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Short communication

Neuroprotective and neurotoxic effects of cyclosporine A on transient focal ischemia in mdr1a knockout mice

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Abstract

The proper dose of cyclosporine A as a neuroprotective agent was investigated using the middle cerebral artery occlusion model of mdr1a knockout mice. After a 30-min occlusion period, reperfusion was performed and the vehicle or cyclosporine A (1 mg/kg or 10 mg/kg×2) was intraperitoneally administered to each animal model group. Forty eight hours after reperfusion, infarction volume in the 1 mg/kg cyclosporine A group was significantly less than that seen in the vehicle group, although, in the high dose cyclosporine A group, infarction volumes were significantly higher than those seen in the vehicle group. These results demonstrate that cyclosporine A shows not only anti-ischemic effects, but also neurotoxic effects depending on the dosage penetrating into the brain.

Keywords: Cyclosporine A; mdr1a; (Knockout mouse); Brain ischemia; NeuN

1. Introduction

Several reports have demonstrated that cyclosporine A confers neuroprotection and can be administered by various routes and repetition (Matsumoto et al., 1999, 2002). The blood–brain barrier is considered to be impermeable to cyclosporine A (Cefalu and Pardridge, 1985). Therefore, precise elucidation of the anti-ischemic effects of cyclosporine A remains illusive. On the contrary, in vitro studies show that cyclosporine A induces neuronal apoptosis and encephalopathy due to injury of brain capillary endothelial cells, neurons, oligodendrocytes, among other cell types (McDonald et al., 1996), and that neurotoxicity is induced by high concentrations of cyclosporine A.

P-glycoprotein, which possesses the multidrug resistance (MDR) gene, and acts as a membrane active efflux system for a variety of drugs, positively inhibits the uptake of cyclosporine A by the brain (Sakata et al., 1994). Mammalian P-glycoproteins are encoded by distinct but closely related genes; MDR1 and MDR2 in humans (Gottesman and Pastan, 1993) and mdr1a, mdr1b and mdr2 in mice (Hsu et al., 1989). Among these P-glycoproteins, human MDR1 and mouse mdr1a and mdr1b P-glycoproteins confer MDR, and are described as drug-transporting or mdr1-type P-glycoproteins. Mdr1-type P-glycoprotein is abundantly found in various tissues, such as the small intestines, adrenal gland, liver and kidney (Thiebaut et al., 1987). P-glycoprotein also localizes at the luminal membrane of brain capillary endothelial cells (Cordon-Cardo et al., 1989). Schinkel et al. (1995) showed that the absence of mdr1-type P-glycoprotein, which conducts the active efflux of several drugs, results in markedly elevated cyclosporine A levels in the brain.

This study investigates how cyclosporine A acts on focal cerebral ischemia, using mdr1a knockout mice, in which high concentrations of cyclosporine A easily pass into the brain, by assessing infarction volume.

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2. Materials and methods

2.1. Animals and preparation of transient focal cerebral ischemia

Adult male mdr1a-deficient mice (mdr1a−/−) generated by gene targeting were purchased from Taconic (Germantown, NY). All animals were allowed free access to food and water. They were anesthetized with 2% isoflurane in 67% N_2O and 33% O_2 and the body temperature was maintained by a heating pad. A 6–0 siliconized filament (PDS®II, Ethicon, NJ, USA) was inserted into the incision in the external carotid artery. The filament was advanced through the origin of the anterior cerebral artery, so as to occlude to the middle cerebral artery. After a 30-min occlusion period, reperfusion was accomplished by withdrawing the intraluminal filament from the external carotid artery. The experimental protocol was approved by the Tokyo Medical University Institutional Review Committee for the Use of Animal Subjects.

2.2. Drug administration and experimental group

Experimental animals were divided into three groups: a vehicle (Cremophore® EL, BASF, Germany; diluted 50 times with saline)-treated group (control, n=13), a 1 mg/kg cyclosporine A-treated group (low dose cyclosporine A, n=12), and a 10 mg/kg cyclosporine A-twice-treated group (high dose cyclosporine A, n=6). In the 1 mg/kg cyclosporine A-treated group, cyclosporine A (Novartis, Sweden) was administered into each animal intraperitoneally immediately after recirculation. According to our previously reported paper (Matsumoto et al., 1999), in the 10 mg/kg cyclosporine A-treated group, cyclosporine A was administered intraperitoneally twice, i.e., immediately and 24 h after recirculation.

2.3. Neurological assessment

In order to ensure successful placement of the intraluminal suture, the functional effects of ischemia were measured at 2 h after ischemia by a masked observer. We estimated the severity of the neurological deficit by using a modified four-point scale as follows: 0—no observable neurological deficits; 1—failure to extend left forepaw; 2—circling to the left or right side; 3—loss of walking ability or right reflex.

2.4. Assessment of cerebral blood flow

In order to monitor regional cerebral blood flow continuously, a laser-Doppler flowmetry probe (EG probe, Omegawave, Tokyo) was fixed to the intact skull (2 mm posterior and 5 mm lateral from the bregma) by surgical glue and connected to the accelerator (Loctite®, Takpak, Sweden). Steady-state baseline values were recorded before middle cerebral artery occlusion, and regional cerebral blood flow during and after occlusion was expressed as a percentage of the baseline values.

2.5. Immunostaining and evaluation of brain infarction

The evaluation of brain infarction was performed as described in our previously reported "NeuN" method, which selectively stains the nuclei of neuronal cells (Sakai et al., 2001). Briefly, 48 h after reperfusion, all mice were deeply anesthetized with 5% isoflurane with 67% N_2O and 33% O_2 in a box, and then their brains were fixed with 4% paraformaldehyde through the heart. Forty-micrometer coronal sections of brain were stained with a mouse monoclonal antibody against mouse neuronal nuclei (NeuN, Chemicon, CA) for identification of the infarct area.

The infract areas and normal areas were examined under a light microscope, and the infarct areas were compared with the same sections using NIH image version 1.55. Infarct volumes were calculated using three-dimensional reconstruction software as calculated in the following equation: damaged area (%)=(infarct volumes of damaged hemisphere÷volume of undamaged hemisphere)×100. Infarct volumes of damaged areas were calculated by two masked observers.

2.6. Statistical analysis

One-factor analysis of variance followed by Scheffe's test was used to compare physiological parameters among groups. Infarct sizes in treated and untreated groups were compared using Kruskal–Wallis test followed by Mann–Whitney U-test.

3. Results

During the 48-h experimental period after reperfusion, mortality rates were represented by the following ratios of dead to live mice in the vehicle group, the low dose (1 mg/kg)

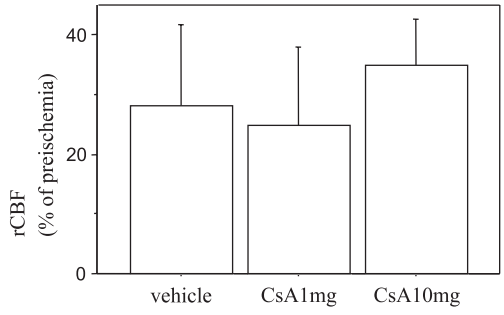

Fig. 1. Effects of transient middle cerebral artery occlusion on mean regional cerebral blood flow in mdr1a knockout mice treated with vehicle, 1-mg cyclosporine A and 10-mg cyclosporine A. Data are presented as a percentage of regional cerebral blood flow during pre-ischemia. Error bars indicate mean±S.D.

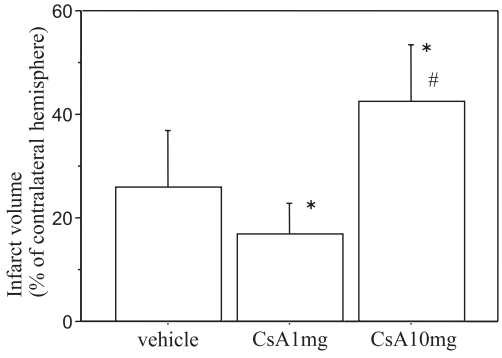

Fig. 2. Mean infarct volume of ischemic damage in mdr1a knockout mice treated with vehicle, 1-mg cyclosporine A and 10-mg cyclosporine A, at 48 h after 30-min middle cerebral artery occlusion. Data are presented as a percentage of the contralateral hemisphere. Error bars indicate mean±S.D. Asterisks indicate significant differences compared to vehicle group ($P<0.05$). Sharp indicates significant differences compared to 1-mg cyclosporine A group ($P<0.05$).

cyclosporine A group and the high dose (10 mg/kg) cyclosporine A group: 3:13, 2:12 and 1:6, respectively. These findings were not significantly different. All mice exhibited at least grade 1 deficit after middle cerebral artery occlusion, indicating successful placement of the intraluminal suture. Rectal temperature of each animal was monitored before, during and after ischemia for 48 h. No significant differences in rectal temperature among groups were observed (data not shown).

Fig. 1 shows the regional cerebral blood flow of each group during the surgical period. Regional cerebral blood flow in all animals decreased to 25–35% of the baseline immediately after middle cerebral artery occlusion. The decreased regional cerebral blood flow continued for 30 min during middle cerebral artery occlusion. After reperfusion, regional cerebral blood flow immediately increased to 60–120% of the baseline value. There were no significant differences during or after ischemia among groups.

After 48 h of reperfusion, infarct areas basically developed in the striatum of both vehicle and low dose cyclosporine A groups. In the high dose cyclosporine A group, however, developed infarctions were seen in the cortex and striatum. Fig. 2 shows the calculated infarct sizes. The mean infarction volumes were 26%, 16% and 40% for vehicle animals, low dose cyclosporine A animals and high dose cyclosporine A animals, respectively. Infarction volumes in the low dose cyclosporine A group were significantly less than those seen in the vehicle group ($P<0.05$). In the high dose cyclosporine A group, infarction volumes were significantly higher than those seen in the vehicle and low dose cyclosporine A groups ($P<0.05$).

4. Discussion

In the present study, we used mdr1a knockout mice, since the central effects of cyclosporine A on brain ischemia can be better evaluated in the absence of a cyclosporine A-related efflux pump in the blood–brain barrier.

The mdr1a knockout mouse was developed by Schinkel et al. (1995) and the functions of the blood–brain barrier are partially impaired in this model. Schinkel et al. (1995) also found that the mdr1a gene produces the major p-glycoprotein in the blood–brain barrier, and that its absence in knockout mice results in elevated drug levels in many tissues, especially in the brain. For instance, the levels of radiolabeled cyclosporine A in the brain of such knockout mice are 50-fold higher than those of wild-type mice, and these levels in knockout mice are increased earlier and sustained longer than those of wild-type mice. The anti-ischemic effects of cyclosporine A, and at what dosages, can be shown using this mdr1a knockout mouse model. In fact, we confirmed that the infarction volumes in the knockout mice group with 1 mg/kg of cyclosporine A was significantly smaller than those seen in the vehicle-treated group, although this dose is not expected to exhibit the significant neuroprotective effects in middle cerebral artery occlusion and other ischemic animal models (Matsumoto et al., 1999, 2002). These results show that 1 mg/kg cyclosporine A does have an anti-ischemic effect in cases of transient focal cerebral ischemia, when cyclosporine A passes through the blood–brain barrier. In contrast, however, we also detected a neurotoxic effect of cyclosporine A in the present study when mice were treated with two administrations of 10 mg/kg cyclosporine A. As shown in our previously reported paper, this double treatment revealed the expected anti-ischemic effects on cerebral ischemia without any toxic effects in brain ischemia rats (Matsumoto et al., 1999). Additionally, in both cases, the same dosage of cyclosporine A was intraperitoneally administered immediately after recirculation in both rats and mdr1a knockout mice, and after 48 h of recirculation the effects of cyclosporine A on cerebral ischemia were examined. These results suggest that cyclosporine A induces dose-dependent anti-ischemic effects in brain ischemia with intact blood–brain barrier function, and that cyclosporine A has neurotoxic effects depending on the dose of cyclosporine A in the brain. In addition, it may be assumed that larger dosages of cyclosporine A induce severe damage to neuronal cells of intact mdr1a knock out mice, as seen in the renal cells and neuronal cells of intact animals, and as evaluated in previous papers by us and other investigators (Gerkens, 1989; Shuto et al., 1999).

The usefulness of this cerebral ischemia model in knockout mice was demonstrated in our earlier report (Sakai et al., 2001). Although the occlusion time of 30 min in the present experiment was shorter than the time used in other reports, damage to the brain was appropriate for estimating brain ischemia compared to other ischemic brain models in mice (Connolly et al., 1996; Kitagawa et al., 1998). Moreover, NeuN staining has been shown to be a very specific and sensitive pathological method for detecting neuronal nuclei (Mullen et al., 1992). Therefore, this staining method can

clearly detect even mild damage to brain areas induced by short-term brain ischemia, i.e., 30 min.

In conclusion, cyclosporine A shows not only anti-ischemic effects, but also neurotoxic effects, depending on the dosage penetrating into the brain, and is potentially beneficial for the treatment of brain ischemia. These results indicate that cyclosporine A has a "double-edged" effect, which requires further investigation for a more precise understanding of brain ischemia.

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