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Full-length article

Caffeic acid ameliorates early and delayed brain injuries after focal cerebral ischemia in rats[1]

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Key words

caffeic acid; focal cerebral ischemia; neuron; astrocyte; 5-lipoxygenase; leukotriene

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Abstract

Aim: To investigate the effects of caffeic acid on early and delayed injuries after focal cerebral ischemia in rats, and the possible relation to 5-lipoxygenase inhibition. **Methods**: Transient focal cerebral ischemia was induced by middle cerebral artery occlusion in Sprague-Dawley rats. Caffeic acid (10 and 50 mg/kg) was ip injected for 5 d after ischemia. The brain injuries were observed, and the levels of cysteinyl leukotrienes and leukotriene B_4 in the brain tissue were measured. **Results:** Caffeic acid (50 mg/kg) ameliorated neurological dysfunction and neuron loss, and decreased infarct volume 24 h after ischemia; it attenuated brain atrophy, infarct volume, and particularly astrocyte proliferation 14 d after ischemia. In addition, it reduced the production of leukotrienes (5-lipoxygenase metabolites) in the ischemic hemispheres 3 h and 7 d after ischemia. **Conclusion:** Caffeic acid has protective effect on both early and delayed injuries after focal cerebral ischemia in rats; and this effect may partly relate to 5-lipoxygenase inhibition.

Introduction

Caffeic acid (3,4-dihydroxycinnamic acid) is one of the natural phenolic compounds widely distributed in plant materials such as vegetables, fruits, coffee and tea[1–3]. As a potent antioxidant[4,5], caffeic acid exerts anti-inflammatory effects[6]. One of caffeic acid derivatives, caffeic acid phenethyl ester (CAPE), suppressed cerebral lipid peroxidation[7] and reduced brain infarct[8] after cerebral ischemia in rats. In contrast, caffeic acid is an inhibitor of 5-lipoxygenase (5-LOX)[9,10] and can inhibit the biosynthesis of pro-inflammatory leukotrienes. We have recently found that 5-LOX is activated after oxygen-glucose deprivation (OGD)-induced *in vitro* ischemic injury and caffeic acid attenuated this injury in PC12 cells[11] and cultured rat cortical neurons[12]. However, whether caffeic acid affects brain injury via inhibiting 5-LOX activity after focal cerebral ischemia *in vivo* is unknown.

Ischemic brain injury can be separated into three serial phases: metabolic stress and excitotoxicity (acute, minutes to hours), inflammation and apoptosis (subacute, hours to days), and repair and regeneration (chronic, days to months)[13,14]. Neuron injury, including necrosis and apoptosis, is the main lesion in the acute or subacute phase; while one of the chronic changes after cerebral ischemia or other brain injuries is the formation of a glial scar that results from reactive gliosis (mainly consisting of proliferated astrocytes)[15,16]. During the chronic phase of cerebral ischemia, gliosis (astrogliosis) is detected in the infarct boundary[17], which may be a physical and biochemical barrier for the regeneration of axons[15]. Whether caffeic acid has an effect on brain ischemia in acute and subacute/chronic phases is still unclear.

In the present study we evaluate the effects of caffeic acid on injuries after focal cerebral ischemia in rats, and determined the relation to 5-LOX inhibition. We defined that injury occurs at 24 h after ischemia as the early injury (including acute and subacute phases), and at 14 d as the delayed injury (including subacute and chronic phases).

Materials and methods

Measurements of physiological variables Male Sprague-Dawley rats weighing 250–300 g (Experimental Animal Center, Zhejiang Academy of Medical Sciences) were used in this

study. Rats were housed under a controlled temperature (22±1 °C), 12 h light/12 h dark cycle and allowed free access to food and water. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Rats were anaesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). A polyethylene tube was inserted into the right femoral artery for continuous monitoring of blood pressure using a computer-assisted system (PC-Lab, Kelong, Nanjing, China) and for measuring Pao_2, $Paco_2$, and arterial blood pH (Blood Gas Analyzer ABL 330, Leidu, Copenhagen, Denmark). Blood glucose was monitored by one touch basic blood glucose monitoring system (Lifescan, Los Angeles, CA, USA). Rectal (core) temperature was monitored and maintained at 37.0±0.5 °C with a heating pad and a heating lamp during the surgery. Percent changes in regional cerebral blood flow (rCBF) over the middle cerebral arterial (MCA) territory (2 mm in diameter; 6 mm lateral and 2 mm caudal to bregma) were recorded as in a previous study[18] using a laser Doppler flowmeter (ML191, AD Instruments, New South Wales, Australia), as the steady state baseline of rCBF value before ischemia was defined to be 100%.

Focal cerebral ischemia Transient focal cerebral ischemia was induced by a modified method of middle cerebral artery occlusion (MCAO)[19] according to a previously reported method[20]. Briefly, after anesthesia, a midline incision was made in the neck, the right external carotid artery (ECA) and the right internal carotid artery (ICA) were carefully exposed and dissected, and a 3-0 monofilament nylon suture was inserted from the ECA into the ICA to occlude the origin of the right MCA. Thirty minutes after occlusion, the suture was withdrawn to allow reperfusion, the ECA was ligated and the incision was closed. Sham-operated rats underwent identical surgery, except for inserting the intraluminal filament. Achievement of MCAO was confirmed by a reduction of 50% or more in rCBF to the baseline value[21]. After surgery, rats were kept for approximately 2 h in a warm box heated by lamps to maintain their body temperature.

Caffeic acid administration Caffeic acid (Aldrich-Sigma, Saint Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO); the solution was freshly diluted with saline to final concentrations of 10 mg/mL and 50 mg/mL before use. Caffeic acid (10 and 50 mg/kg, 0.1 mL per 100 g bodyweight)[22] or 20% (*v*/*v*) DMSO-saline solution was injected intraperitoneally 30 min before MCAO and 0, 1, 2 h after reperfusion in the first day, and twice daily in the 2nd to 5th day. For evaluating the early injury 24 h after reperfusion, caffeic acid was injected only for 1 d; for evaluating the effect on rCBF, it was only injected 30 min before and immediately after MCAO.

Neurological examination Neurological deficit scores were evaluated 24 h and 14 d after MCAO, according to a reported method[23], as follows: 0, no deficit; 1, flexion of contralateral forelimb upon lifting of the whole animal by the tail; 2, decrease of thrust toward contralateral plane; 3, circling to the contralateral side. An inclined board test was performed to assess balance and coordination[24] based on modification of a previously reported method[25]. Rats were placed on a board (50 cm×30 cm); once they stayed stably the board was inclined from horizontal to vertical. The degree at which the animal fell from the board was recorded. The test was repeated three times and the average degree was used.

Histological examination Rats were anesthetized after neurological examination and perfused transcardially with 4% paraformaldehyde after pre-washing with saline. Brains were removed and photographed by a digital camera (FinePix S602 Zoom, Fuji, Japan). The surface area of ipsilateral (ischemic) or contralateral hemisphere was calculated and compared using an image analysis program (AnalyPower 1.0, Zhejiang University, Hangzhou, China). Then, six serial coronal sections (20 μm) at 2 mm intervals from the frontal to the occipital poles were cut by cryomicrotomy (CM1900, Leica, Germany) for gross photographic examination after being stained with 1% toluidine blue. The infarct area and hemisphere area were measured, and the infarct volume and ipsilateral/contralateral hemisphere ratio were calculated as reported[24].

Simultaneously, 12-μm coronal sections (1.8–2.0 mm caudal from bregma) were cut for histological examination. Apparently survival neurons in similar regions of temporoparietal cortex III, IV layers were counted in five randomly selected fields of the sections stained with 1% toluidine blue by an image analysis program (AnalyPower1.0, Zhejiang University, Hangzhou, China). Astrocytes were stained with a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP), a specific marker of astrocyte (1:200, Zhongshan Biotechnology, Beijing, China). Brain sections were sequentially incubated overnight at 4 °C with the antibody against GFAP, biotinylated goat anti-rabbit IgG (1:200), and with horseradish peroxidase streptavidin (1:200, Zhongshan Biotechnology, Beijing, China) for 2 h; finally visualized using diaminobenzidine (DAB Kit, Zhongshan Biotechnology). Negative control sections were treated by identical procedure, except that the primary antibodies were omitted. The stained sections were observed by light microscopy. To evaluate astrocyte proliferation, the optic density of GFAP staining in an area of 1 mm×1 mm in the boundary zone adjacent

to ischemic core was compared to that in the same location of contralateral non-ischemic cortex. The percentage increase was calculated.

Leukotriene measurement Brain samples were removed from ischemic or contralateral non-ischemic cortex at 3 h and 7 d after reperfusion (which were the peak times for leukotriene production as indicated in our previous study), and prepared according to a previously reported method[26]. The tissue was freshly homogenized in 100% ethanol. After centrifugation of the homogenates at 15 000×*g*, 4 °C for 30 min, the supernatant was collected and applied to a 0.2-μm filtrator. The filtrate was dried under nitrogen and resuspended with an enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI, USA). Tissue concentrations of cysteinyl leukotrienes (CysLTs, including LTC_4, LTD_4, and LTE_4) and leukotriene B_4 (LTB_4) were measured using an enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's instructions. All measurements were carried out in duplicate, and calculated as pg/g wet tissue.

Statistical analysis Values are expressed as mean±SD. Statistical analyses were performed using one-way ANOVA according to the experimental design, followed by Bonferroni test (Prism 4 for windows, 2003, GraphPad Software , San Diego, CA, USA). $P<0.05$ was considered to be statistically significant.

Results

Physiological variables Mean blood pressure, arterial blood pH, Pa_{O_2}, Pa_{CO_2}, and glucose were not changed before and after the surgery, and there was no differences between sham operation, ischemia and caffeic acid groups. However, in all the ischemic rats, rCBF of the MCA territory was reduced by approximately 50%–60% during 30-min MCAO, and recovered to nearly baseline levels 15 min after reperfusion. Treatment with caffeic acid (50 mg/kg) did not alter rCBF reduction and recovery (Table 1). No rats died of ischemic injury in all groups 24 h after MCAO, whereas 20% of rats in each ischemic group died (2/10 rats) within 14 d after MCAO but no sham-operated rats died.

Ischemic injury Neurological deficit scores were maximal at 24 h, and disappeared 14 d after ischemia. Similarly, holding angle in the inclined board test significantly decreased 24 h then recovered 14 d after ischemia. Treatment with caffeic acid 50 mg/kg, not 10 mg/kg, for 5 d significantly reduced neurological deficit scores and increased holding angle 24 h after ischemia (Figure 1).

The gross photographs of whole brains showed the changes in the surface area (Figure 2A), and the coronal slices stained with toluidine blue showed the infarct tissue (Figure 2B) in the ischemic hemispheres 24 h and 14 d after ischemia. The area of brain surface was significantly in-

Table 1. Summary of selected physiological parameters before and after surgery. Mean±SD. $^cP<0.01$ *vs* sham operation, one-way ANOVA followed by the Bonferroni test.

Valuables		Sham operation	Ischemia	Caffeic acid
		n=6	*n*=6	*n*=6
MABP (mmHg)	Baseline	121±19	118±17	122±21
	30 min after reperfusion	117±16	116±20	127±22
pH	Baseline	7.37±0.03	7.38±0.06	7.37±0.05
	30 min after reperfusion	7.36±0.03	7.36±0.05	7.36±0.03
Pa_{O_2} (mmHg)	Baseline	90±11	93±12	94±14
	30 min after reperfusion	88±14	90±16	89±19
Pa_{CO_2} (mmHg)	Baseline	41.8±2.5	40.9±3.5	43.0±3.2
	30 min after reperfusion	42.4±3.8	42.1±4.1	44.1±5.0
Glucose (g/L)	Baseline	6.28±1.82	6.31±1.67	6.11±1.28
	30 min after reperfusion	6.19±1.33	6.16±0.89	6.21±1.47
		n=4	*n*=4	*n*=4
rCBF (%)	Baseline	100	100	100
	5 min after ischemia	103.1±1.5	49.3±6.8^c	44.5±9.5^c
	30 min after ischemia	98.5±1.8	48.8±7.8^c	39.6±10.9^c
	15 min after reperfusion	97.5±1.7	97.0±5.7	97.7±12.3

MABP, mean arterial blood pressure.

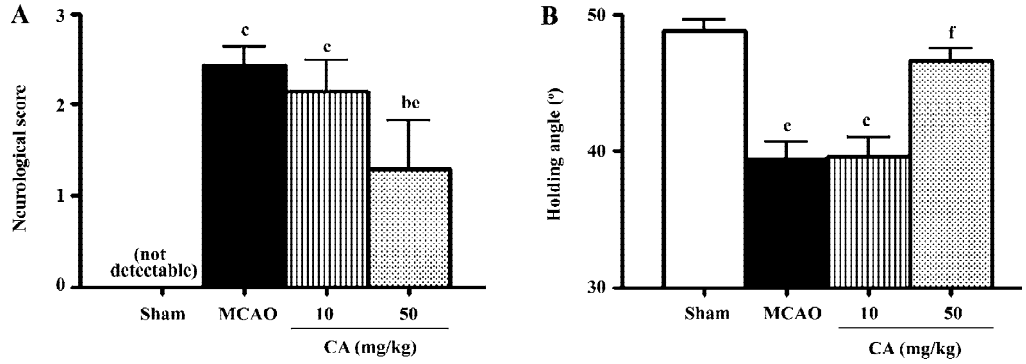

Figure 1. Effects of caffeic acid on neurological dysfunction 24 h after ischemia/reperfusion in rats. Neurological deficit score (A) and holding angle in inclined board test (B) were determined 24 h after MCAO. Caffeic acid (CA, 10 and 50 mg/kg, ip for 5 d) reduced neurological deficit score and increased holding angle. Mean±SD. n=8. ^{b}P<0.05 and ^{c}P<0.01 *vs* sham operation; ^{e}P<0.05 and ^{f}P<0.01 *vs* MCAO control, one-way ANOVA followed by the Bonferroni test.

creased 24 h indicating brain edema, and reduced in the ischemic hemisphere 14 d after ischemia indicating brain atrophy. The brain atrophy was primarily localized in cortical and subcortical regions of the ipsilateral hemisphere as indicated in brain sections (Figure 2B). Infarct volume was obviously increased 24 h and 14 d after reperfusion. Caffeic acid (50 mg/kg) did not significantly inhibit the changes in hemisphere ratio but reduced infarct volume 24 h after reperfusion, whereas it ameliorated both the changes 14 d after reperfusion (Figure 2C and 2D).

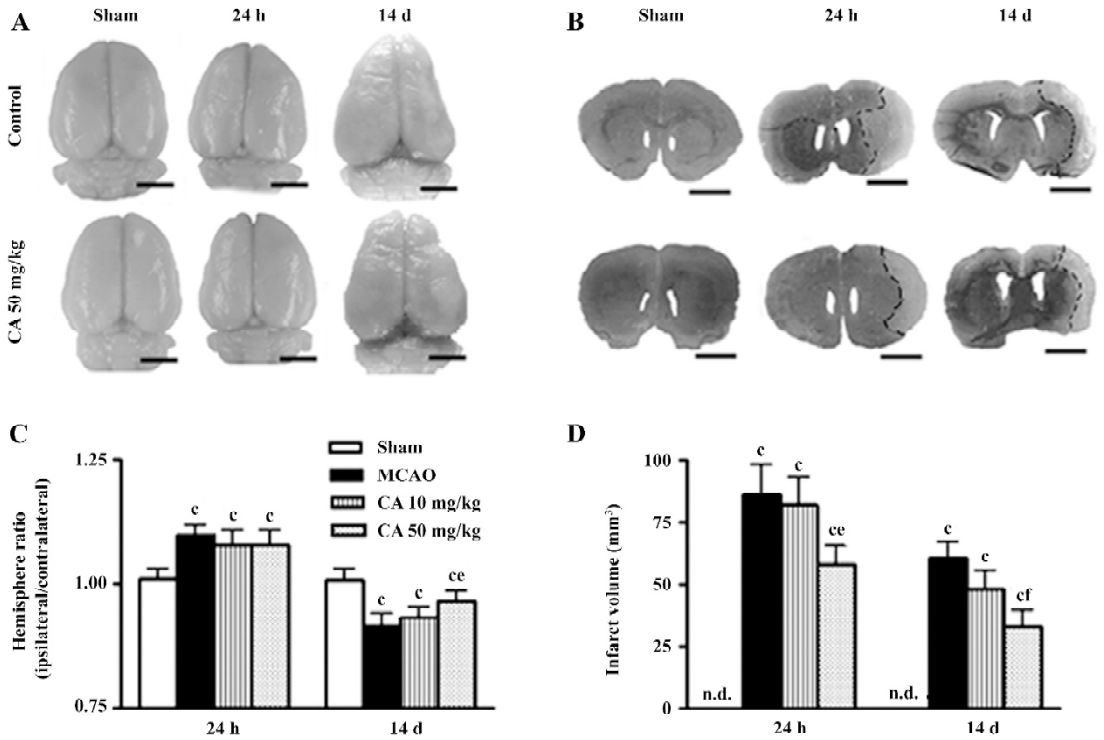

Figure 2. Effects of caffeic acid on the changes of brain surface area and infarct volume after ischemia/reperfusion in rats. Gross photographs of whole brains (A) were used to detect surface areas of both hemispheres and ipsilateral/contralateral hemisphere ratio (C); 20-mm thick coronal slices stained with 1% toluidine blue (B) were used to calculate infarct volume (D). Caffeic acid (CA, 50 mg/kg) did not alter hemisphere ratio (C) but reduced infarct volume (D) 24 h after MCAO; whereas it attenuated both the changes 14 d after MCAO. Mean±SD. n=8. ^{c}P<0.01 *vs* sham operation; ^{e}P<0.05 and ^{f}P<0.01 *vs* MCAO control, one-way ANOVA followed by the Bonferroni test. Bars=5 mm.

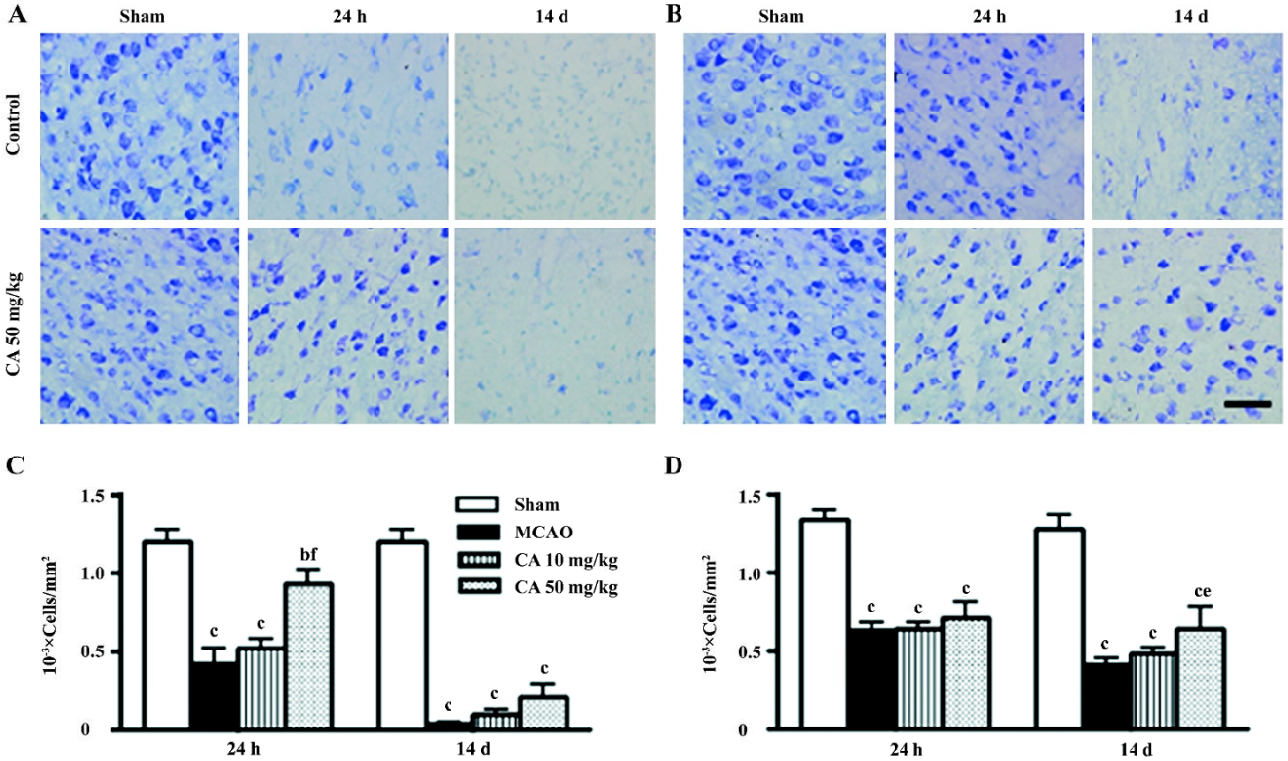

Figure 3. Effects of caffeic acid on the changes in neuron density after ischemia/reperfusion in rats. Representative photographs show the densities of apparently survival neurons in the ischemic core (A) and the boundary zone (B). Neuron densities were reduced in the ischemic core and the boundary zone 24 h and 14 d after reperfusion. Caffeic acid (CA, 50 mg/kg) attenuated the neuron loss in the ischemic core 24 h, but not 14 d, after reperfusion (C); conversely it attenuated neuron loss in the boundary zone 14 d, but not at 24 h, after reperfusion (D). Mean±SD. n=8 rats for each group. $^{c}P<0.01$ *vs* sham operation; $^{e}P<0.05$ and $^{f}P<0.01$ *vs* MCAO control, one-way ANOVA followed by the Bonferroni test. Bar=50 μm.

In the ischemic core, the density of the apparently surviving neurons was significantly reduced 24 h, and almost disappeared 14 d after ischemia (Figure 3A). In the boundary zone adjacent to the ischemic core, the neurons were reduced by approximately 60%–70% 24 h and 14 d after reperfusion (Figure 3B). Caffeic acid (50 mg/kg) attenuated neuron loss in the ischemic core, but not in the boundary zone, 24 h after ischemia; however, it did not reverse neuron loss in the ischemic core but attenuated neuron loss in the boundary zone 14 d after ischemia (Figure 3C and 3D). On the other hand, in the ischemic core, GFAP-positive astrocytes were increased at 24 h, but almost disappeared 14 d after reperfusion (data not shown). However, in the boundary zone, the number of GFAP-positive astrocytes was greatly increased and the intense astrocytic fibers surrounded the ischemic core 14 d after ischemia (Figure 4A). The astrocyte proliferation was partially inhibited by caffeic acid (50 mg/kg, Figure 4B).

5-LOX enzymatic activity To evaluate whether the effects of caffeic acid is mediated by inhibiting 5-LOX enzymatic activity, we measured the levels of CysLTs and LTB_4 in the ischemic cortex 3 h and 7 d after reperfusion, the peak time points for leukotriene production. Caffeic acid administered for 5 d reduced the levels of both CysLTs and LTB_4 at 3 h, but only reduced the level of CysLTs at 7 d after reperfusion (Figure 5).

Discussion

In the present study, we found that caffeic acid attenuated both early and delayed ischemic injuries, and also inhibited enzymatic activation of 5-LOX as reducing the production of the metabolites, CysLTs and LTB_4, 3 h and 7 d after ischemia. Its neuroprotective effect on early injury is consistent with previously published reports[7,8,27], but our findings further indicate its effect on the delayed ischemic injury. The effects of caffeic acid on 5-LOX activation confirms such an effect in PC12 cells or neurons after *in vitro* ischemic injury[11,12].

In the early injury after focal cerebral ischemia, caffeic

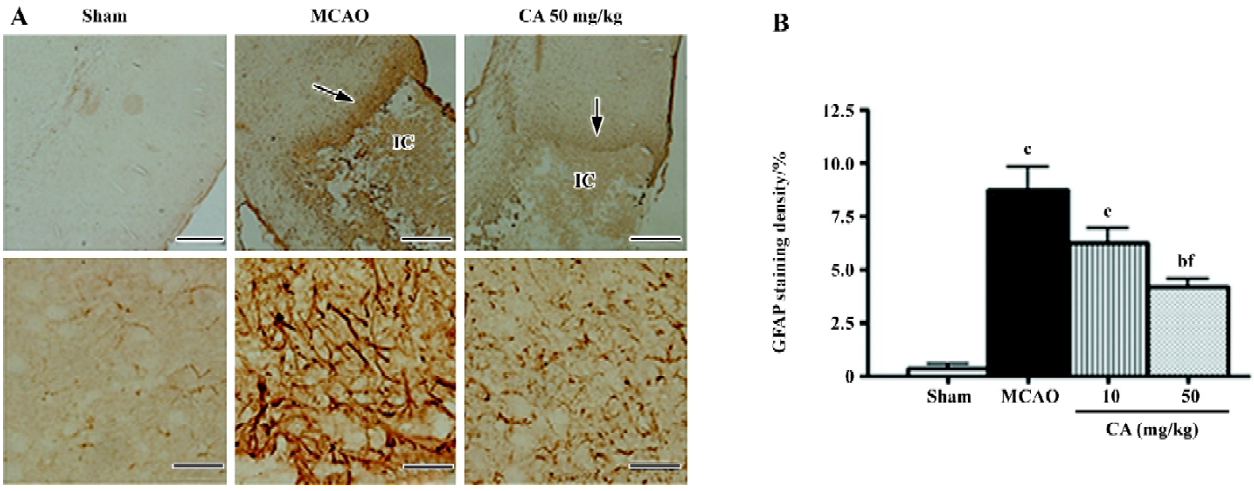

Figure 4. Effects of caffeic acid on the changes in astrocytes 14 d after ischemia/reperfusion in rats. Representative photographs show glial fibrillary acidic protein (GFAP)-immunopositive astrocytes in the ischemic hemispheres (A) 14 d after reperfusion. Intense proliferated astrocytes are localized in the boundary zone (upper panels in A), and the amplifications show the hypertrophed astrocytes (lower panels in A). Caffeic acid (CA, 50 mg/kg) inhibited astrocyte proliferation and reduced GFAP staining density (B). Mean±SD. n=8 rats for each group. ^{b}P <0.05 and ^{c}P<0.01 *vs* sham operation; ^{f}P<0.01 *vs* MCAO control, one-way ANOVA followed by the Bonferroni test. Bars in A=50 μm (upper panels) or 200 μm (lower panels). IC, ischemic core.

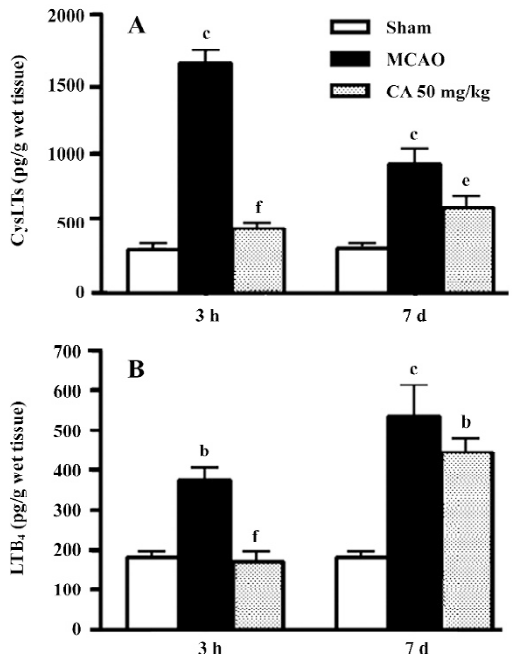

Figure 5. Effects of caffeic acid on the levels of cysteinyl leukotrienes (CysLTs) and leukotriene B_4 (LTB_4) in the ischemic cortices after ischemia/reperfusion in rats. Brain samples were obtained from the ischemic cortex 3 h and 7 d after reperfusion. Caffeic acid (CA, 50 mg/kg) reduced the levels of CysLTs and LTB_4 3 h, but only reduced the level of CysLTs 7 d after reperfusion. Mean±SD. n=4 rats for each group. ^{b}P<0.05 and ^{c}P<0.01 *vs* sham operation; ^{e}P<0.05 and ^{f}P <0.01 *vs* MCAO control, one-way ANOVA followed by the Bonferroni test.

acid attenuated neurological symptoms, the neuron loss in the ischemic core and the infarct volume, but not the brain edema. Because inflammation is one of the events occurring in early ischemic injury, pro-inflammatory mediators play an important role[13]. Among the mediators, the production of 5-LOX metabolites, CysLTs and LTB_4, is increased after cerebral ischemia[28–31]. Our present result showed that caffeic acid inhibited the production of both CysLTs and LTB_4 in the brain 3 h after ischemia (a peak time as indicated in our recent study), which indicated the inhibition of the enzymatic activity of 5-LOX by caffeic acid. This finding suggests that 5-LOX inhibition may be one of the mechanisms of its neuroprotective effect on early ischemic injury. However, the potent antioxidant effect of caffeic acid may be another explanation for its effect against acute ischemic injury. Caffeic acid and its derivative CAPE can scavenge oxygen radicals *in vitro*[5] and inhibit the reactive oxygen species (ROS) production in rats[32]. However, in the present study caffeic acid did not inhibit the brain edema in early injury although most ischemia injuries were ameliorated. This incomplete protection may be because post-ischemic brain edema is determined by a complex system and caffeic acid only inhibits parts of the system; for example, caffeic acid does not inhibit prostaglandin production even at higher concentrations[9].

The most important finding in the present study is that caffeic acid primarily inhibits astrocyte proliferation in

addition to other delayed injuries (14 d after ischemia). We found that caffeic acid inhibited brain atrophy, brain infarct volume and neuron loss in the boundary zone. Whereas caffeic acid did not recover the neuron loss in the ischemic core, indicating that it only postponed neuron death in this region because an amelioration of neuron loss was observed here in the early injury. Because neurological dysfunction (neurological deficit scores and holding angles in the inclined board test) was already recovered 3–5 days after transient ischemia in the present study, no change was observed 14 d after ischemia. Furthermore, caffeic acid significantly inhibited the proliferated astrocytes in the boundary zone, suggesting a beneficial effect on delayed injury after cerebral ischemia. Because 5-d administration of caffeic acid also inhibited the production of 5-LOX metabolites, CysLTs but not LTB_4, 7 d after ischemia (a second peak time as indicated in our recent study), the effects of caffeic acid might also relate to 5-LOX activation. As supporting evidence, we found that 5-LOX expression was increased in the boundary zone accompanied with astrocyte proliferation 7–14 d after focal cerebral ischemia (Zhou Y, *et al* 2006; unpublished observations). Moreover, the 5-LOX metabolites, CysLTs, mediate astrocyte proliferation via activating $CysLT_1$ receptor (one of the receptors for CysLTs) as shown in the cultured astrocytes[26]. We recently found that $CysLT_1$ receptor antagonist pranlukast inhibits glial scar formation in the chronic phase of cerebral ischemia[24], supporting the role of 5-LOX in astrocyte proliferation. However, the antioxidant effect of caffeic acid may be another mechanism. Since cerebral ischemic injury is a temporally continuous process[13], inhibition of oxidant stress in early injury may reasonably attenuate the secondarily delayed injuries like astrocyte proliferation and glial scar formation. Until now, few effective agents on post-ischemic astrocyte proliferation are reported, although heparin oligosaccharides or phosphodiesterase inhibitor rolipram inhibited glial scar formation after traumatic brain or spinal cord injury[33,34], and A_{2A} adenosine receptor antagonists, dexamethasone and isoproterenol inhibited astrocyte proliferation *in vitro*[35,36]. Therefore, the effect of caffeic acid may represent a novel approach for ameliorating astrocyte proliferation and glial scar formation in the ischemic brain.

In conclusion, the present study shows that caffeic acid ameliorates both early and delayed injuries, especially the astrocyte proliferation, after focal cerebral ischemia in rats; one mechanism of the neuroprotection may be the inhibition of 5-LOX. Our findings indicate that caffeic acid may be a prototype compound of neuroprotective agents against cerebral ischemia injury; however its effective dose is relatively larger so further investigations will be necessary to search its derivatives and fully clarify the properties and mechanisms.

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