volume (mm^3) was determined by integrating the appropriate area and the section thickness. To reduce errors associated with processing of tissue for histological evaluation, the lesion area and volume were corrected using the method of Swanson *et al.*[19]

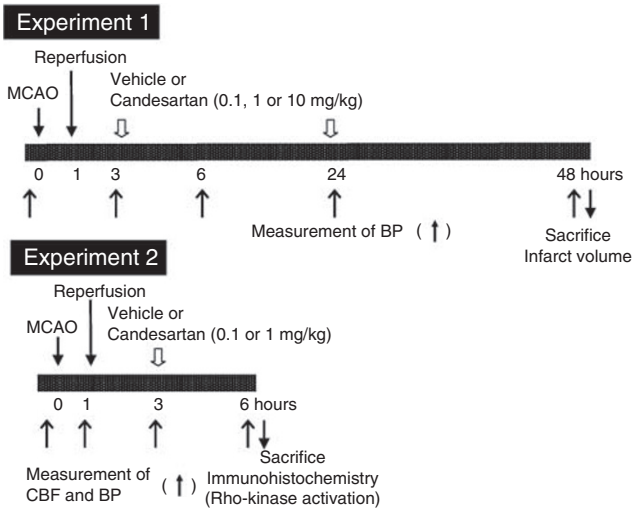

Figure 1 Experimental protocols. (Experiment 1) Vehicle or candesartan (0.1, 1, or 10 mg kg^{-1}) was applied 3 and 24 h after middle cerebral artery occlusion (MCAO). Systolic blood pressure (BP) and neurological deficits were evaluated before ischemia and at 3, 6, 24, and 48 h after ischemia. (Experiment 2) Vehicle or candesartan (0.1 or 1 mg kg^{-1}) was applied 3 h after MCAO. Systolic BP and cerebral blood flow were evaluated before ischemia and at 1, 3, and 6 h after ischemia.

Experiment 2

Measurement of cerebral blood flow. General anesthesia was induced using 4.0% halothane and maintained with 0.5% halothane with an open facemask (Figure 1). A polyacrylamide column with an inner diameter of 0.8 mm, for the measurement of cortical microperfusion by laser Doppler flowmetry (Laser Doppler Blood Flow Meter, model TBF-LNIT; Unique Medical Co. Ltd. Tokyo, Japan), was attached with dental cement to the intact skull at 1 mm posterior to the bregma and 5 mm from the midline. Animals received left MCA occlusion and reperfusion 60 min later, and we measured the ipsilateral regional cerebral blood flow (rCBF) before and after MCA occlusion and after reperfusion under anesthesia to confirm ischemia and reperfusion. At 3 h after MCA occlusion, rats recovered fully from anesthesia, and their blood pressure (BP) and rCBF were evaluated. Then, vehicle (n=5) or candesartan at 0.1 mg kg^{-1} (n=5) or 1 mg kg^{-1} (n=5) was given orally using a gavage. After 3 h, BP and rCBF were measured again. The value of rCBF was expressed as percentage of the rCBF measured before treatment with candesartan or vehicle. Laser Doppler flowmetry, though not quantitative, provides a reliable estimate of rCBF.

Immunohistochemistry. After evaluation of BP and rCBF, animals were perfused transcardially with Zamboni's solution (2% paraformaldehyde, 0.2% picric acid), and brains were postfixed in the same fixative overnight at 4 °C. Brains were cut into frozen 10-μm-thick coronal sections, non-specific staining was blocked with 10% normal serum (Vector Laboratories, Burlingame, CA, USA), and the sections were incubated with the primary antibodies, anti-p-adducin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p-MYPT1 (myosin phosphatase target subunit 1) (CycLex Co. Ltd, Nagano, Japan). Adducin and MYPT1 are specific Rho-kinase substrates,[20] and increased phosphorylation of adducin or MYPT1 is related to Rho-kinase activation. The ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) was used after sections were incubated with the appropriate biotinylated secondary antibody.

Statistical analysis. All values reported here are expressed as means ± standard deviation (s.d.). Statistical comparisons among groups were carried out using one-way analysis of variance (ANOVA) with the Bonferroni–Dunn *post-hoc* test using SPSS version 9J (SPSS Japan Inc., Tokyo, Japan). For all procedures, P-values <0.05 were considered statistically significant.

RESULTS

Effects of drug treatments on blood pressure and body temperature

In SHRs subjected to transient MCA occlusion, treatment with 1 and 10 mg kg^{-1} candesartan significantly reduced the systolic blood pressure (SBP) as early as 3 h after administration compared with vehicle, and the reduction was maintained until 21 h after treatment and was more pronounced 24 h after the second administration in a dose-dependent manner (Table 1). There was no significant difference in BP between the 0.1 mg kg^{-1} candesartan and vehicle groups. There was no difference in body temperature between the treatment groups (Table 1).

Ischemic lesions and neurological scores

In the vehicle group, 48 h after 1-h MCA occlusion, lesion areas of the brain extended to the entire MCA territory, including the cerebral cortex and caudoputamen (Figure 2a). The total lesion volume was significantly smaller in rats treated with candesartan at a dose of 0.1 mg kg^{-1} than in those treated with vehicle (109.6 ± 86.6 *vs.* 234.9 ± 59.1 mm^3; $P<0.05$) (Figures 2b and 3a). There was no difference in lesion size between rats treated with 1 or 10 mg kg^{-1} candesartan and vehicle-treated rats (Figures 2 and 3a). The

Table 1 Change of systolic blood pressures and body temperatures

Group						
Time after MCAO		*Pre*	*3 h*	*6 h*	*24 h*	*48 h*
Time after drug application	n	*Pre*	*Pre*	*3 h*	*21 h*	*45 h*
Blood pressure (mm Hg)						
Heart rate (beats per min)						
Vehicle	12	181 ± 10	184 ± 22	181 ± 12	181 ± 22	189 ± 13
		355 ± 45	372 ± 52	380 ± 43	354 ± 53	423 ± 62
Candesartan 0.1 mg kg^{-1}	12	176 ± 9	181 ± 17	167 ± 14	179 ± 12	187 ± 15
		372 ± 54	380 ± 35	393 ± 18	389 ± 59	430 ± 57
Candesartan 1 mg kg^{-1}	12	183 ± 10	191 ± 17	158 ± 12*	154 ± 15*	157 ± 11*
		373 ± 51	400 ± 46	407 ± 24	403 ± 41	407 ± 47
Candesartan 10 mg kg^{-1}	12	177 ± 8	180 ± 14	150 ± 17*	126 ± 21*	117 ± 20*
		341 ± 48	363 ± 50	407 ± 59	417 ± 44	421 ± 29
Body temperature (°C)						
Vehicle	12	37.7 ± 0.4	37.7 ± 0.3	37.9 ± 0.4	38.0 ± 0.5	37.9 ± 0.4
Candesartan 0.1 mg kg^{-1}	12	37.9 ± 0.2	38.0 ± 0.4	38.0 ± 0.4	38.2 ± 0.2	38.1 ± 0.4
Candesartan 1 mg kg^{-1}	12	37.8 ± 0.4	37.7 ± 0.5	37.9 ± 0.4	38.0 ± 0.3	37.8 ± 0.4
Candesartan 10 mg kg^{-1}	12	37.6 ± 0.3	38.0 ± 0.5	37.9 ± 0.5	38.0 ± 0.4	37.9 ± 0.4

Abbreviation: MCAO, middle cerebral artery occlusion.
Values are the mean ± s.d.
The significance of differences was determined using ANOVA followed by Bonferroni's *post hoc* tests.
*$P<0.05$ compared with the vehicle group.

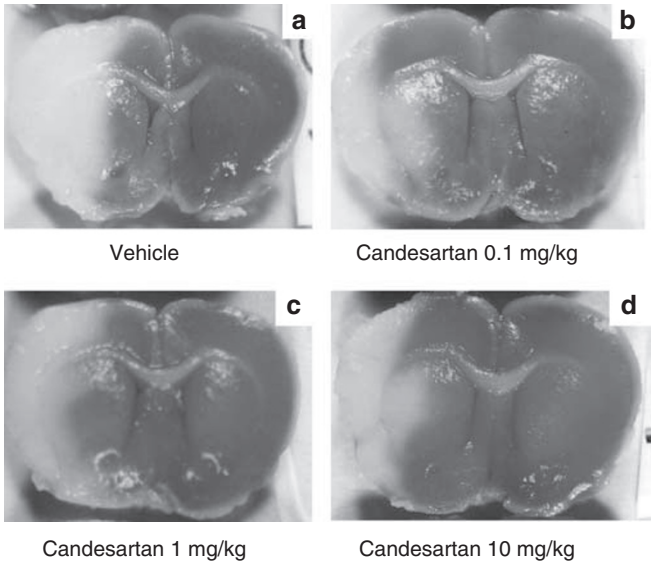

Figure 2 Representative 2,3,5-triphenyltetrazolium chloride staining of the brain 48 h after middle cerebral artery (MCA) occlusion for 1 h. In the rats treated with vehicle, infarct was visible in the entire MCA territory (**a**). However, a smaller infarct area was observed in the rats treated with 0.1 mg kg^{-1} candesartan (**b**). In the rats treated with 1 mg kg^{-1} (**c**) or 10 mg kg^{-1} (**d**) candesartan, the size of infarct was similar to that in vehicle treatment.

neurological score improved in a time-dependent manner (Figure 3b), but there were no significant differences among the treatment groups.

Change of cerebral blood flow after drug administration

MCA occlusion decreased the ipsilateral rCBF to 20% of baseline, and reperfusion recovered rCBF to baseline under anesthesia in all groups. At 3 h after MCA occlusion, the animals were fully awake, and the SBP was about 200 mm Hg. At 3 h after administration of 1 mg kg^{-1} candesartan, the SBP fell to 172 mm Hg. Ipsilateral rCBF expressed as a percent of the values before drug treatment also significantly decreased compared with the vehicle group (Figure 4). In contrast, treatment with 0.1 mg kg^{-1} candesartan showed no change in SBP and no decrease in rCBF compared with the vehicle group (Figure 4).

Suppression of Rho-kinase activity by candesartan

At 6 h after transient MCA occlusion for 1 h, phosphorylation of adducin (p-adducin) and myosin phosphatase target subunit 1 (p-MYPT1), which are specific Rho-kinase substrates, increased in brain vessels in the ipsilateral cortex (Figures 5a and b) compared with the contralateral cortex (Figures 5c and d). Treatment with 0.1 mg kg^{-1} candesartan attenuated the signal for p-adducin and p-MYPT1 in brain microvessels within the ischemic hemisphere (Figures 5e and f).

DISCUSSION

We show that oral administration of candesartan at a low dose (0.1 mg kg^{-1}) starting at 3 h after transient MCA occlusion results in a significant reduction of infarct size without decreasing BPs in SHR. Compared with controls, infarct size in rats treated with low-dose candesartan was nearly 50% smaller. However, candesartan treatment at higher doses (1 or 10 mg kg^{-1}) lowered the BP but failed to show beneficial effects on infarct size. Gohlke *et al.*[21] have shown earlier that oral administration of 0.1 mg kg^{-1} candesartan inhibited the central response to angiotensin II. This finding is in agreement with other studies showing that postischemic low-dose, but not moderate- or high-dose, administration of candesartan is neuroprotective against transient focal ischemia in both normotensive[5] and hypertensive rats.[6] Furthermore, our results show that lowering the BP shortly after reperfusion in SHR results in a decline in cerebral blood flow in the affected cortex, which could overshadow any potential beneficial

effects of ARBs. However, postischemic treatment with another ARB, olmesartan, was shown to be protective in normotensive rats at both low and high doses.[3] Future study of this treatment is required to clarify whether postischemic high-dose treatment with other ARBs is protective in hypertensive rats. In clinical studies, BP reduction during the first 24 h after stroke onset is an independent risk factor for poor outcomes after acute ischemic stroke.[22] The ACCESS study showed a reduction in mortality among patients with acute ischemic stroke who received candesartan within 48 h after onset, whereas the level of BP was similar between candesartan and placebo groups.[23] Both experimental and clinical studies are likely to show that postischemic treatment with the ARB candesartan is neuroprotective only at doses with little effect on BP. It remains unclear whether other antihypertensive drugs have similar protective effects at low doses.

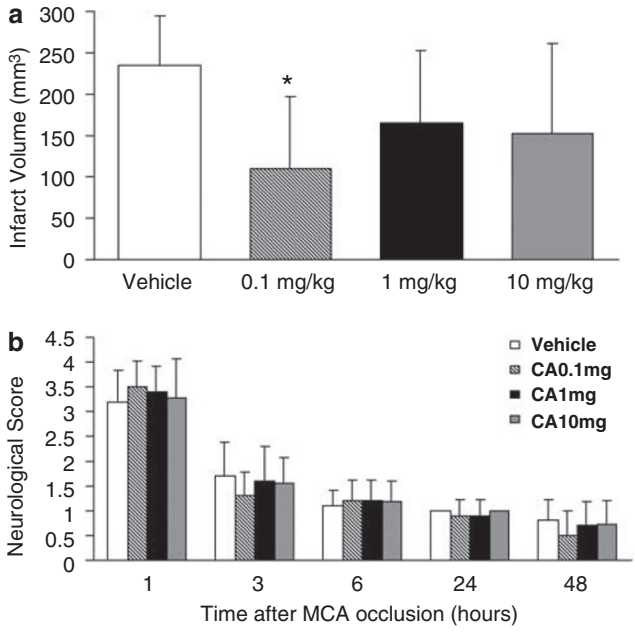

Figure 3 (**a**) Infarct volume after middle cerebral artery (MCA) occlusion. Treatment with candesartan (CA) at a dose of 0.1 mg kg^{-1} reduced the total lesion volume, but no significance was shown in the treatment groups at doses of 1 or 10 mg kg^{-1} compared with the vehicle group. (**b**) Neurological scores after MCA occlusion. Neurological findings improved in a time-dependent manner after MCA occlusion, but there was no difference among treatment groups. Values are mean ± s.d. *$P<0.05$ compared with the vehicle group.

There are several mechanisms underlying the neuroprotective effects of ARBs in the ischemic brain. Chronic treatment with ARBs before ischemia has been shown to reverse remodeling of cerebral vessels,[24,25] promote angiogenesis,[26] and attenuate the severity of ischemia after vessel occlusion. However, the effects on blood flow or vascular remodeling are unlikely to explain the beneficial effects of postischemic treatment with ARBs. Besides lowering of BP and relaxation of vascular tonus, ARBs have been shown to have potentially beneficial effects such as anti-inflammatory,[3,7–9] anti-oxidative,[10] anti-apoptotic,[11] and stimulation of angiotensin II type 2 receptors[27] in cerebral ischemia models. Here, in addition to these pleiotropic effects of ARBs, we show that activation of endothelial Rho-kinase in the ischemic brain is suppressed after treatment with candesartan. In the ischemic brain of acute stroke, brain angiotensin II is upregulated,[3] and stimulation of the AT1 receptor activates Rho-kinase.[15] Rho-kinase was identified as an effector of the small GTPase rho[28,29] and was implicated in smooth-muscle contraction, cell migration, and endothelial function.[15,30] In brain endothelial cells, activated Rho-kinase can cause damage to the endothelial cells and can also cause microcirculatory disturbances.[14] Postischemic treatment with the Rho-kinase inhibitor fasudil inhibits the expansion of cerebral infarction.[14] Our results show that administration of candesartan suppresses endothelial Rho-kinase activation. This effect may be one of the explanations for the neuroprotective effects of ARBs.

In conclusion, we show that postischemic oral administration of the ARB candesartan at doses with little effect on BP reduces infarct volume in a transient MCA occlusion model of SHR. We also show that treatment with an ARB suppresses Rho-kinase activation in brain microvessels after ischemia. The significance of the neuroprotective

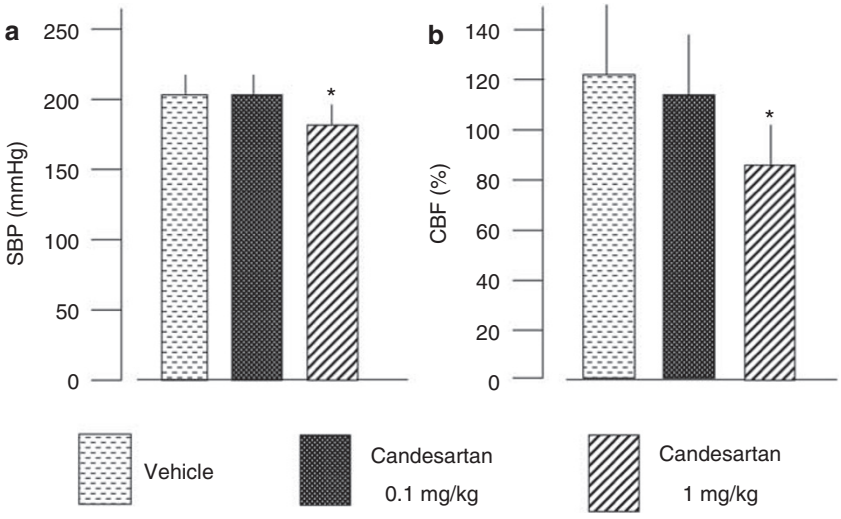

Figure 4 (**a**) Systolic blood pressure (SBP) after candesartan treatment. Administration of candesartan at 1 mg kg^{-1} lowered SBP from 200 to 172 mm Hg 3 h later, but administration of vehicle or 0.1 mg kg^{-1} candesartan did not decrease SBP. (**b**) Ipsilateral regional cerebral blood flow (rCBF) changed after candesartan administration. CBF decreased 3 h after 1 mg/kg candesartan treatment compared with that in vehicle, but treatment with 0.1 mg kg^{-1} candesartan did not reduce CBF. Values are mean ± s.d. *$P<0.05$ compared with the vehicle group.

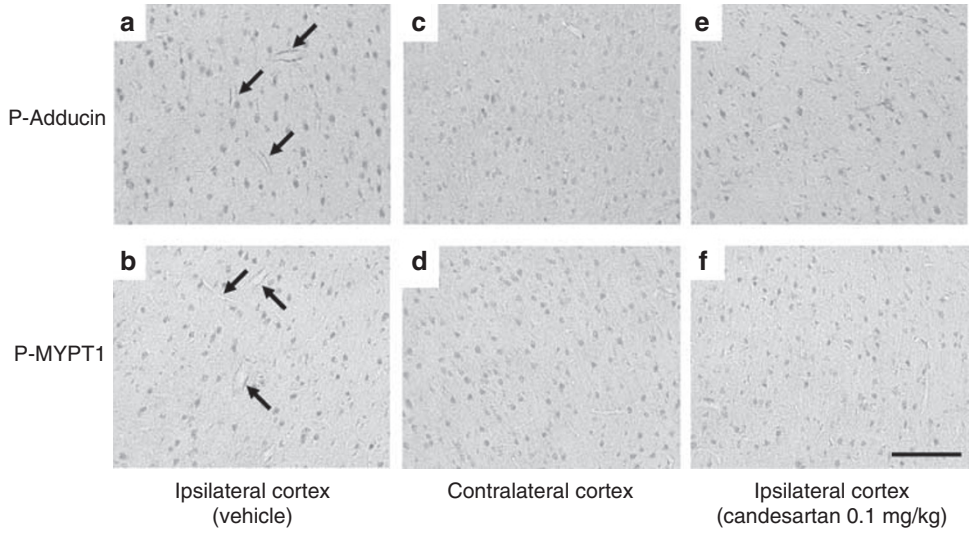

Figure 5 Suppression of Rho-kinase activity in the brain vessels of ischemic cortex by candesartan treatment. Immunohistochemistry of p-adducin (**a**, **c** and **e**) and p-myosin phosphatase target subunit 1 (MYPT1) (**b**, **d** and **f**) indicates Rho-kinase activation. At 6 h after middle cerebral artery occlusion (MCAO), the signals for p-adducin and p-MYPT1 were increased in brain vessels in ipsilateral cortex (arrows in **a** and **b**) compared with those in the contralateral cortex (**c** and **d**). Decreased staining of p-adducin (**e**) or p-MYPT1 (**f**) in the ipsilateral brain vessels of rats treated with 0.1 mg kg^{-1} candesartan indicated suppression of Rho-kinase activity.

action of the ARBs will have to be clarified in the future from both basic and clinical aspects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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