

Inhibition of Excitatory Amino Acid Efflux Contributes to Protective Effects of Puerarin Against Cerebral Ischemia in Rats¹

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Objective To investigate whether the protective effects of puerarine (Pur) against cerebral ischemia is associated with depressing the extracellular levels of amino acid transmitters in brain of rats. **Methods** Male Sprague-Dawley rats were subjected to transient middle cerebral artery occlusion (MCAO) for 60 min followed by 24 h reperfusion. Pur (50, 100 mg/kg, *i.p.*) was administered at the onset of MCAO. The infarct rate and edema rate were detected on TTC (2,3,5-triphenyltetrazolium chloride)-stained coronal sections. The extracellular levels of amino acid transmitters were monitored in striatum of rats with ischemic/reperfusion injury using *in vivo* microdialysis technique. Furthermore, the protective effects of Pur against glutamate-induced neurotoxicity were detected. Glutamate-induced apoptotic and necrotic cells in hippocampus were estimated by flow cytometric analysis of Annexin-V and PI labeling cells. **Results** Pur (100 mg/kg) significantly decreased infarct size by 31.6% ($P<0.05$), reduced edema volume ($P<0.05$), and improved neurological functions ($P<0.05$) following MCAO. In these rats, the ischemia-induced extracellular levels of aspartate (Asp), glutamate (Glu), γ -aminobutyric acid (GABA), and taurine (Tau) were significantly reduced in striatum of vehicle-treated animals by 54.7%, 56.7%, 75.8%, and 68.1% ($P<0.01$ and $P<0.05$). Pur reduced the peak values of Glu and Asp more obviously than those of GABA and Tau, and the rate of Glu/GABA during MCAO markedly decreased in Pur-treated MCAO rats, compared with the vehicle-treated MCAO rats. Meanwhile, apoptosis and necrosis induced by Glu in cultured hippocampal neurons were significantly reduced after Pur treatment. **Conclusion** Acute treatment with Pur at the onset of occlusion significantly depresses ischemia-induced efflux of amino acids, especially, excitotoxicity in the striatum, a mechanism underlying the neuroprotective effect on cellular survival.

Key words: Cerebral ischemia; Puerarin; Microdialysis; Amino acid transmitter; Neuroprotection

INTRODUCTION

In cerebral ischemia, the severe reduction of blood flow initiates a complex series of interconnected pathophysiological processes, including marked reduction in energy metabolism, loss of ionic homeostasis, excessive release of neurotransmitters, and advancement of injury cascades. Microdialysis studies have confirmed the release of various excitatory amino acids (EAA) in the extracellular space in rats subjected to middle cerebral artery occlusion (MCAO)^[1]. The release of EAA is thought to play an important role in the pathogenesis of neuronal damage and death in acute cerebral ischemia. In particular, it has been postulated that the overflow in extracellular glutamate and aspartate underlies an excitotoxic mechanism for

neuronal damage^[2].

Puerarin (Pur), 8-C-C-glucopyranosyl-4'-7-dihydroxyisoflavone, is an active component extracted from the dried root of *Pueraria lobata* (Wild) Ohwi, a traditional Chinese herbal medicine. A number of investigations showed that Pur attenuates injury caused by cerebral ischemia and arterial obstruction of retina^[3-4]. However, the cellular and molecular mechanisms underlying the protective effects of Pur are currently unclear. Recent studies showed that Pur possesses protective effects against cerebral cortical neuron and astrocyte damage induced by oxygen-glucose deprivation or glutamate excitotoxicity *in vitro*^[5-6]. However, there is little information about the *in vivo* effects of Pur on the amino acid efflux during different phases of stroke (occlusion and reperfusion). Combined with

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intracerebral microdialysis, the MCAO model was employed in the present study to investigate the acute effects of Pur on the temporal modifications in amino acid transmitters during ischemic and reperfusion periods.

MATERIALS AND METHODS

Preparation of Middle Cerebral Artery Occlusion (MCAO) and Reperfusion Model

All animal handling and surgery were performed in accordance with the Care Standard of the Laboratory Animals (China Ministry of Health publication, 1998). Male Sprague-Dawley rats, weighing 300-350 g, were from Zhejiang Academy of Medical Sciences. The rats were subjected to MCAO as described by Zea-Longa *et al.*^[7] with modifications. Briefly, after anaesthetized with chloral hydrate (400 mg/kg, *ip*), a midline incision was made, the right common carotid artery (CCA) and the external carotid artery (ECA) as well as the internal carotid artery (ICA) were exposed. The distal ECA was coagulated completely. The CCA and ICA were temporarily clamped with microvascular clips. A monofilament nylon filament (diameter 0.234 mm) with a blunted tip was introduced into the ECA lumen. The filament was then gently advanced to the distal ICA until it reached the clipped position. After the microvascular clip was removed, the filament was inserted until resistance was felt, which ensured the occlusion of the origin of the middle cerebral artery. The distance between the CCA bifurcation and the resistive point was about 18.5-19.5 mm. During the entire stroke procedure, rectal temperature was kept at approximately 37.0°C-37.5°C with a heating pad and light. The filament was withdrawn from the ICA after 60 min to allow MCA reperfusion.

Drug Treatment

The rats were randomized into drug- and vehicle-treated groups ($n=6$ in each group). Drug-treated groups: MCAO for 60 min followed by reperfusion, and treatment with puerarin (50, 100 mg/kg, *ip*; 98.5% Pur provided by Conba Pharmaceutical Co., China) or MK801 (dizocilpine, 3 mg/kg, *ip*; Sigma) at the onset of MCAO. The dose of drugs used in the present experiment was selected based on our preliminary experiments. Both drugs were dissolved in 10% methyl glycol. Vehicle-treated group: MCAO for 60 min followed by reperfusion, and treatment with vehicle at the onset of MCAO. MK801 is a non-competitive NMDA receptor antagonist. There is evidence showing the effectiveness of MK801 in focal models of cerebral

ischemia^[8].

Measurement of Neurological Function, Edema Ratio, and Infarct Size

Neurological deficits were evaluated in all MCAO animals 24 h following reperfusion. Rats were scored on a four-point scale (0-4) described by Zea-Longa *et al.*^[7] (0=no deficit, 1=failure to extend left forepaw fully, 2=circling to the left, 3=falling to the left, 4=no spontaneous walking with a depressed level of consciousness).

For infarct volume measurement, brains were isolated 24 h after MCAO, and sectioned into six coronal sections with 2-mm thickness from the frontal tip. The sections were stained with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC), and fixed with 10% phosphate-buffered formalin. Infarct areas of all the sections were calculated using image analysis software. The total infarct area was multiplied by thickness of brain sections to get infarct volume. Volumes of ipsilateral and contralateral hemispheres were calculated. Edema correction for infarct volume was done using the formula: corrected infarct volume=(infarct volume \times contralateral hemisphere volume)/ipsilateral hemisphere volume. The percentage of corrected infarct (infarct ratio) was calculated by dividing the corrected infarct volume by the total volume of the bilateral hemisphere. Edema volume was obtained by subtracting the contralateral volume from the ipsilateral volume. Edema ratio was then obtained by dividing the edema volume by the contralateral volume.

Intracerebral Cannula Guide of Microdialysis Placement

The rats were anaesthetized with chloral hydrate (400 mg/kg, *ip*). An intracerebral cannula guide (BAS MD-2251, USA) was implanted into the right striatum. The stereotaxic coordinates used for implantation were 0.2 mm anterior and 3.5 mm lateral to the bregma and 5.8 mm ventral from the brain surface^[9]. The cannula guide was fixed to the skull with anchor screws and acrylic dental cement. After operation, rats were allowed to recover for 3 days.

Microdialysis Procedure

A microdialysis probe (2 mm membrane, BAS, MD-2200, USA) was inserted through the cannula guide into the right striatum of rats under chloral hydrate (400 mg/kg, *ip*) anaesthesia. Artificial cerebrospinal fluid (composition in mmol/L: 126 NaCl, 27.5 NaHCO₃, 2.4 KCl, 5 KH₂PO₄, 5 Na₂HPO₄, 0.5 Na₂SO₄, 0.82 MgCl₂·6H₂O, 1.1 CaCl₂·2H₂O, 5

glucose, pH 7.4) was continuously perfused at a rate of 2 $\mu\text{L}/\text{min}$ by a microdialytic pump (BAS, MD-1001, USA). Following an equilibration period of 120 min, the basal sample was collected. After the second basal sample was collected, surgery for MCAO was performed. Pur (100 mg/kg, *ip*) was administered at the onset of MCAO. This dose of Pur was selected because the results of neurological function, edema ratio, and infarct size demonstrated that 100 mg/kg Pur was an optimal anti-infarction dose in rats. The microdialytic samples were continuously collected every 15 min into an ice-cold tube, from 30 min before MCAO to 120 min after reperfusion. The samples were immediately frozen and kept at -40°C until analysis. Anesthesia was maintained with an additional dose of 20 mg/kg chloral hydrate every 2 h during experiment.

Amino Acid Transmitters Detection

The concentrations of aspartate (Asp), glutamate (Glu), γ -aminobutyric acid (GABA), and taurine (Tau) in the microdialytic samples were determined by high performance liquid chromatography (HPLC) using a fluorescence detector (RF-10AXL, Shimadzu, Japan) after precolumn derivatization with OPA- β -mercaptoethanol^[10-11]. The mobile phases were (A) 0.1 mol/L KH_2PO_4 buffer (pH 6.6): methanol=65:35 (v/v) and (B) 0.1 mol/L KH_2PO_4 buffer (pH 6.6): methanol=10:90 (v/v). All HPLC chemicals were obtained from Sigma. The two-buffer HPLC system (Shimadzu-10AVP, Japan) was coupled to a fluorescent detector.

Separation was achieved on a C18 column (Hypersil, BDS, 5 μm , 4.0×200 mm). Twenty μL of the reaction mixture was injected onto the column and separated with a gradient from 0% B to 40% B within 12 min, then eluted with 100% B for 5 min to elute other components. The flow rate was 1.0 mL/min (E_X : 357 nm; E_M : 455 nm). Amino acid transmitter levels in the samples were evaluated by converting peak area units into μmol using an external standard calibration curve.

Histological Observations

At the end of each experiment, the rat was sacrificed with an overdose of urethane. The brain was dissected, cut into 60- μm thick sections and stained. The location of the microdialysis probe was histologically verified.

Primary Hippocampal Cell Culture and Drug Treatment

Primary hippocampal cultures were prepared from 2 day-old Sprague-Dawley rats as described by

Isaev *et al.* with modifications^[12]. Briefly, the tissue was digested with 0.25% trypsin in phosphate-buffered saline (PBS) at 37°C for 20 min, followed by mechanical dissociation. Hippocampal cells were seeded in poly-L-lysine-coated plates (120 000 cells/ cm^2) and grown in neurobasal medium with B-27 serum-free supplement (Gibco), 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 2 mmol/L L-glutamine. The cultures were maintained at 36.5°C in an incubator containing 5% CO_2 , 95% air. The medium was changed starting from day 4 *in vitro* by replacing half of the medium twice a week. Serum-free hippocampal cultures were utilized for the experiments after 9 days *in vitro*.

On day 9, glutamate (Glu, 0.1, 0.5, 1.0 mmol/L) was added into the cultures for 30 min. After Glu treatment, the cultures were replaced into neurobasal medium and maintained in a CO_2 -incubator for 24 h. The percentages of cell apoptosis and necrosis were calculated. Based on the results that 0.5 mmol/L Glu obviously damaged hippocampal neurons, we investigated the protective effects of Pur against neurotoxicity induced by 0.5 mmol/L Glu. Pur (40, 100 $\mu\text{mol}/\text{L}$), or MK801 (10 $\mu\text{mol}/\text{L}$) was added into the cultures during Glu (0.5 mmol/L) treatment and for an additional 24 h.

Flow Cytometry Studies

Apoptotic detection kit (Caltag Laboratories, USA) was used to distinguish necrotic from apoptotic cells. As an early event of apoptosis, cells translocate phosphatidylserine from the inner site of their plasma membrane to the outer surface while the membrane remains physically intact^[13]. Early apoptotic cells therefore were stained with annexinV-FITC which binds to phosphatidylserine, and necrotic cells were detected by use of propidium iodide (PI), a DNA binding dye excluded from cells with intact plasma membranes. The cells which were neither apoptotic nor necrotic were not stained with either dye as previously described^[14].

For flow cytometric analysis, cells were harvested and washed with Ca^{2+} - and Mg^{2+} -free PBS. After centrifugation at 1500 rpm for 5 min, the pellet was resuspended in $1\times$ binding buffer at a density of 1×10^6 cells/mL. Cells (100 μL) were transferred to a culture tube, and 5 μL of annexin V-FITC and 10 μL of PI were added. After gentle vortex, the tube was incubated for 15 min at room temperature (20°C - 25°C) in the dark, and 400 μL of $1\times$ binding buffer was then added into it. The sample was analyzed using flow cytometer (FACSort, Becton Dickinson) as soon as possible (within 1 hour). The percentages of apoptotic and necrotic cells of each sample were

estimated.

Statistical Analysis

All results were expressed as $\bar{x} \pm s$. For neurological deficits Kruskal-Wallis one-way analysis of variance on ranks was performed. The other results were compared using Student's *t*-test following one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Infarct Size, Edema Ratio, and Neurological Score of MCAO Rats

Sixty min of MCAO and 24 h of reperfusion resulted in significant infarct (27.5%) as shown in

TTC-stained coronal brain sections (Fig. 1). Neurological deficit score of 2.5 ± 0.5 was observed in the vehicle-treated MCAO rats (Table 1). Pur treatment at 100 mg/kg significantly decreased the total infarct volume by 31.6% ($P < 0.05$), compared with the vehicle-treated MCAO rats (Fig. 1). Meanwhile, Pur (100 mg/kg) treatment produced obvious improvement in neurological functions ($P < 0.05$) and 22.6% reduction in edema volume ($P < 0.05$, Table 1). The lower dose of Pur (50 mg/kg) treatment mildly reduced infarct size, which was not statistically significant different from the vehicle-treated group. MK801 significantly decreased infarct size by 36.4% ($P < 0.05$) and edema volume by 28.4% ($P < 0.05$), and improved neurological functions ($P < 0.01$), compared with the vehicle-treated MCAO rats.

TABLE 1

Edema and Neurological Scores in Vehicle- and Puerarin-treated MCAO Rats ($\bar{x} \pm s$)

Parameters	n	Vehicle	Puerarin		MK801 3 mg/kg
			50 mg/kg	100 mg/kg	
Edema (%)	6	19.0 ± 2.9	17.5 ± 2.2	14.7 ± 1.8*	13.6 ± 1.8*
Neurological Score	6	2.5 ± 0.5	1.8 ± 0.3	1.5 ± 0.5*	1.3 ± 0.4**

Note. Pur (100 mg/kg) treatment significantly decreased edema rate and neurological score. **The value differs from the control (vehicle) at $P < 0.01$ or * $P < 0.05$.

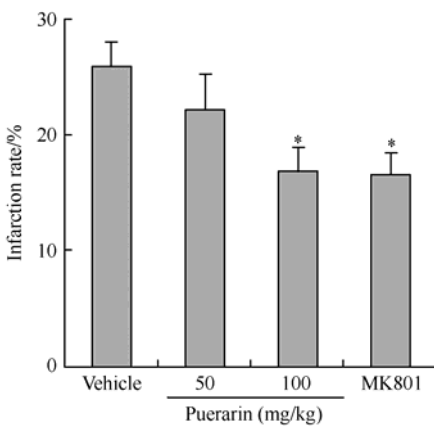


FIG. 1. Evaluation of total infarct rate after transient focal cerebral ischemia in puerarin- and vehicle-treated groups. The percentage of infarct (infarct ratio) was calculated by dividing the corrected infarct volume by the total volume of the bilateral hemisphere. Data represent $\bar{x} \pm s$, $n = 6$. *The value differs from the control (vehicle) at $P < 0.05$.

Amino Acid Efflux in MCAO Rats

The basal levels of amino acids in the striatum

dialysate liquid of the vehicle-treated animals before MCAO were: Asp, $0.893 \pm 0.172 \mu\text{mol/L}$; Glu, $2.405 \pm 0.509 \mu\text{mol/L}$; GABA, $0.677 \pm 0.11 \mu\text{mol/L}$; and Tau, $5.18 \pm 0.952 \mu\text{mol/L}$. A 60-min period of ischemia resulted in significant increases in striatum dialysate levels of Asp, Glu, GABA, and Tau. The maximal percentage increases observed during ischemia period were: Asp, 799%; Glu, 1315%; GABA, 822%; and Tau, 383%. The levels of these amino acids subsequently declined towards their pre-ischemic values during the 120-min reperfusion period (Fig. 2).

Basal levels of amino acids in the Pur-treated animals were comparable (Fig. 2) and not affected by the administration of Pur. However, ischemia-evoked effluxes of Asp, Glu, GABA, and Tau were depressed in the Pur-treated animals. The maximal values of Asp, Glu, GABA, and Tau declined to 54.7%, 56.7%, 75.8%, and 68.1% in the vehicle-treated animals, respectively ($P < 0.01$, $P < 0.05$). Pur reduced the peak values of Glu and Asp more remarkably than those of GABA and Tau. The maximal rate of Glu/GABA was 5.51% in the vehicle-treated animals, and reduced to 4.26% in the Pur-treated animals during MCAO.

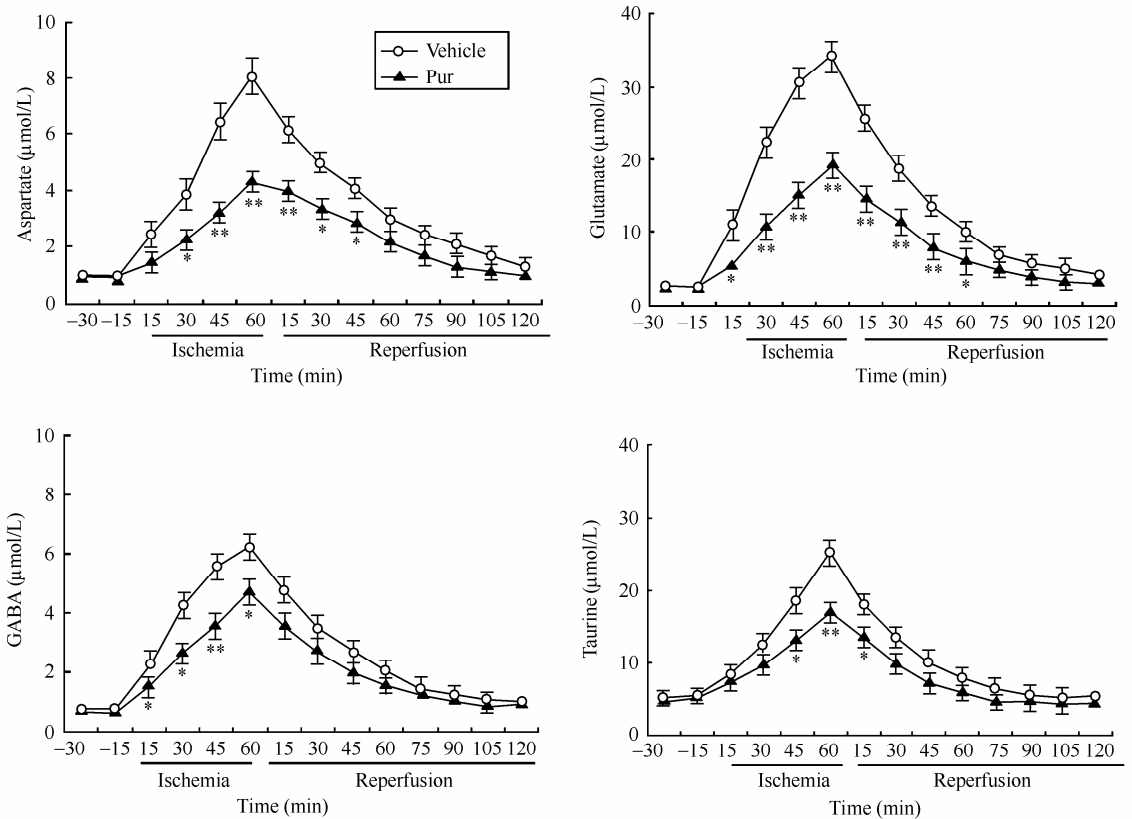


FIG. 2. Effects of puerarin (100 mg/kg, *ip*) on ischemia/reperfusion-evoked amino acid efflux in striatum. Line plots show the concentrations ($\mu\text{mol/L}$) of amino acids before, during and after 60 min of MCAO. Puerarin was administered at the onset of MCAO. The data represent $\bar{x} \pm s$, $n=6$. **The value differs from the control (vehicle) at $P < 0.01$ or * $P < 0.05$ for puerarin.

Damage of Hippocampal Cultures Induced by Excitotoxicity of Glu

In the culture system, dead cells in hippocampus were estimated by flow cytometric analysis of annexin-V and PI labeling cells. Exposure of hippocampal neurons to Glu for 30 min resulted in a dose-dependent cell damage on the next day. Compared with the control, there was no obvious injury in hippocampal neurons by 0.1 mmol/L Glu, while 0.5, 1.0 mmol/L Glu significantly promoted cell apoptosis by 134% and 262%, and increased necrosis by 112% and 358%, respectively (Fig. 3). In the presence of 100 $\mu\text{mol/L}$ Pur, cell death induced by 0.5 mmol/L Glu was dramatically suppressed, and the percentages of apoptosis and necrosis were reduced by 33.1% and 35% ($P < 0.01$ and $P < 0.05$), compared with the vehicle-treated cultures. Likewise, MK801 also significantly decreased the percentages of apoptosis and necrosis in the Glu-treated hippocampal cultures. The lower concentration of Pur (40 $\mu\text{mol/L}$) exhibited mild effects on both cell apoptosis and necrosis ($P > 0.05$) (Fig. 4).

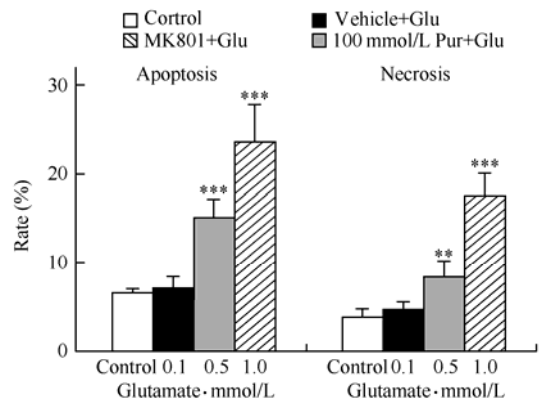


FIG. 3. Dose-dependent neurotoxic effects of L-glutamate on rat primary hippocampal neurons. After 8 days *in vitro*, rat hippocampal neurons were exposed to vehicle or 0.1, 0.5, 1.0 mmol/L L-glutamate (Glu) for 30 min. Apoptosis and necrosis were determined with annexin V and PI 24 h after treatment with Glu. Data are expressed as $\bar{x} \pm s$ from six different experiments. ***The value differs from the control (vehicle) at $P < 0.001$ and ** $P < 0.01$.

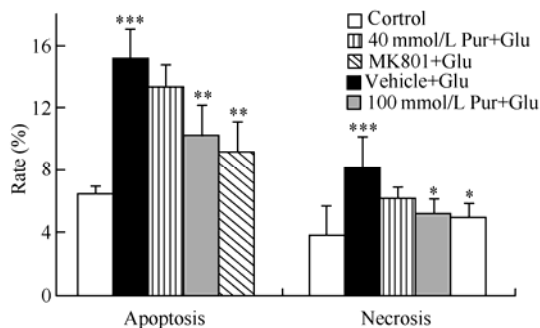


FIG. 4. The percentage of apoptosis or necrosis in cultured hippocampal neurons of puerarin- and vehicle-treated groups during and after 0.5 mmol/L L-glutamate (Glu) treatment. Apoptosis and necrosis were determined with annexin V and PI 24 h after treatment with Glu. $n=6$. Data represent $\bar{x} \pm s$. ***The value differs from the control at $P<0.001$; **The value differs from the vehicle-treated Glu group at $P<0.01$ or * $P<0.05$.

DISCUSSION

Puerarin (Pur), one of the puerarine isoflavones, is a primary component of most functional extracts from *Pueraria lobata*. It has been reported that Pur increases cerebral blood flow in dogs^[14] and attenuates cerebral or spinal cord injury resulting from ischemia/reperfusion in rats or rabbits^[4,15]. In this study, Pur (100 mg/kg) treatment decreased the infarct size. Meanwhile, the edema volume was reduced, and the neurological functions were improved after Pur treatment.

Evaluation of extracellular levels of amino acids play a role in the pathogenesis of stroke^[1]. In order to clarify the mechanism by which Pur reduces infarction size after transient focal cerebral ischemia, in the current study, microdialysis and allied techniques were used for continuous sampling and analysis of amino acids in the extracellular fluid of striatum. These techniques also provide dynamic information about amino acids in the brain during MCAO and after reperfusion. The elevated levels of Asp, Glu, GABA, and Tau obtained in this study during cerebral ischemia are consistent with other reports^[1]. However the ischemia-evoked efflux of these amino acids was reduced after Pur treatment, compared with the vehicle-treated MCAO animals. As amino acid efflux into the extracellular space is considered to be indicative of ischemic injury, these results suggest that Pur protects the striatum against ischemia/reperfusion injury.

MK801, a non-competitive NMDA receptor antagonist, has neuroprotective effects on cerebral ischemia. The effects of the drug are attributed to

inhibition of glutamate-mediated excitotoxicity and intracellular Ca^{2+} overload. Our study has confirmed that MK801 not only reduces infarct size induced by MCAO but also inhibits glutamate neurotoxicity in hippocampal cultures. Meanwhile, Pur possesses protective effects against hippocampal neuron injury induced by glutamate *in vitro*, as shown by flow cytometric analysis of cell apoptosis and necrosis. Dong *et al.*^[5] reported that Pur reduces astrocytic swelling and LDH leakage *in vitro* induced by oxygen-glucose deprivation or sodium glutamate, suggesting that Pur possesses the protective effects as potent as MK801 against cerebral ischemic damage, and these effects are correlated to the action of excitatory amino acid transmitter system, including inhibition of the EEA efflux and attenuation of excitotoxicity.

The elevated levels of excitatory amino acids (EEA) are thought to contribute to ischemia-evoked neuron injury and death. Increased extracellular Glu and Asp levels may be due to the initial calcium-dependent exocytosis followed by a reduced uptake and later release caused by the reversal of Glu/Asp transporters^[16], contributing to excitotoxic mechanisms. Recovery of amino acids to pre-ischemic levels requires their uptake by high affinity Na^+ -dependent transporters, operating in their normal mode, following restoration of energy metabolism, cell resting potentials and ionic gradients^[17]. Therefore the possible mechanisms can explain the protective effects of Pur against ischemia/reperfusion-induced cerebral injury. Firstly, Pur may act on the glial cells, reducing the release of Glu and Asp by inhibiting the activity of Na^+ -dependent amino acid transporters operating in a reversed mode and the opening of swelling-induced chloride channels in the plasma membrane, and enhancing the removal of amino acids, especially Glu and Asp, from the extracellular space by high affinity Na^+ -dependent transporters operating in their normal mode, because there are various ion channels and ion transporters in the plasma membrane of astrocytes^[5]. It was reported that Pur inhibits sodium current in rat dorsal root ganglion neurons^[18]. Secondly, Pur can attenuate free radical generation, which promotes release of Glu in the presynaptic sites during cerebral ischemia. It has been found that Pur protects against myocardial reperfusion injury^[3]. A previous study showed that Pur acts as a radical scavenger^[19], which attenuates oxidative stress after ischemia reperfusion. Finally, Pur attenuates the neurotoxicity resulting from EEA. The effects of Pur may be associated with inhibition of NMDA receptor activity induced by the EEA efflux in neurons.

In the present study Pur not only reduced

extracellular levels of both excitatory and inhibitory amino acids in the striatum of rats during ischemia. Furthermore, attenuation of the EEA efflux was more significant than that of the inhibitory amino acids efflux by pur. It was reported that the increased extracellular levels of GABA and Tau during ischemia are beneficial for attenuating both ischemic brain damage and excitotoxicity induced by EAA in the brain. GABA inhibites Glu release *via* pre-synaptic receptors and attenuates neuronal injury^[20]. Tau has been shown to reduce calcium influx in ischemia and antagonize the calcium overload induced by Glu or hypoxia in the hippocampal neurons^[21]. Therefore, the effects of Pur on reducing the rate of excitatory/inhibitory amino acids protect against neuron death in the brain during ischemia/reperfusion.

In conclusion, Pur protects against ischemia-induced damage by depressing ischemia-evoked amino acid efflux and attenuating neurotoxicity of EEA.

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