A Dissertation for the Degree of Doctor of Philosophy

The therapeutic effects of protease-defective S478A of tissue plasminogen activator and Pyruvate in rat brain ischemia model

랫드 뇌 허혈성 모델에서 프로테아제 결손
조직플라스미노겐 활성인자와 피루브산의 치료효과

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August, 2014

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The therapeutic effects of protease-defective S478A of tissue plasminogen activator and Pyruvate in rat brain ischemia model

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A Dissertation submitted to the Faculty of the Graduate School of Seoul National University
In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

April, 2014

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Abstract

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Despite intensive investigation over the last couple of decades, there is no standard parenchymal neuroprotective treatment available for ischemic stroke. So, we find to possible agent for ischemic stroke treatment. The purpose of this study was to examine the therapeutic effects of candidate agents in rat brain ischemia model.

At the first, we examined parenchymal effects of tPA and its non-protease mutant S478A-tPA in permanent focal cerebral ischemia in rats. Before doing *in vivo* experiments, effects of tPA and S478A-tPA on zinc or NMDA toxicity were first studied in cortical cultures. Like tPA, which has protease-independent cytoprotective effects, the non-protease mutant S478A-tPA blocked zinc toxicity in cortical cell cultures, but did not affect calcium-mediated NMDA toxicity. Then, effects of tPA and S478A-tPA on infarcts induced by permanent occlusion of middle cerebral artery (MCA) were investigated. tPA and S478A-tPA were administered into the cerebral ventricle 15 min or 1 h after MCA occlusion. Both tPA and its non-protease mutant S478A-tPA, when given 15 min after ischemia, substantially reduced infarcts and

ameliorated motor deficits in the MCA occlusion model of focal cerebral ischemia. However, when administered 1 h after MCA occlusion, neither showed protective effects. The protective effects of tPA or S478A-tPA remained unchanged at 7 days after MCA occlusion.

At the second, we examined the therapeutic effects of pyruvate in focal cerebral ischemia in rats. Pyruvate markedly reduces neuronal death following transient global ischemia. In the present study, we investigated the possible neuroprotective effect of pyruvate in focal ischemia. Pyruvate (62.5–250 mg/kg) treatment, regardless of whether given intraperitoneally (ip) or intravenously (iv), decreased infarct volume by more than 50% in both transient (1 h) and permanent occlusion models. The infarct reducing effects of pyruvate were maintained for 14 days after MCAO. Interestingly, higher doses failed to reduce the infarct size. Pyruvate administration also reduced motor deficits. Magnetic resonance (MR) spectroscopy revealed that protective doses of pyruvate, but not the non-protective doses, were associated with a reduction in the level of lactate compared with saline controls. Diffusion-weighted MR images further confirmed infarct reduction in pyruvate-treated rats.

Since S478A-tPA lacks protease activity, which has been implicated in causing cerebral hemorrhage or aggravating excitotoxicity, its parenchymal neuroprotective effect may be useful in treatment of ischemic stroke. In addition, pyruvate is an endogenous metabolite of glycolysis, and hence is unlikely to have serious side effects. The combined use of a neuroprotective agent would enhance neuron survival and thereby extend the window of opportunity for thrombolytic treatment to potentially benefit more patients.

Keywords : Stroke, Rat brain ischemia, Tissue plasminogen activator, Pyruvate, Neuroprotection, Magnetic resonance

Student Number : 2008-30472

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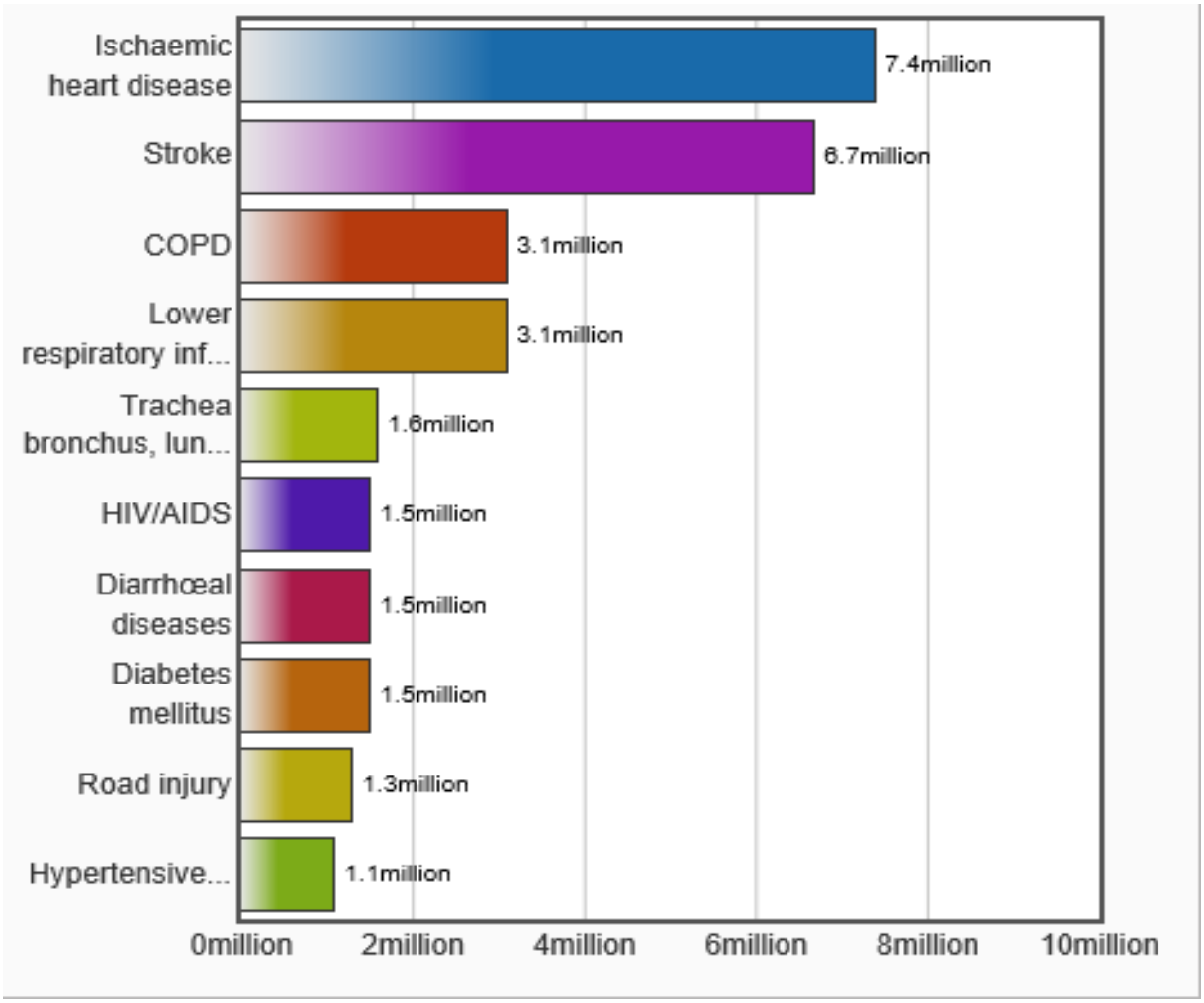
Chapter 1. General Introduction

1. The occurrence of stroke

Stroke was the second most frequent cause of death worldwide in 2011, accounting for 6.2 million deaths (~11% of the total). Approximately 17 million people had a stroke in 2010 and 33 million people have previously had a stroke and were still alive (Feigin et al., 2013).

A stroke is the loss of brain function due to a disturbance in the blood supply to the brain. This disturbance is due to either ischemia (lack of blood flow) or hemorrhage (Sims and Muyderman., 2009). Ischemia is caused by either blockage of a blood vessel via thrombosis or arterial embolism, or by cerebral hypoperfusion. Hemorrhagic stroke is caused by bleeding of blood vessels of the brain, either directly into the brain parenchyma or into the subarachnoid space surrounding brain tissue (Donnan et al., 2008).

A stroke is a medical emergency and can cause permanent neurological damage or death. Risk factors for stroke include old age, high blood pressure, previous stroke or transient ischemic attack (TIA), diabetes, high cholesterol, tobacco smoking and atrial fibrillation (Donnan et al., 2008). Ischemic stroke occurs because of a loss of blood supply to part of the brain, initiating the ischemic cascade (Deb et al., 2010). Brain tissue ceases to function if deprived of oxygen for more than 60 to 90 seconds, and after approximately 3 h will suffer irreversible injury possibly leading to death of the tissue.



Images from WHO. 2014.

Fig. 1. The 10 leading causes of death in the world 2012.

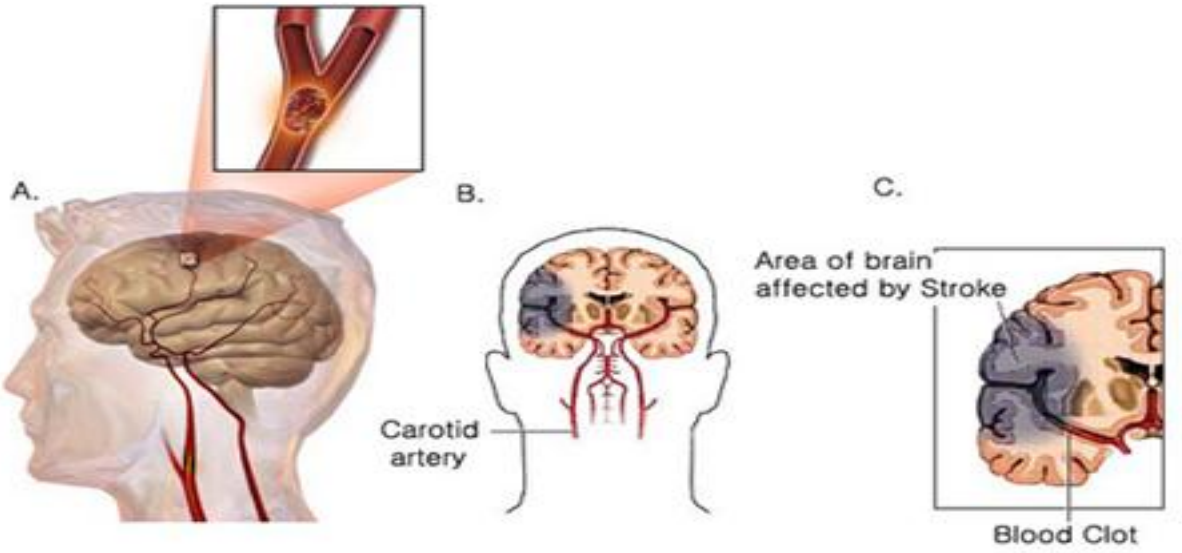


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Fig. 2. Illustration of stroke. (A) A blockage lodged in a blood vessel. (B, C) Area of brain attected by stroke.

2. Medical uses of tissue plasminogen activator

Tissue plasminogen activator (tPA) is a protein involved in the breakdown of blood clots. It is a serine protease found on endothelial cells, the cells that line the blood vessels. As an enzyme, it catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. Because it works on the clotting system, tPA is used in clinical medicine to treat embolic or thrombotic stroke. Use is contraindicated in hemorrhagic stroke and head trauma. tPA may be manufactured using recombinant biotechnology techniques. tPA created this way may be referred to as recombinant tissue plasminogen activator (rtPA). tPA is used in some cases of diseases that feature blood clots, such as pulmonary embolism, myocardial infarction, and stroke, in a medical treatment called thrombolysis. The most common use is for ischemic stroke. It can either be administered systemically, in the case of acute myocardial infarction, acute ischemic stroke, and most cases of acute massive pulmonary embolism, or administered through an arterial catheter directly to the site of occlusion in the case of peripheral arterial thrombi and thrombi in the proximal deep veins of the leg (Fugate and Rabinstein, 2014).

There is significant debate regarding recombinant tPA's effectiveness in ischemic stroke. There have been twelve relevant, large scale, high-quality trials of rtPA in acute ischemic stroke. A prominent meta-analysis of these trials concluded that rtPA given within 6 h of a stroke significantly increased the odds

of being alive and independent at final follow-up, particularly in patients treated within 3 h. However, there was an excess of mortality in treated patients in the first week after the event, mostly from intracranial haemorrhage (Wardlaw et al., 2012).

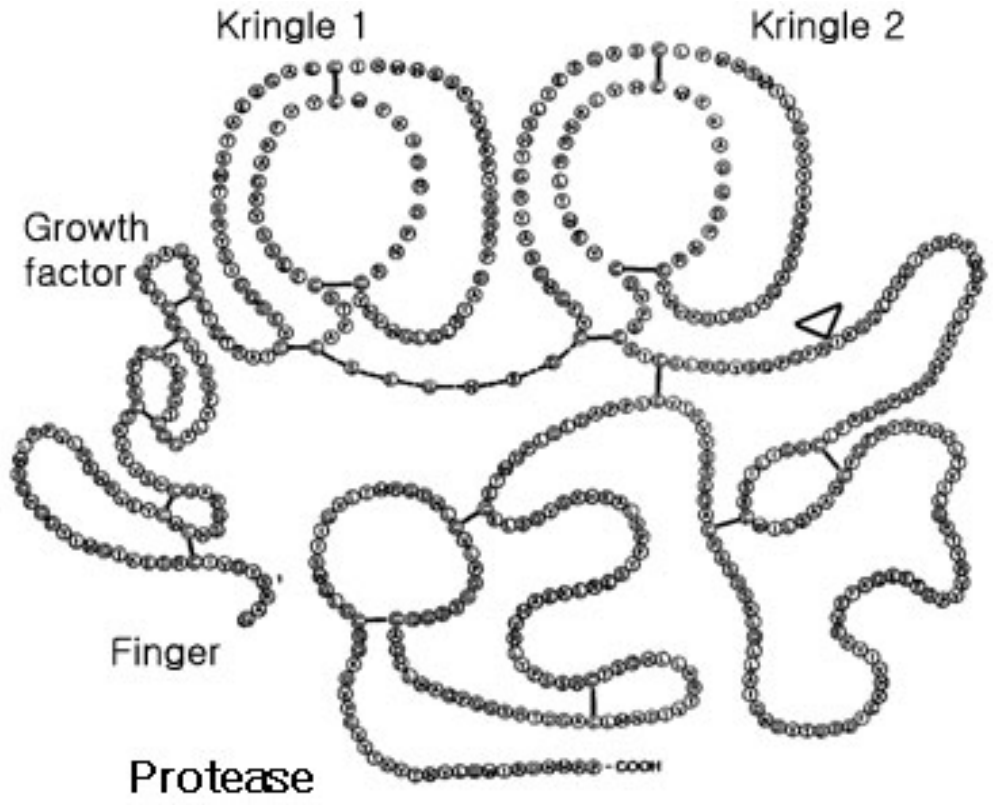


Fig. 3. Protein structure of tissue plasminogen activator (tPA). Arrow denotes site on serine 478 substitution with alanine (S478A-tPA) .

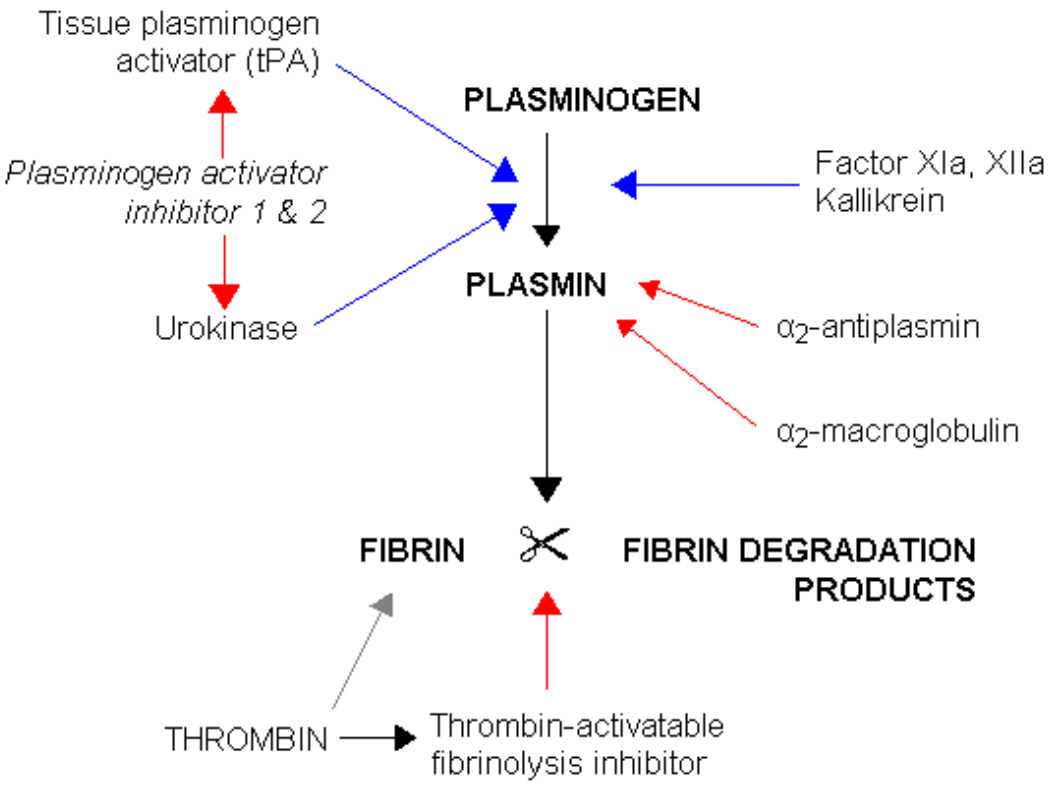


Fig. 4. A simplified illustration demonstrates clot breakdown. Blue arrows denote stimulation, and red arrows denote inhibition.

3. Properties of pyruvate

Pyruvate is a natural metabolite of the glycolytic pathway and hence should not lead to serious side effects. Pyruvic acid ($CH_3COCOOH$) is an organic acid, has a carboxylic acid and a ketone functional group, and is the simplest of the alpha-keto acids. Pyruvic acid can be made from glucose through glycolysis, converted back to carbohydrates via gluconeogenesis. Pyruvic acid supplies energy to living cells through the citric acid cycle (also known as the Krebs cycle) when oxygen is present (aerobic respiration), and alternatively ferments to produce lactic acid when oxygen is lacking (fermentation).

The antioxidative cytoprotective effects of pyruvate have been demonstrated in diverse cell systems (Desagher et al., 1997; Chung et al., 2004; Yoo et al., 2004; Frenzel et al., 2005; Panda et al., 2014; Hamburger et al., 2013). Sheline et al. (2000, 2002) reported that pyruvate has remarkable protective effects against zinc toxicity in cultured cortical cells. Pyruvate protects rat brains against transient global ischemia (Lee et al., 2001). Considering that zinc accumulation and oxidative stress may contribute to injury in both global and focal cerebral ischemia (Koh et al., 1996; Love, 1999; Lee et al., 2002), the neuroprotective capacity of pyruvate has the potential to extend to focal ischemia.

One potential concern is the possibility that excess pyruvate in the ischemic area may lead to accumulation of lactate, which may aggravate acidosis and cell injury associated with focal cerebral ischemia (Rehncrona et al. 1981, 1985).

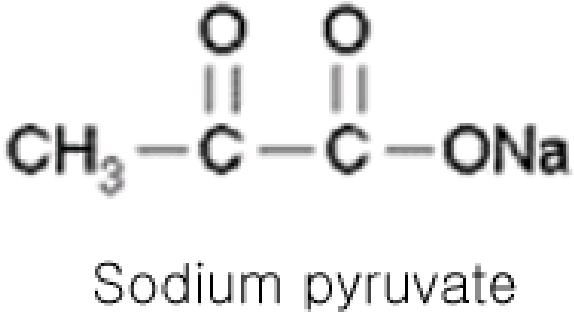


Fig. 5. Chemical structure of sodium pyruvate.

Chapter 2. Infarct reduction in rats following intraventricular administration of either tissue plasminogen activator (tPA) or its non-protease mutant S478A-tPA

1. Abstract

Human recombinant tissue plasminogen activator (tPA) is clinically used as a thromobolytic agent in vasculoocclusive conditions such as myocardial and cerebral ischemia (Adams et al., 1996; The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). In ischemic stroke, if introduced within 3 h of symptom onset, tPA has been demonstrated to cause clinical improvement (Levine and Brott, 1992).

In addition to its thrombolytic effect, human recombinant tissue plasminogen activator (tPA) may have parenchymal effects such as protease-dependent neurotoxic and protease-independent neuroprotective effects. The purpose of this study was to examine parenchymal effects of tPA and its non-protease mutant S478A-tPA in permanent focal cerebral ischemia in rats. However, before doing in vivo experiments, effects of tPA and S478A-tPA on zinc or NMDA toxicity were first studied in cortical cultures. Like tPA, which has protease independent cytoprotective effects, the non-protease mutant S478A-tPA blocked zinc toxicity in cortical cell cultures, but did not affect calcium-mediated NMDA toxicity. Then, effects of tPA and S478A-tPA on infarcts induced by permanent occlusion

of middle cerebral artery (MCA) were investigated. tPA and S478A-tPA were administered into the cerebral ventricle 15 min or 1 h after MCA occlusion. Both tPA and its non-protease mutant S478A-tPA, when given 15 min after ischemia, substantially reduced infarcts and ameliorated motor deficits in the MCA occlusion model of focal cerebral ischemia. However, when administered 1 h after MCA occlusion, neither showed protective effects. The protective effects of tPA or S478A-tPA remained unchanged at 7 days after MCA occlusion. Indicating that the native protein conformation is necessary for the protective effect of tPA and S478A-tPA, heat-denatured tPA did not exhibit any protective effect. Since S478A-tPA lacks protease activity, which has been implicated in causing cerebral hemorrhage or aggravating excitotoxicity, its parenchymal neuroprotective effect may be useful in treatment of ischemic stroke.

2. Introduction

Human recombinant tissue plasminogen activator (tPA) is clinically used as a thromobolytic agent in vasculoocclusive conditions such as myocardial and cerebral ischemia (Adams et al., 1996; The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). In ischemic stroke, if introduced within 3 h of symptom onset, tPA has been demonstrated to cause clinical improvement (Levine and Brott, 1992).

In addition to its thrombolytic effect, tPA has diverse parenchymal effects. For instance, local proteolytic effect of tPA may contribute to neurite outgrowth and synaptic plasticity (Baranes et al., 1998; Seeds et al., 1999; Tsirka, 2002). Furthermore, tPA may mediate or aggravate calcium excitotoxicity by degrading components of extracellular matrix such as laminin (Chen and Strickland, 1997; Indyk et al., 2003; Nagai et al., 1999). Consistent with this, tPA null mice exhibited less vulnerability to MCA occlusion than wild type mice (Wang et al., 1998).

Although most reported biological effects of tPA is mediated by its protease activity, more recently, a growing body of evidence indicates that tPA has biological effect independent of its protease activity. tPA increases proliferation rate of canine endothelial cells through receptor-mediated mechanism (Welling et al., 1996) and microglial cell activation through annexin II (Siao and Tsirka, 2002). Furthermore, tPA protects cultured cortical neurons against zinc toxicity and oxidative injury, and hippocampal neurons from oxygen-glucose deprivation

in a protease-independent manner (Flavin and Zhao, 2001; Kim et al., 1999a,b). Consistent with in vitro neuroprotection against zinc toxicity, tPA administered into the cerebral ventricle in rats markedly attenuated neuronal death induced by prolonged seizures (Kim et al., 1999a,b), where zinc toxicity has been implicated (Frederickson et al., 1988).

Since tPA is clinically used as a thrombolytic agent in the setting of acute ischemic stroke, it is crucial to understand its parenchymal effects in focal cerebral ischemia. More specifically, the protease-independent effect of tPA may provide insights into its overall effects in focal cerebral ischemia. Serine 478 of human recombinant tPA is essential for catalytic activity, and its substitution with alanine (S478A-tPA) completely abolishes protease activity. In the present study, to focus on parenchymal effects, we introduced tPA and S478A tPA into the lateral ventricle of rats, and examined changes in infarct size in a suture model of permanent middle cerebral artery occlusion (Aspey et al., 1998).

3. Materials and Methods

3. 1. *Cortical cell culture*

Mixed cortical cell cultures containing both neurons and astrocytes were prepared as described previously (Kim et al., 1999a, b) from fetal mice at 14–16 days gestation. Briefly, dissociated cortical cells were plated onto a previously established astroglial cell monolayer at 2.5 hemispheres per 24-well plate (Nunc, Naperville, IL) in a plating medium (Dulbecco's modified Eagle medium supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 2 mM glutamine, 5% fetal bovine serum and 5% horse serum). Cytosine arabinoside (10 uM) was added 5–6 days after plating to halt growth of non-neurons. Astroglial cultures were prepared from neocortices of newborn mice (postnatal days 1–2) and plated at 0.5hemispheres per 24-well plate, in the same plating medium as above, but supplemented with 10% fetal bovine serum and 10% horse serum. Glial cultures were used for neuron plating between DIV 14 and 28, when they formed a confluent monolayer.

3. 2. *Exposure of cortical cultures to toxins*

Exposure of cortical cultures for 24 h to NMDA or zinc was preformed in serum-free MEM (Eagle's minimal essential medium, Earle's salts, supplied glutamine-free). Before treatments, pre-existing medium containing serum was removed by multiple rinsing and replaced with serum-free medium. When mature (>DIV 10), the survival of neurons in mixed cortical cultures was not

compromised by switching to serum-free MEM. Each experiment was repeated four times using cultures from different dissection

3. 3. *Zymographic analysis*

SDS-PAGE electrophoresis and zymographic analyses were carried out as previously described (Park et al., 1999) with minor modifications. Following electrophoresis, polyacrylamide gels were gently shaken in 2.5% Triton-X 100 for 30 min to remove SDS, washed with distilled water three times, and incubated for 30 min at 37℃ in PBS. Gels were then laid on a zymogram containing 12.5 ug/ml human plasminogen, 1% low-melting agarose, 2.5% non-fat dry milk, 0.65 mM CaCl2, 50 mM glycine, 2.5 mM sodium azide, and 19 mM Tris (pH 7.5), and incubated at 37℃ overnight to allow development of lytic bands. Protease activity was detected as translucent bands and visualized using the Autochemi Analysis System with Image Acquisition and Analysis Software (UVT Bioimaging System, Cambridge, UK).

3.4. *LDH efflux assay*

Overall neuron injury in cortical cell cultures was quantitatively assessed by measuring lactate dehydrogenase (LDH) activity released from damaged cells into the medium (Koh and Choi, 1987). Each LDH value, after subtracting mean background values in sister cultures that underwent sham wash only (= 0), was scaled to the mean value in sister cultures after 24 h exposure to 300 umol/l

NMDA (= 100), where near complete neuron death with no glial damage occurs.

3. 5. *Measurement of nicotinamide adenine dinucleotide (NAD+) levels*

NAD^+ levels were measured with an enzymatic cycling assay (Paschen et al., 2000; Kim and Koh, 2002). Six hours after the onset of ischemia, both hemispheres (ipsilateral and contralateral) were separated. Tissues containing both cortex and striatum in the middle of the MCA territory, were dissected out, weighed, and treated with 0.5 M perchloric acid (Merck, Germany) for 15 min at 4 ℃. Tissues were homogenized and neutralized with 0.5 M KOH for 1 h on ice.

Homogenates were centrifuged at 2000 × g for 15 min at 4 ℃, and the supernatants were used. Twenty-five microliters of supernatant or 0 to 4 μM standard NAD+ solutions (Sigma-Aldrich) were reacted with 100 μl of freshly prepared reagent mixture containing 0.2 mM phenazine ethosulfate, 0.5 mM MTT (3-[4,5-dimethylthiazol-2-yo]-2,5-diphenyltetrazolium bromide), 600 mM ethanol (Merck,Darmstadt, Germany), 0.5 mM EDTA and 4 U alcohol dehydrogenase (Sigma-Aldrich) in 120 mM sodium/bicine buffer (pH 7.8) in the dark at 37 ℃ for 1 h. The absorbance at 570 nm was measured.

3. 6. *Permanent middle cerebral artery (MCA) ischemia*

Ninety-two male Sprague–Dawley (SD) rats (321 ± 34 g, 9–10 weeks) were obtained from Charles River Laboratories (Yokohama, Japan). All rats were allowed free access to food and water. Experiments were performed in

accordance with the Guideline for Care and Use of Laboratory Animals (University of Ulsan, Seoul, South Korea).

Each rat was anesthetized with halothane in a 1:3 mixture of O_2 and N_2O. The left femoral artery was cannulated for monitoring arterial blood pressure (Stoelting, Wood Dale, Illnosis, USA) and sampling of blood. Hemoglobulin, hematocrit, pH, arterial oxygen pressure (pO_2), and partial pressure of carbon dioxide (pCO2) in arterial blood samples were analyzed with an Opti critical care analyzer (Roche Diagnostics, Indiana, Indianapolis). Rectal and temporalis muscle temperature (Physitemp, Clifton, NJ) was maintained at 36.4 ± 0.5℃ with a temperature control unit (Harvard, South Natick, MA) and a heat lamp during and for 2 h after surgery. Permanent ischemia in the MCA territory was induced as previously described (Aspey et al., 1998; Longa et al., 1989; Memezawa et al., 1992). Under a surgical microscope, the right common (CCA), external (ECA), and internal carotid (ICA) arteries were identified, and separated from the vagus nerve. ECA and CCA were ligated with 4–0 silk suture, and the ICA temporarily clipped. A small incision in the CCA was made 1 mm proximal to the bifurcation, and 4–0 suture (Ethylon surgical monofilament polyamide, Ethicon, UK), the tip of which was firepolished to a thickness of 0.25–0.3 mm in diameter, was inserted into the ICA. After releasing the clip at the ICA, the suture was advanced into the proximal portion of the MCA to about 20 mm from the carotid bifurcation. The whole surgical operation was completed within 15 min.

3.7. *Drug administration for Tissue plasminogen activator (tPA) or its non-protease mutant S478A-tPA*

A 10- ul microsyringe (Hamilton, Reno, NV) was stereotactically introduced through a 2-mm burr hole into the right lateral ventricle (0.8 mm posterior and 1.2 mm lateral from bregma, 3.8 mm deep), through which tPA (Boehringer-Ingelheim, 3 ug in 6 ul), S478A-tPA (Genentech, 3 ug in 6 ul), or normal saline (6 ul) was injected after MCA occlusion. For controls, heat-denatured tPA (boiled for 20 min, 3 ug in 6 ul) was injected. The microsyringe was removed 3 min after injection. Operators were blinded to the drug identity.

3.8. *Estimation of motor deficit*

Motor deficit in rats was quantified on a scale from 0 to 3 as described by Longa et al. (1989). No deficit was graded as normal (0), failure to the extend the forepaw when suspended vertically as mild (1), circling to the contralateral side as moderate (2), and loss of circling or righting reflex as severe deficit (3).

3.9. *Measurement of cerebral infarction*

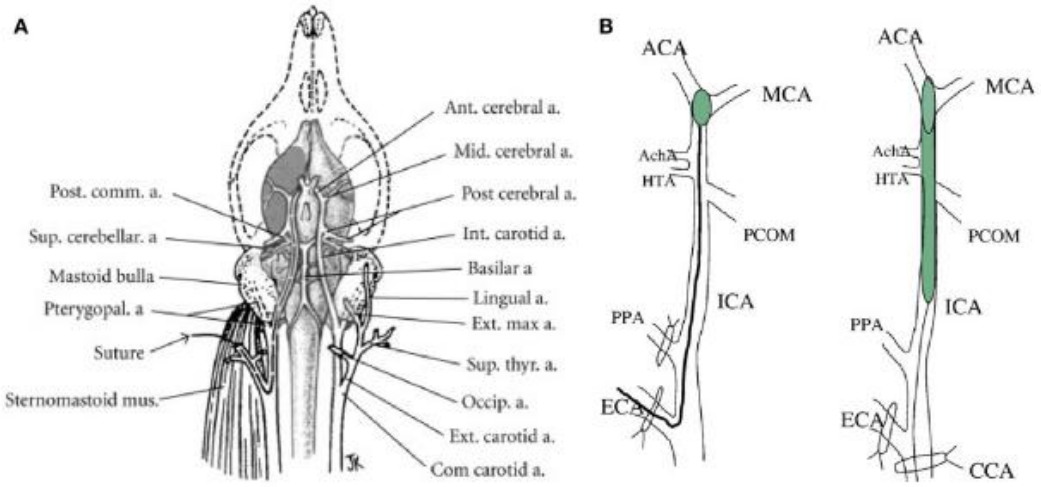
At 24 h or 7 days or 14 days after ischemia induction, rats were euthanized and brains collected for analysis. Two-millimeter- thick brain slices (RBM-4000C, ASI, Warren, MI) were incubated in 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma, St. Louis, MO) in normal saline at 37°C for 30 min, and

then stored in 4% paraformaldehyde (Longa et al., 1989; Aspey et al., 1998; Lee et al., 2002; Yi et al., 2004). Digital images of brain slices were obtained with a flatbed digital scanner. Infarction volumes were quantified using image analysis software (ImagePro, Media cybernetics, Silver spring, Maryland). Total infarction volume was estimated by adding infarct volume of each coronal slice along the AP axis axis (Aspey et al., 1998; Lee et al., 2002; Yi et al., 2004). In addition, to correct for possible contribution by edema, infarct volumes were normalized to the volume of the intact contralateral hemisphere (Swanson et al., 1990; Yi et al., 2004).

Hematoxylin and eosin (H&E) staining was additionally used for brain sections obtained 7 days or14 days after ischemia, since infiltrated inflammatory cells may take up TTC. Analysis of infarct area was the same in the case with TTC staining. Infarct margins were identified in all sections by closely examining sections at high power.

3.10. *Statistics*

For statistical comparisons, two-tailed t test with Bonferroni correction for multiple comparisons was used.



Canazza et al., 2014. Front Neurol.

Fig. 1. Experimental Models of brain ischemia. Intra-luminal suture model. **(A)** Vascular brain anatomy of the rat. **(B)** Induction of middle cerebral artery occlusion (MCAO) by insertion of thread into the external carotid artery (left) or common carotid artery (right). ACA, anterior carotid artery; Ach A, anterior choroidal artery; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotidartery; HTA, hypothalamic artery; PPA, pterygopalatin eartery; PCOM, posterior communicating artery.

4. Results

Consistent with previous reports (Rogove et al., 1999), S478A-tPA showed no protease activity in PAGE-plasminogen zymography assays, unlike wild-type human recombinant tPA (Fig. 2A). However, we found that like wild-type tPA, S478A-tPA (10 ug/ml) markedly attenuated cell death in cultured neurons exposed to 35 uM zinc for 24 h (Fig. 2B). These findings are in agreement with data from others showing that tPA protection against zinc toxicity is independent of protease activity (Kim et al., 1999a, b). Calcium-mediated neuronal death following incubation with 30 AM NMDA (Kim et al., 1999a,b) for 24 h was not attenuated by either tPA or S478A-tPA.(Fig. 2B).

In both global and focal models of cerebral ischemia, zinc neurotoxicity has been proposed as a contributing mechanism of brain injury (Choi and Koh, 1998; Flavin and Zhao, 2001; Koh et al., 1996; Lees et al., 1998). Since both tPA and S478A-tPA markedly protected cultured cortical neurons against zinc toxicity, we tested their protective effects in a permanent middle cerebral artery (MCA) occlusion model in the rat. As a protein of substantial molecular size, tPA may not easily cross the intact blood brain barrier into the brain parenchyme. Therefore, in these studies we directly injected 6 ul of either normal saline solution, tPA or S478A-tPA (3 ug) dissolved in normal saline, into the lateral ventricle 15 min after occlusion of the MCA. We found that systemic parameters such as rectal and scalp temperature, systolic pressure, arterial pH, pCO_2, pO2, and hemoglobin/hematocrit values measured before and 10 min after MCA

occlusion did not differ between the three groups (Table 1). However, both tPA and S478A-tPA tended to decrease mortality associated with permanent MCA occlusion (Table 1). In both global and focal models of cerebral ischemia, zinc neurotoxicity has been proposed as a contributing mechanism of brain injury (Choi and Koh, 1998; Flavin and Zhao, 2001; Koh et al., 1996; Lees et al., 1998). Since both tPA and S478A-tPA markedly protected cultured cortical neurons against zinc toxicity, we tested their protective effects in a permanent middle cerebral artery (MCA) occlusion model in the rat. As a protein of substantial molecular size, tPA may not easily cross the intact blood brain barrier into the brain parenchyme. Therefore, in these studies we directly injected 6 ul of either normal saline solution, tPA or S478A-tPA (3 ug) dissolved in normal saline, into the lateral ventricle 15 min after occlusion of the MCA. We found that systemic parameters such as rectal and scalp temperature, systolic pressure, arterial pH, pCO_2, pO_2, and hemoglobin/hematocrit values measured before and 10 min after MCA occlusion did not differ between the three groups (Table 1). However, both tPA and S478A-tPA tended to decrease mortality associated with permanent MCA occlusion (Table 1). Twenty-four hours after permanent MCA occlusion, brains were removed and infarcts examined using TTC staining. Control rats that received intraventricular saline developed large infarcts in the frontoparietal cortex and caudate nucleus (Fig. 3), while rats that received tPA or S478A-tPA 15 min after occlusion developed much smaller infarcts throughout the anteroposterior (AP) axis (Fig. 3 and 4). In contrast, injection of heat-denatured

(boiled) tPA (3 ug) was not protective (Figs. 3 and 4). Infarct volume (mm^3) was obtained by integrating the areas over the APaxis. In addition, to compensate for possible contribution from edema formation, infarct volume was normalized to the total volume of the infarcted hemisphere. Both estimations showed that mean total infarct volume in tPA and S478A-tPA groups was reduced by about 75% and 60%, respectively, compared with that in the control group (Figs. 5A, 5B). In contrast, heat-denatured tPA did not have anyprotective effect. The protective effects were dependent onthe time of administration since neither tPA nor S478A-tPA reduced infarct volume when injected 1 h after occlusion (Fig. 5C).

Finally, we examined whether the protective effect of tPA and S478A-tPA would last for more than 24 h. MCA occlusion experiments were performed as described above with agents added 15 min after induction of ischemia. Brains were harvested 7 days later, sectioned and stained with TTC or H&E. We found both tPA and S478A-tPA reduced the size of infarcts by about 65–70% (Figs. 6A, 6B), indicating that the protective effects observed at 24 h lasted for at least 7 days. In addition to this anatomical evidence for brain protection by tPA and S478A-tPA, the motor deficit scores recorded 7 days after ischemia induction were significantly better intPA- and S478A-tPA-treated rats compared to controls (Fig. 7A). Furthermore, weight loss, a functional parameter of ischemic stroke in rats, was less in tPA- and S478A-tPA-treated rats 7 days after ischemia (Fig. 7B). At 24 h after ischemia, there was no difference among groups in either motor deficit scores or weight reduction (Figs. 7A and 7B).

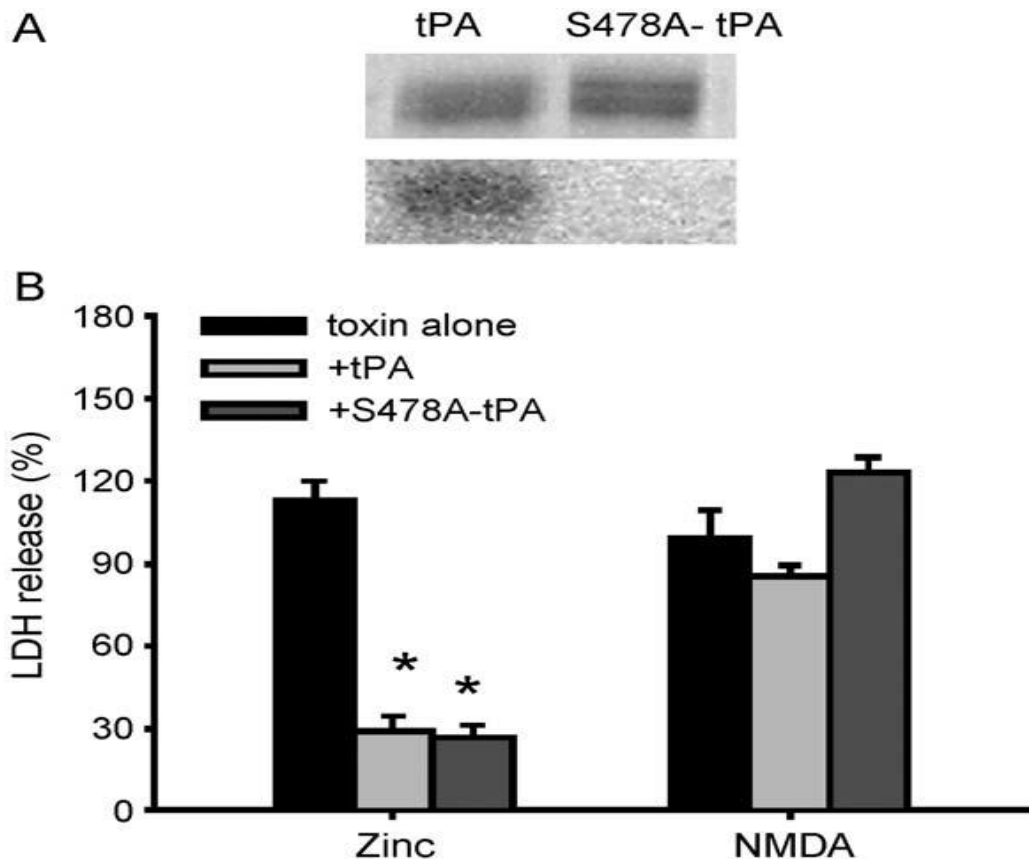


Fig. 2. Neuroprotection by tPA and S478A-tPA in cortical cell cultures. (A) tPA and S478A tPA (1 ug/ml) were electrophoresed on 10% SDS polyacrylamide gels and stained with Coomassie Brilliant Blue (upper). The protease activity of 20 ng/ml tPA and S478A-tPAwas determined using PAGE-plasminogen zymography (lower). (B) LDH release (mean ± SEM, n = 4 cultures) in cortical cell cultures exposed to 35 umol/l zinc or 30 umol/l NMDA for 24 h in the absence or presence of 10 ug/ml tPA (+ tPA) or 10 ug/ml S478A-tPA (+ S478A tPA). * denotes difference from toxin alone ($P < 0.01$, two-tailed t test with Bonferroni correction for two comparisons).

Table. 1.

Physiological parameters (mean ± SEM) and mortality

Parameter	Saline control (n = 42)		tPA (n = 24)		S478A-tPA (n = 25)	
	Pre	Post	Pre	Post	Pre	Post
Rectal temperature (°C)	36.3 ± 0.0	36.2 ± 0.0	36.4 ± 0.0	36.2 ± 0.0	36.5 ± 0.0	36.1 ± 0.0
Temporalis temperature (°C)	36.2 ± 0.0	36.3 ± 0.0	36.3 ± 0.0	36.3 ± 0.0	36.5 ± 0.0	36.4 ± 0.0
Systolic pressure (mm Hg)	111.2 ± 0.3	111.0 ± 0.5	118.2 ± 0.4	117.4 ± 0.5	117.1 ± 0.4	113.0 ± 0.6
pH	7.4 ± 0.0	7.4 ± 0.0	7.4 ± 0.0	7.4 ± 0.0	7.4 ± 0.0	7.4 ± 0.0
pCO_2 (mm Hg)	42.6 ± 0.1	42.9 ± 0.3	40.8 ± 0.2	41.6 ± 0.4	42.2 ± 0.2	41.7 ± 0.7
pO_2 (mm Hg)	158.6 ± 0.8	154.8 ± 0.9	157.6 ± 1.0	160.3 ± 1.3	145.3 ± 1.3	137.6 ± 2.3
Hemoglobin (g/dl)	12.9 ± 0.0	12.8 ± 0.0	13.0 ± 0.0	12.2 ± 0.2	13.2 ± 0.0	12.6 ± 0.1
Hematocrit (%)	38.7 ± 0.1	38.3 ± 0.1	39.0 ± 0.1	38.6 ± 0.2	39.5 ± 0.1	38.0 ± 0.2
Mortality (24 h)	10/42 (23.8%)		1/24 (4.2%)		2/25 (8.0%)	

Fig. 3. tPA and S478A-tPA protect against permanent focal cerebral ischemia. Fifteen minutes after MCA occlusion, rats were intraventricularly injected with 6 ul volumes of either saline (n = 12), tPA (n = 10, 3 ug), S478A-tPA (n = 8, 3 ug) or heat-denatured (boiled) tPA (n = 7, 3 ug). Twenty-four hours later, rat brains were removed and 2-mm-thick coronal sections stained with TTC and analyzed. Scale bar, 3 mm.

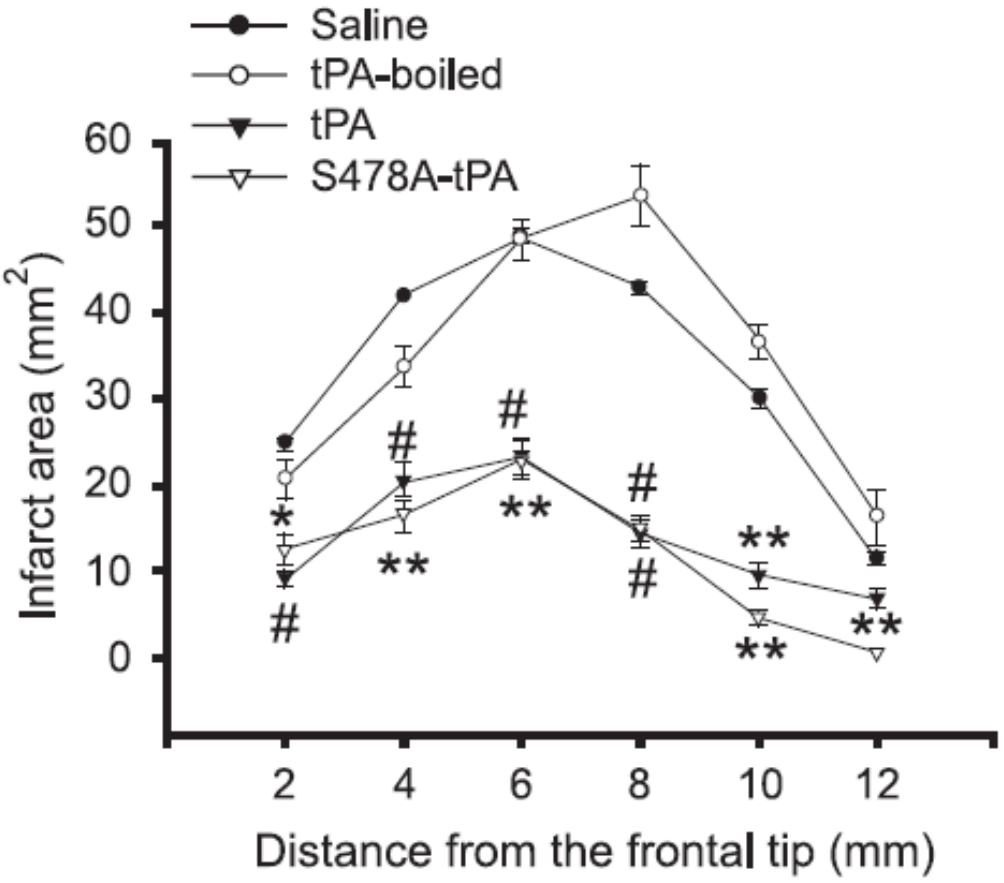
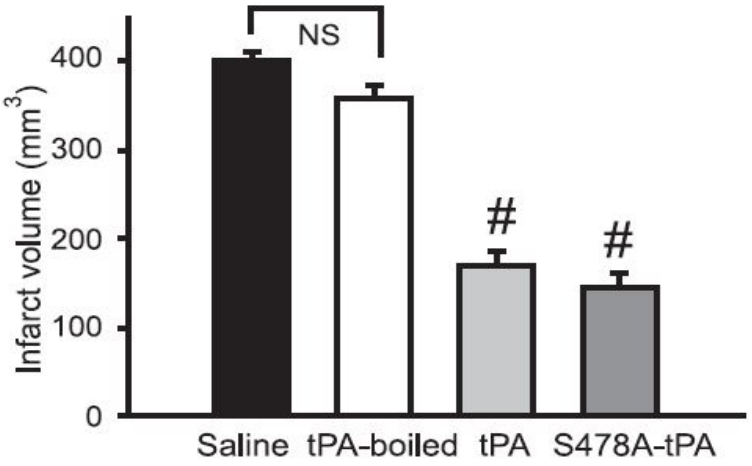
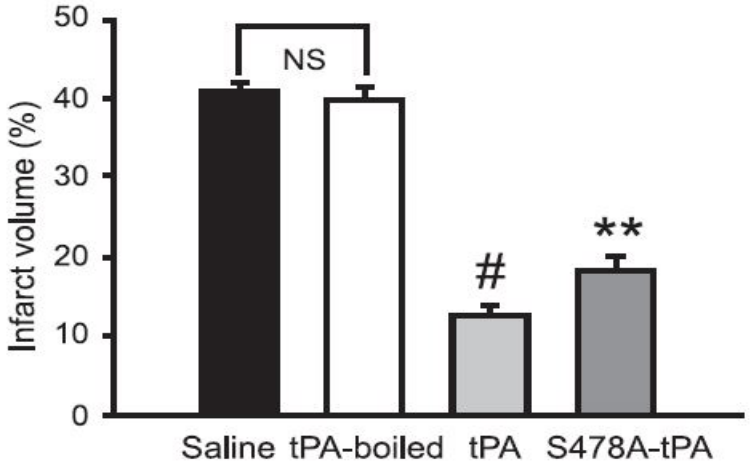


Fig. 4. tPA and S478A-tPA protect against permanent focal cerebral ischemia. Area (mm^2) of infarction in six consecutive 2-mm thick coronal sections of the brain. Saline, tPA or S478A-tPA was given 15 min after the occlusion. Twenty-four hours later, rat brains were removed and 2-mm-thick coronal sections stained with TTC and analyzed. Bars represent infarct volume (mm^3). *P < 0.05, **P < 0.01, #P < 0.001 (two-tailed t test with Bonferroni correction for two comparisons).

A.


NS
400
300
200
100
0
Infarct volume (mm3)
#
#
Saline
tPA-boiled
tPA
S478A-tPA

B.


NS
50
40
30
20
10
0
Infarct volume (%)
#
**
Saline
tPA-boiled
tPA
S478A-tPA

C.

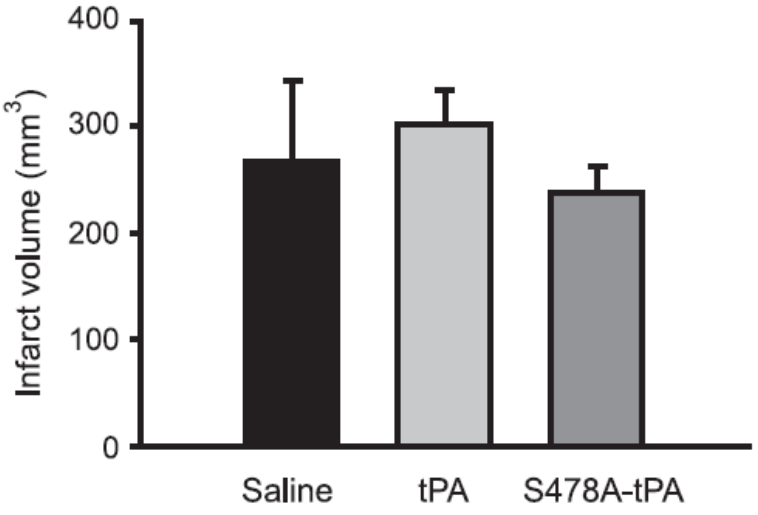
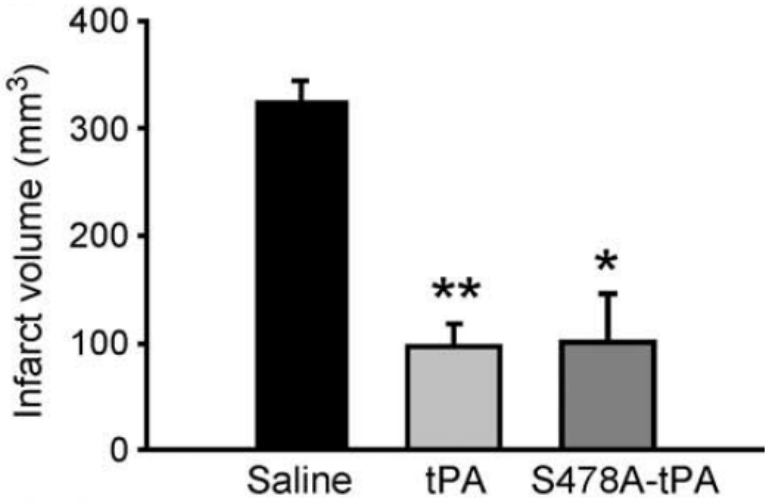

400
300
200
100
0
Infarct volume (mm3)
Saline
tPA
S478A-tPA

Fig. 5. tPA and S478A-tPA protect against permanent focal cerebral ischemia. Infarct volume 24 h after occlusion. Absolute (mm^3, left bars, A). Relative (% of the hemisphere, B). Saline, tPA or S478A-tPA was given 15 min after the occlusion. (C) One hour after MCA occlusion, rats were intraventricularly injected with 6 ul volumes of either saline, tPA (3 ug) or S478A-tPA (3 ug) Twenty-four hours later, rat brains were removed and 2-mm-thick coronal sections stained with TTC and analyzed. Bars represent infarct volume (mm3). $^{**}P < 0.01$, $\#P < 0.001$ (two-tailed t test with Bonferroni correction for two comparisons).

A.



B.

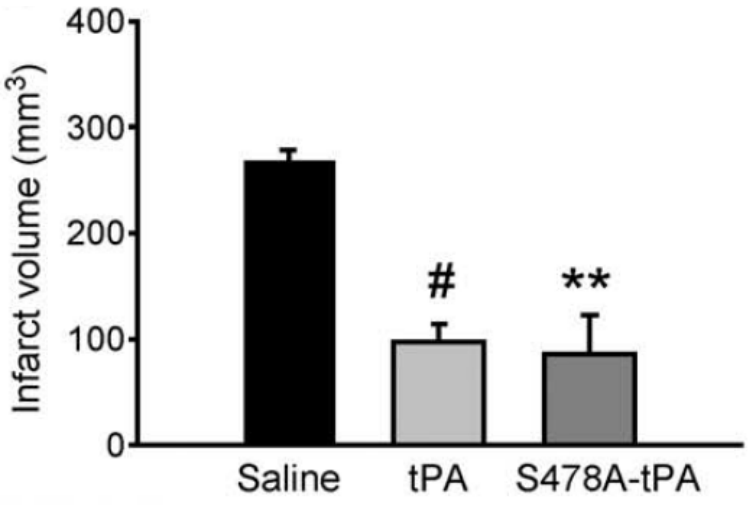
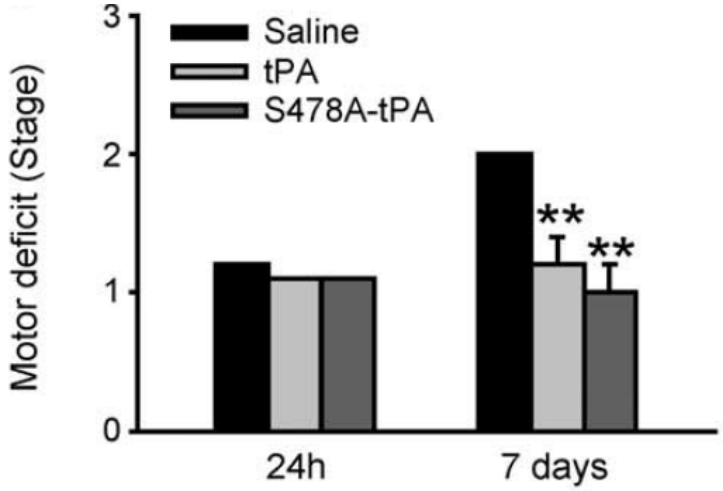


Fig. 6. Sustainability of protection. Fifteen minutes after MCA occlusion, rats were intraventricularly injected with 6 ul volumes of either saline, tPA (3 ug) or S478A-tPA (3 ug). Seven days later, rat brains were removed and 2-mm-thick coronal sections stained with TTC analyzed (A) or H and E analyzed (B), Bars denote infarct volume (mm^3). * denotes difference from saline control (*$P < 0.05$, **$P < 0.01$, #$P < 0.001$ two-tailed t test with Bonferroni correction for two comparisons.

A.



B.

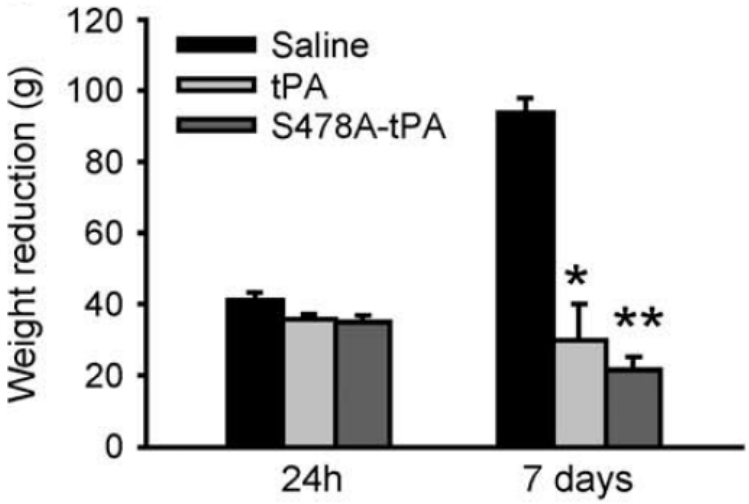


Fig. 7. Sustainability of protection. Fifteen minutes after MCA occlusion, rats were intraventricularly injected with 6 ul volumes of either saline, tPA (3 ug) or S478A-tPA (3 ug). (A) Motor deficit scores 24 h and 7 days after occlusion. (B) Whole rat weight loss 24 h and 7 days after occlusion compared to pre-ischemia values. * denotes difference from saline control (**$P < 0.01$ two-tailed t test with Bonferroni correction for two comparisons).

5. Discussion

The present study demonstrated that intraventricular administration of tPA or its non-protease mutant, S478-tPA, 15 min after MCA occlusion, provides substantial brain protection in rats. The fact that tPA completely loses its protective efficacy after heat denaturation indicates that the protective effect of tPA or S478- tPA is entirely dependent upon the native protein conformation.

At first glance, the protective effect of tPA seems at odds with other reports that tPA can mediate excitotoxic injury in seizures and stroke (Tsirka et al., 1995; Wang et al., 1998). However, the pro-injurious effect of tPA is dependent upon its proteolytic effect. Degradation of laminin mainly via generation of plasmin may be a crucial event for the toxic effect of tPA (Chen and Strickland, 1997; Tsirka et al., 1997). In contrast, the protective effect of tPA shown here is not mediated by the proteolytic effect of tPA. Since levels of plasminogen in CSF are much lower than those in blood (Whitelaw et al., 1997), administration of tPA into ventricles may have reduced its potential pro-injurious effect as compared with its administration into the blood circulation.

Reduction of infarct volume by more than 60% is notable considering permanent MCA occlusion inflicts substantially more severe injury than transient occlusion (Zhang et al., 1994). Since arterial blood flow was permanently occluded with sutures, thrombolysis and reperfusion cannot be the basis of the infarct reduction. That both tPA and S478A-tPA protected to a similar degree also makes this explanation unlikely. Since in permanent occlusion the infarct core

with complete lack of arterial supply cannot be salvaged by any means, the volume that was rescued by tPA likely represents the penumbral area, which has been estimated to be quite substantial even in the permanent MCA model (Hakim, 1998, Lipton, 1999, Chelsea et al., 2004, Andrei and Marta, 2009).

A time point of 24 h after ischemia induction has been widely used for quantitative assessments of infarct formation, with some studies demonstrating little change in infarct size after this time (Alonso et al., 2001; Aspey et al., 1998; Lipton, 1999; Zhang et al., 1994). However, more recent studies demonstrated that infarcts can develop later in certain cases. For example, mild focal ischemia can induce highly delayed infarct formation (Du et al., 1996). Thus, it is significant that the protective effect of S478A-tPA persisted 7 days after ischemia induction, when most brain damage is completed (Du et al., 1996; Lipton, 1999). The functional improvement in motor deficit scores and the degree of weight reduction 7 days after ischemia is also notable.

Like tPA, S478A-tPA showed remarkable neuroprotective effects against zinc toxicity, but not against calciumoverload excitotoxic neuron death in cortical cell cultures. Since zinc toxicity has been proposed as a contributing mechanism in global and focal brain ischemia (Koh et al., 1996; Lee et al., 2002), it is possible that protection by tPA and S478A-tPA against zinc toxicity contributed to infarct reduction in the present study, although other possibilities cannot be excluded.

Although remarkably effective in infarct reduction in this rat model, intraventricular injection may not be easily employed in the context of human

ischemic stroke. In the case of tPA, an alternative method of administration such as intravenous injection may not be safe as much larger doses of tPA would be required to attain protective concentrations in the brain parenchyma. The risk of brain hemorrhage steeply rises with increasing doses of tPA when given intravenously to patients with ischemic stroke (Hacke et al., 1995). Furthermore, tPA has been shown to exacerbate calcium excitotoxicity via plasmin generation when given intravenously (Wang et al., 1998). In contrast, due to a lack of protease activity, S478A-tPA carries no hemorrhage or calcium excitotoxicity risks. Therefore, it may be that substantial amounts of S478A-tPA can be safely introduced into the brain or systemic circulation. A future study to determine the safety and efficacy of intravenous S478A-tPA administration appears warranted.

Chapter 3. Systemic pyruvate administration markedly reduces infarcts and moter deficits in rat models of transient and permanent focal cerebral ischemia

1. Abstract

In the present study, we investigated the possible neuroprotective effect of pyruvate in focal ischemia. Pyruvate (62.5–250 mg/kg) treatment, regardless of whether given intraperitoneally (ip) or intravenously (iv), decreased infarct volume by more than 50% in both transient (1 h) and permanent occlusion models. The infarct reducing effects of pyruvate were maintained 14 days (d) after MCAO. Interestingly, higher doses failed to reduce the infarct size. Pyruvate administration also reduced motor deficits. Magnetic resonance (MR) spectroscopy revealed that protective doses of pyruvate, but not the non-protective doses, were associated with a reduction in the level of lactate compared with saline controls. Diffusion-weighted MR images further confirmed infarct reduction in pyruvate-treated rats. Pyruvate is an endogenous metabolite of glycolysis, and hence is unlikely to have serious side effects. Considering its substantial neuroprotective capacity in focal cerebral ischemia, a clinical trial is warranted.

2. Introduction

Despite intensive investigation over the last couple of decades, there is no standard parenchymal neuroprotective treatment available for ischemic stroke (Dirnagl et al., 1999). Nonetheless, efforts to find clinically effective neuroprotectants continue as demand for such agents increases, particularly with the advent of thrombolytic treatment. Intravenous tPA treatment is currently indicated for ischemic stroke patients that present to hospitals within 3 hours (h) of the onset of symptoms (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Adams et al., 1996; Zivin, 1997; Vatankhah et al., 2005). A time limit of 3 h for the instigation of tPA treatment is warranted as after this time period vulnerable neurons are considered irreversibly injured, and the risk for hemorrhage increases (Hacke et al., 1995; Clark et al., 2000). This short window of opportunity allows only a small percentage of patients to benefit from tPA treatment (Clark et al., 1999; Barber et al., 2001). The combined use of a neuroprotective agent would enhance neuron survival and thereby extend the window of opportunity for thrombolytic treatment to potentially benefit more patients.

While the infarct volume measured in postmortem brain tissues is the standard quantitative parameter for examining neuroprotective effects in focal cerebral ischemia, recent advances in MR spectroscopy and brain imaging techniques, provide additional information about metabolic changes, neuronal

injury and infarct formation in living animals. MR spectroscopy can measure Nacetylaspartate (NAA), creatine, lipid and lactate in the selected volume (voxel of interest) of the brain. Of these, the increase of lactate doublet peak and the decrease of NAA peak have been linked to permanent neuronal injury (Gyngell et al., 1995). On the other hand, diffusion-weighted MR imaging can delineate areas with cytotoxic edema, which correlate well with the infarct formation (Gyngell et al., 1995; Neumann-Haefelin et al., 2000).

The mechanism underlying ischemic neuronal death is complex. Calcium overload excitotoxicity, oxidative stress, apoptosis, acidosis, cytokines and other inflammatory reactions, all may contribute to ischemic cell death in the brain (Choi, 1995; Siesjo and Siesjo, 1996; Kristian and Siesjo, 1998; Kato and Kogure, 1999; Lipton, 1999; Love, 1999; Mattson et al., 2000; Iadecola and Alexander, 2001). Further adding to the complexity, recent evidence suggests that zinc contributes to cell death following brain ischemia (Koh et al., 1996; Frederickson et al., 2005).

If zinc contributes to ischemic neuronal death (McCord et al., 2014 Shuttleworth et al., 2011), measures to reduce zinc toxicity may be useful in ischemic stroke. In this regard, pyruvate appears promising as a zinc toxicity reducing neuroprotectant. An advantage of pyruvate is that being a natural metabolite, it is unlikely to be toxic or lead to serious side effects. The antioxidative cytoprotective effects of pyruvate have been demonstrated in diverse cell systems (Desagher et al., 1997; Chung et al., 2004; Yoo et al., 2004;

Frenzel et al., 2005). Recently, Sheline et al. (2000, 2002) reported that pyruvate has remarkable protective effects against zinc toxicity in cultured cortical cells. In a previous report, we demonstrated that pyruvate protects rat brains against transient global ischemia (Lee et al., 2001). Considering that zinc accumulation and oxidative stress may contribute to injury in both global and focal cerebral ischemia (Koh et al., 1996; Love, 1999; Lee et al.,2002), the neuroprotective capacity of pyruvate has the potential to extend to focal ischemia. A conflicting investigation, however, reported that pyruvate provided little protection against permanent focal ischemia (Gonzalez-Falcon et al., 2003).

To address this discrepancy, in the present study, we examined the potential protective effect of pyruvate in models of focal cerebral ischemia in rats. This was achieved by examining the effects of various doses of pyruvate on the infarct size in rats that had undergone transient and permanent middle cerebral artery occlusion (Aspey et al., 1998; Yi et al., 2004).

3. Materials and Methods

3. 1. *Permanent middle cerebral artery (MCA) ischemia*

Male Sprague–Dawley (SD) rats (298 ± 24.2 g, 8–9 weeks) were obtained from Charles River Laboratories (Yokohama, Japan). Total of 402 rats were used for experiments. All rats were allowed free access to food and water. Experiments were performed in accordance with the Guideline for Care and Use of Laboratory Animals (University of Ulsan, Seoul, South Korea).

Each rat was given 50 mg/kg zoletil [1:1 mixture (weight base) of tiletamine and zolazepam; Virbac, France] by intramuscular injection. The left femoral artery was cannulated for monitoring arterial blood pressure (Stoelting, Wood Dale, Illinois) and sampling blood. For hemoglobin, hematocrit, pH, arterial oxygen pressure (pO_2) and partial pressure of carbon dioxide (pCO_2), arterial blood samples were analyzed with an Opti critical care analyzer (Roche Diagnostics, Indiana, Indianapolis). The rectal and temporalis muscle temperature (Physitemp, Clifton, NJ) was maintained at 36.4 ± 0.5 ℃ with a temperature control unit (Harvard, South Natick, MA) and a heat lamp during surgery and for 2 h after. Permanent ischemia in the MCA territory was induced as previously described (Longa et al., 1989; Memezawa et al., 1992; Aspey et al., 1998; Yi et al., 2004). Under a surgical microscope, the right common (CCA), external (ECA) and internal carotid (ICA) arteries were identified and separated from the vagus nerve. ECA and CCA were ligated with 4-0 silk suture, and the ICA was temporarily clipped. A small incision in the CCA was made 1 mm

proximal to the bifurcation, and 4-0 suture (Ethylon surgical monofilament polyamide, Ethicon, UK), the tip of which was fire-polished to a thickness of 0.38–0.40 mm in diameter, was inserted into the ICA. After releasing the clip at the ICA, the suture was advanced into the proximal portion of the MCA to about 20 mm from the carotid bifurcation. The surgical operation was completed within 15 min. Transient ischemia was induced by removing the filament 1 h after occlusion.

3.2. *Laser-Doppler flowmetry*

The principles and technical details of laser-Doppler flowmetry (LDF) (BLF21D, Transonic instrument, Ithaca, NY) have been described in detail elsewhere (Dirnagl et al., 1989). For placement of the LDF probe, a burr hole (2 mm diameter) was drilled 2 mm posterior and 6mmlateral to the bregma, with care being taken not to injure the underlying dura mater. The animals were placed in the supine position, and the skull was firmly immobilized in a stereotaxic frame. Cerebral blood flow (CBF) was recorded 10 min before MCAO, after 20 min of MCAO and after 30 min of reperfusion. The CBF values were expressed as a percentage of the baseline value.

3.3. *Drug administration for pyruvate*

Na-Pyruvate (Sigma, St. Louis, MO) (31.3, 62.5, 125, 250 and 500 mg/kg, pH 7.4) (Mongan et al., 1999) or NaCl (Sigma, St.Louis, MO) (16.7, 33.2, 66.3,

132.5 and 265 mg/kg, pH 7.4) dissolved in distilled water were given to rats by ip or iv administration (tail vein, 0.11, 0.25, 0.40, 0.9 and 1.7 ml/h) 30 min after the onset of pMCAO or reperfusion of tMCAO.

We evaluated the effects of 125 mg/kg of pyruvate when administered in a longterm administration regime (30 min, 1 and 3 h). Operators were blinded to the drug identity.

3.4. *Magnetic resonance imaging*

All MR experiments were performed in a horizontal 4.7T/30 magnet (Bruker, Fllanden, Switzerland) and 25 mT/m active shield gradients. MRI was acquired using a multi-slice Diffusion-weighted image (DWI) sequence (TR/TE=2000/80 ms). MRI was acquired, 1, 2, 6 and 24 h after the onset of ischemia (Levy et al., 1986).

3.5. *^{1}H-magnetic resonance spectroscopy (^{1}H-MRS)*

Spectra were acquired 1, 2, 6 and 24 h after the onset of ischemia (Gyngell et al., 1995; van der Toorn et al., 1996).Water suppressed ^{1}H-MRS spectra were acquired using a VOSY sequence with detection of the double-refocused spin echo signal from the selected voxel (3×3×2.5) using the following acquisition parameters: SW=5000 Hz, SI=4096 pts, NS=128, TR/TE=3000/30 and 135 ms. To identify the peak at 1.3 ppm, the spectra were acquired at two echo times of TE=30 and 135 ms to differentiate the lactate peak from the lipid peak. All raw

spectroscopic data were processed using the XWIN-NMR software. We normalized lactate peaks to creatine peaks, as the creatine peaks did not differ between pyruvate and saline groups (1.916+0.149 vs. 1.905+ 0.164).

3.6. *Estimation of motor deficit*

Motor deficit in rats was quantified on a scale from 0 to 3 as described by Longa et al. (1989). No deficit was graded as normal (0), failure to the extend the forepaw when suspended vertically as mild (1), circling to the contralateral side as moderate (2), and loss of circling or righting reflex as severe deficit (3).

3.7. *Motor performance test*

One week before MCA occlusion, rats were conditioned to the accelerating rota-rod (Ugo Basile, Model 47700, Italy). Each animal received a training session on the rota-rod set at a constant speed of 4 rpm. Animals were tested on the rota-rod until they achieved a certain level for remaining on the rotating spindle. Each animal then received a single baseline trial on the accelerating rotarod in which the spindle increased in speed from 4 to 40 rpm over a period of 5 min (Rogers et al., 1997). After ischemia, they received daily 5 min test trials on the accelerating rota-rod until day 14 when brains were harvested. Control rats were subjected to the same training routines, but with anesthesia and sham operation. All measurements were carried out in a condition-blinded manner.

3. 8. *Measurement of cerebral infarction*

At 24 h or 7 days or 14 days after ischemia induction, rats were euthanized and brains collected for analysis. Two-millimeter- thick brain slices (RBM-4000C, ASI, Warren, MI) were incubated in 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma, St. Louis, MO) in normal saline at 37℃ for 30 min, and then stored in 4% paraformaldehyde (Longa et al., 1989; Aspey et al., 1998; Lee et al., 2002; Yi et al., 2004). Digital images of brain slices were obtained with a flatbed digital scanner. Infarction volumes were quantified using image analysis software (ImagePro, Media cybernetics, Silver spring, Maryland). Total infarction volume was estimated by adding infarct volume of each coronal slice along the AP axis axis (Aspey et al., 1998; Lee et al., 2002; Yi et al., 2004). In addition, to correct for possible contribution by edema, infarct volumes were normalized to the volume of the intact contralateral hemisphere (Swanson et al., 1990; Yi et al., 2004).

Hematoxylin and eosin (H&E) staining was additionally used for brain sections obtained 7 days or14 days after ischemia, since infiltrated inflammatory cells may take up TTC. Analysis of infarct area was the same in the case with TTC staining. Infarct margins were identified in all sections by closely examining sections at high power.

3. 9. *Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) staining*

To quantify neuronal cell death in brain sections, brain sections were processed for TUNEL staining with the in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN). Brains were harvested 24 h after ischemia, immediately frozen in isopentane and then stored at −70 °C. Coronal brain sections (10-μm-thick) were prepared using a cryostat and mounted on prechilled glass slides coated with poly-L-lysine. Maximum ischemia lesions were detected in the caudatoputamen (striatum) at the level of the optic chiasm. Subsequent brain sections were collected through the optic chiasm. Brain sections were fixed with 4% paraformaldehyde, incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and incubated with TUNEL reaction mixture at 37 ℃ (Lee et al., 2001). The TUNEL stained sections were examined under a fluorescent microscope and photographed. The number of TUNEL positive cells was counted in four randomly chosen fields (100×) of the cortex or striatum. Imaging-Pro Plus software was used to determine the TUNEL-positive cell count.

3.10. *Statistics*

For statistical comparisons, two-tailed t test with Bonferroni correction for multiple comparisons was used.

4. Results

The effects of pyruvate on focal cerebral ischemia were examinedusing the transient and permanent MCA occlusion models. Na- Pyruvate or saline was either ip injected or iv infused over 5–6 h, commencing 30 min after permanent MCA occlusion or 30 min after reperfusion for transient occlusion. Operators were blinded to the identity of the injecting solutions. Physiological parameters, rectal and scalp temperatures, systolic pressure, arterial pH, pCO_2, pO_2 and hemoglobin/hematocrit values, were not significantly different between the pyruvate and saline groups either ip or iv injected (Table 1). There was also no significant difference observed in cerebral blood flow (CBF). In transient focal ischemia, the CBF value was reduced to 31.6 ± 0.4% of the baseline in the NaCl-treated groups and 32.5 ± 0.7% of the baseline in the pyruvate-treated groups.

To measure the infarct size, brains were harvested 24 h later, and stained with TTC. The infarct volume (mm^3) was calculated by integrating the infarct areas in serial coronal sections over the AP axis. The % infarct volume was calculated to correct for the possible contribution from edema. First, we examined pyruvate effects in the transient (1 h) MCA occlusion model. Regardless of the route of administration, ip (Figs. 1A, 1B) or iv (Figs. 2A, 2B), pyruvate produced similar effects; Na-Pyruvate (62.5–250 mg/kg) reduced mean total infarct volume by 56–76% compared with the respective control group, by both direct (Figs. 1A, 2A) and indirect (Figs.1B, 2B) estimations. Interestingly, at 500 mg/kg, pyruvate protection largely disappeared for both ip and iv

administration.

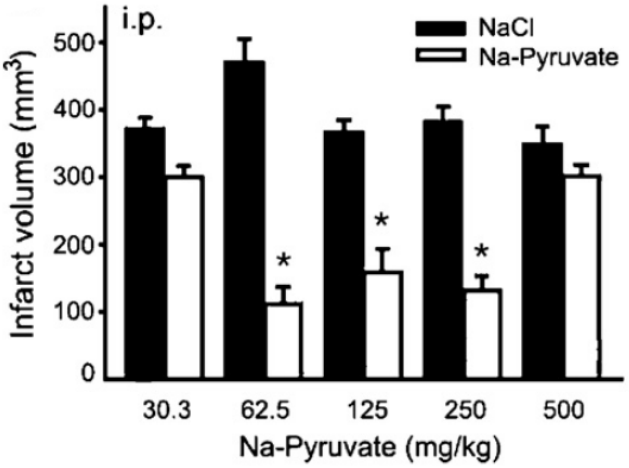
Next, we examined the effects of pyruvate in permanent MCA occlusion. Again, physiological parameters were not significantly different between the pyruvate and saline groups (Table 1). CBF was also not significantly different between the two groups. The CBF decreased to 39.6 ± 0.5% of the baseline in the NaCl-treated group, and 35.6 ± 1.9% of the baseline in the pyruvate-treated group ($p > 0.1$). In the permanent MCA occlusion model for focal ischemia, both ip and iv pyruvate treatment (62.5–250 mg/kg) reduced the infarct volume by 54–85% compared to the control group (Figs. 3, 4). Again, higher dose of pyruvate (500 mg/kg) showed little protective effect (Figs. 3, 4). We then examined cortex and striatum separately (Fig. 5). Although the infarct volume and the extent of pyruvate protection were smaller in striatum than in cortex, pyruvate still significantly reduced the infarct volume in striatum by more than 60%.

To investigate the time window of efficacy, an important issue in stroke neuroprotection, pyruvate (125 mg/kg, ip) was injected at 1 and 3 h after permanent MCA occlusion. We did not examine administration by the iv route because ip and iv did not differ significantly in neuroprotective efficacy. Pyruvate treatment reduced the infarct volume by 60–70% when given 1 h after MCA occlusion (Fig. 6); however, the same dose of pyruvate did not reduce the infarct volume when given 3 h after occlusion.

Table 1. Physiological parameters (mean ± SEM) and mortality

Parameter	NaCl (n=46)		Na-Pyruvate (n=43)	
	Pre	Post	Pre	Post
Rectal temperature (°C)	36.4±0.0	36.5±0.0	36.6±0.0	36.6±0.0
Temporalis temperature (°C)	36.3±0.0	36.7±0.0	36.5±0.0	36.7±0.0
Systolic pressure (mm Hg)	129.8±0.3	130.4±0.4	128.2±0.3	130.4±0.4
pH	7.41±0.0	7.39±0.0	7.41±0.0	7.40±0.0
pCO_2 (mm Hg)	42.1±0.1	42.3±0.2	41.4±0.1	43.2±0.3
pO_2 (mm Hg)	87.8±0.2	80.6±0.3	86.0±0.2	85.1±0.3
Hemoglobin (g/dl)	11.7±0.0	11.4±0.0	11.4±0.0	11.3±0.0
Hematocrit (%)	36.5±0.2	34.1±0.1	34.1±0.1	34.0±0.1

A.



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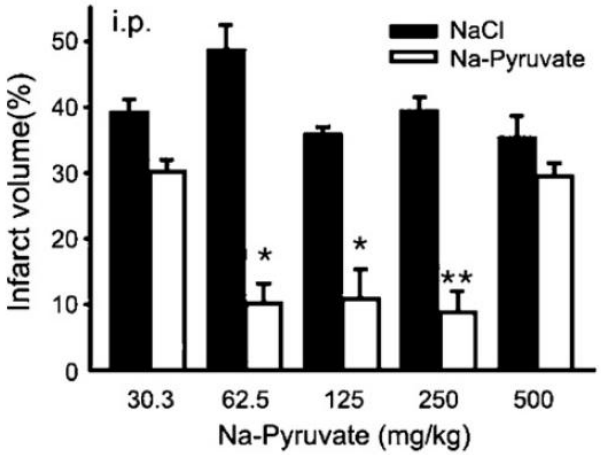
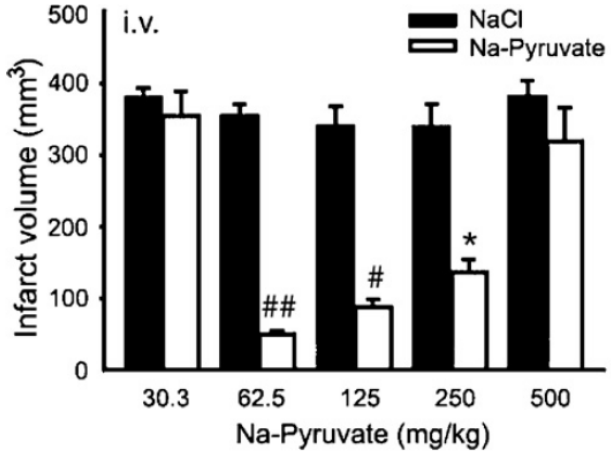


Fig. 1. Pyruvate protects against transient focal cerebral ischemia. Intraperitoneal Na-Pyruvate injection. Bars represent infarct volume (mean+SEM) estimated with TTC staining 24 h after 1 h MCA occlusion, absolute (mm^3, left bars, A) and relative (% of the hemisphere, B). Thirty minutes after the release of MCA occlusion, indicated doses of Na-Pyruvate (n=3–8) were intraperitoneally administered. * denotes difference from saline control (*$P < 0.05$, *$P < 0.01$ two-tailed t test with Bonferroni correction for two comparisons).

A.



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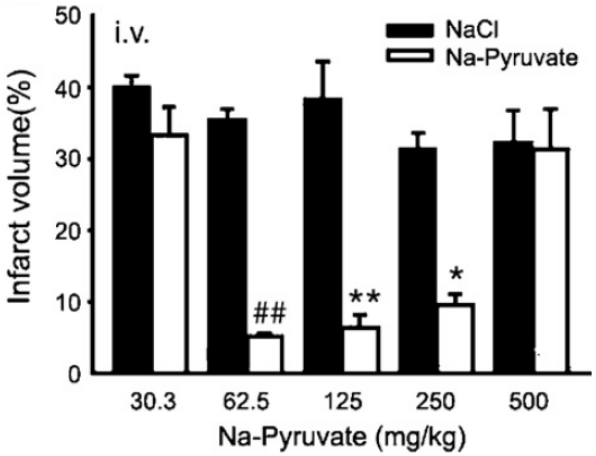
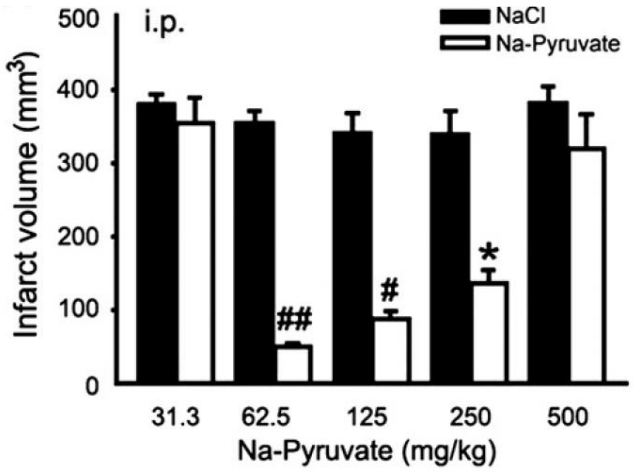


Fig. 2. Pyruvate protects against transient focal cerebral ischemia. Intravenous pyruvate infusion. Infarct volume estimated 24 h after 1 h MCA occlusion, absolute (mm^3, A) and relative (% of the hemispheric volume, B). Thirty minutes after the release of MCA occlusion, rats were injected intravenously with indicated doses of Na-Pyruvate (n=4−7), or osmolarity-matched saline (n=4−7).

* denotes difference from saline control (*P < 0.05 *P < 0.01 #P<0.001, ##P<0.0001, two-tailed t test with Bonferroni correction for two comparisons).

A.



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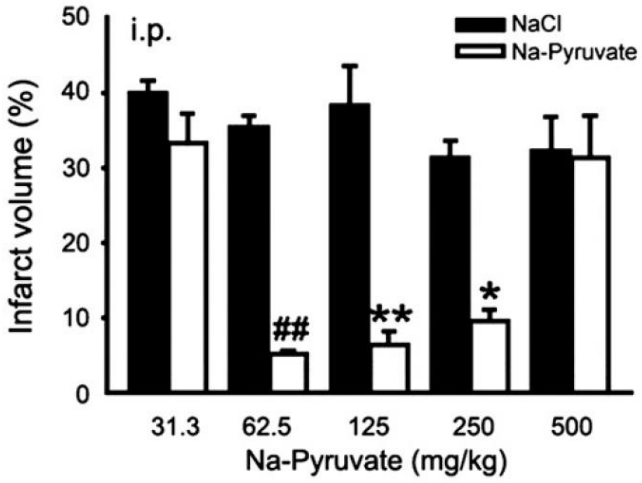
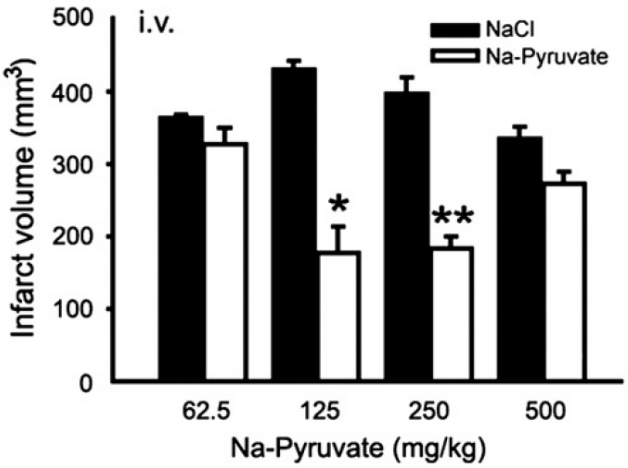


Fig. 3. Pyruvate protects against permanent focal cerebral ischemia. Intraperitoneal pyruvate injection. Bars represent infarct volume estimated with TTC staining 24 h after the onset of permanent MCA occlusion, absolute (mm^3, A) and relative (% of the hemispheric volume, B).Thirty minutes after the onset of MCA occlusion, indicated doses of Na-Pyruvate (n=4−7), or saline (n=4−7), were intraperitoneally administered. * denotes difference from saline control (*$P < 0.05$, # $P < 0.001$, ## $P < 0.0001$, two-tailed t test with Bonferroni correction for two comparisons).

A.



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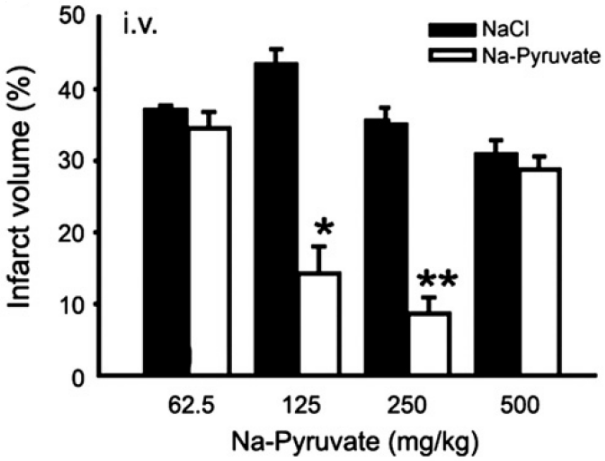


Fig. 4. Pyruvate protects against permanent focal cerebral ischemia. Intravenous pyruvate infusion. Infarct volume estimated 24 h after the onset of permanent MCA occlusion, absolute (mm^3, A) and relative (% of the hemispheric volume, B). Thirty minutes after the onset of MCA occlusion, rats were injected intravenously with indicated doses of Na-Pyruvate (n=6–9), or saline (n=3–6). * denotes difference from saline control (*P < 0.05, **P < 0.001, two-tailed t test with Bonferroni correction for two comparisons).

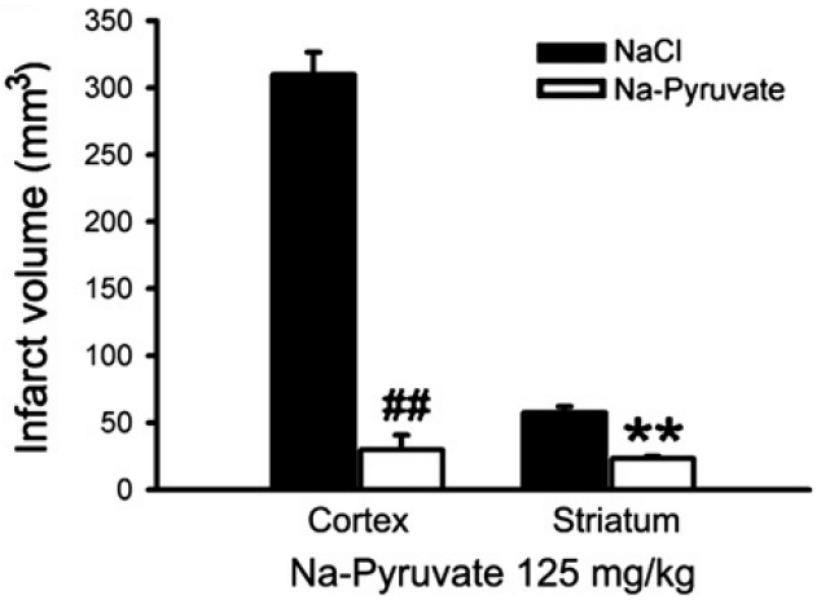
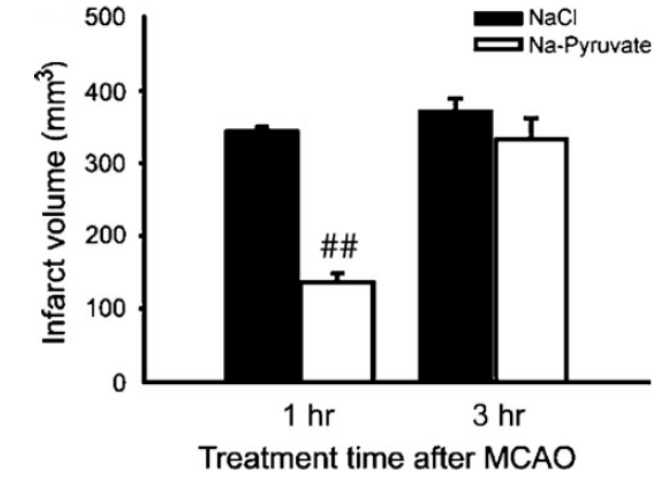


Fig. 5. Pyruvate protects against permanent focal cerebral ischemia. Bars denote infarct volume (mm^3) in cortex and striatum of saline- (n=7) or Na-Pyruvate-treated (125 mg/kg, ip, n=4) rats 24 h after the onset of permanent MCA occlusion.). * denotes difference from saline control (**$P < 0.001$, ## $P < 0.0001$, two-tailed t test with Bonferroni correction for two comparisons).

A.



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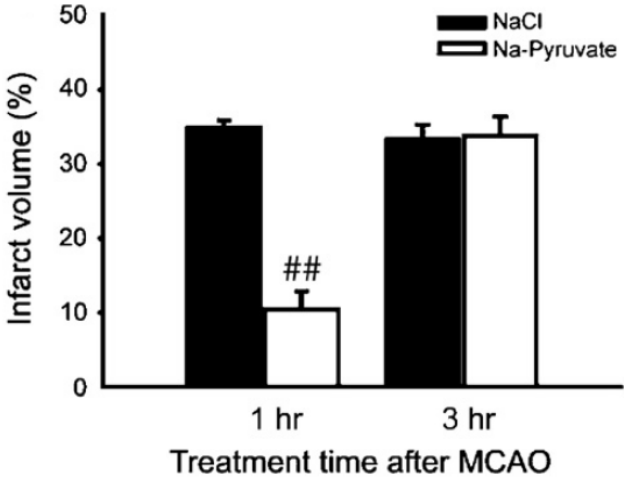
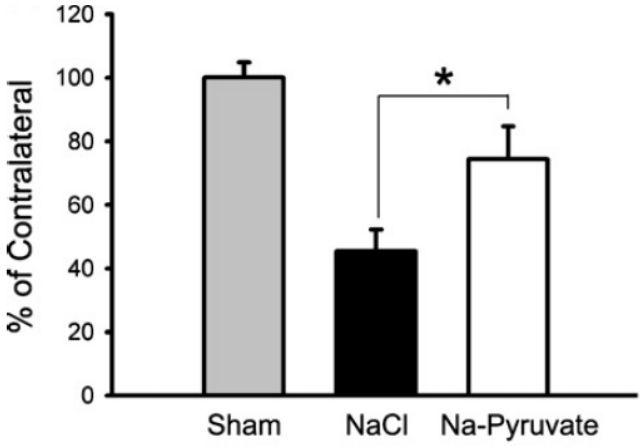


Fig. 6. Delayed treatment with pyruvate. Infarct volume 24 h after permanent MCA occlusion (n=5−11), absolute (mm^3, A) and relative (% of the hemispheric volume, B). One or three hours after MCA occlusion, rats were intraperitoneally injected with Na-Pyruvate (125 mg/kg) or saline. * denotes difference from saline control ## $P < 0.0001$, two-tailed t test with Bonferroni correction for two comparisons).

The mechanism of pyruvate protection is still not completely understood. Sheline and colleagues (2000) proposed that the normalization of NAD^+ levels may underlie the pyruvate protection against zinc toxicity in vitro. Consistent with this idea, we found that pyruvate substantially inhibited the reduction in NAD^+ levels in the ischemic area (Fig. 7A). However, nicotinamide that inhibits NAD^+ depletion, at a dose previously reported to reduce infarcts in Wistar rats (500 mg/kg; Ayoub et al., 1999), showed no protection in permanent MCA occlusion (Fig. 7B), suggesting that additional mechanisms may contribute to marked protective effects of pyruvate in this model.

Another key issue for neuroprotection in brain ischemia is the maintenance of protection. The extent of maintenance of neuroprotection was examined by harvesting brains 24 h and 14 days after MCA occlusion. Pyruvate or saline was given 30 min after the onset of ischemia. Brainswere stained withTTC to estimate infarct size 14 days after ischemia (Fig. 8). Control rats treated with saline developed large infarcts in the frontoparietal cortex and caudate nucleus 24 h after MCA occlusion. Tissue atrophy led to a reduction in the size of the infarct measured at 14 d (Figs. 9A, 9B). Despite the effect of tissue atrophy on infarct measurements, pyruvate treatment had the capacity to significantly reduce the infarct volume directlymeasured at this time point. Furthermore, when the indirect method was used (to correct for tissue atrophy), the infarct volume was similar to that measured at the 24 h time point (Fig. 9B) with pyruvate treatment reducing the infarct volume by approximately 50% at 14 d after MCA occlusion.

A.



B.

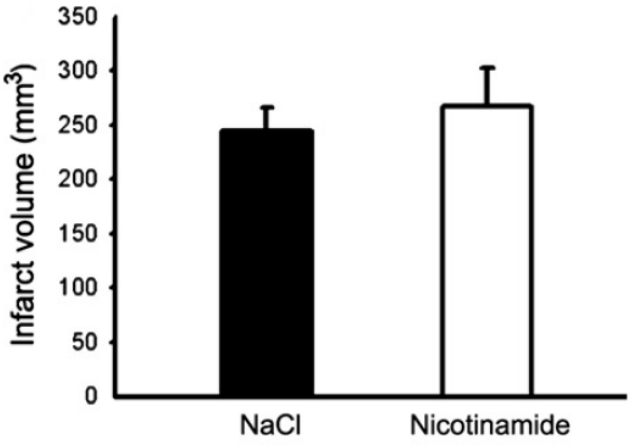


Fig. 7. Possible mechanism of pyruvate protection. (A) Effects of pyruvate on NAD^+ levels in the ischemic area. Bars (n=4–8) denote levels of NAD^+ in ischemic areas of rats (% of contralateral side), saline- or Na-Pyruvate-treated rats 24 h after the onset of permanent MCA occlusion. (B) Effects of nicotinamide on infarct size. Bars (n=4) denote infarct volume (mm^3) of saline- or nicotinamide-treated rats 24 h after the onset of permanent MCA occlusion).

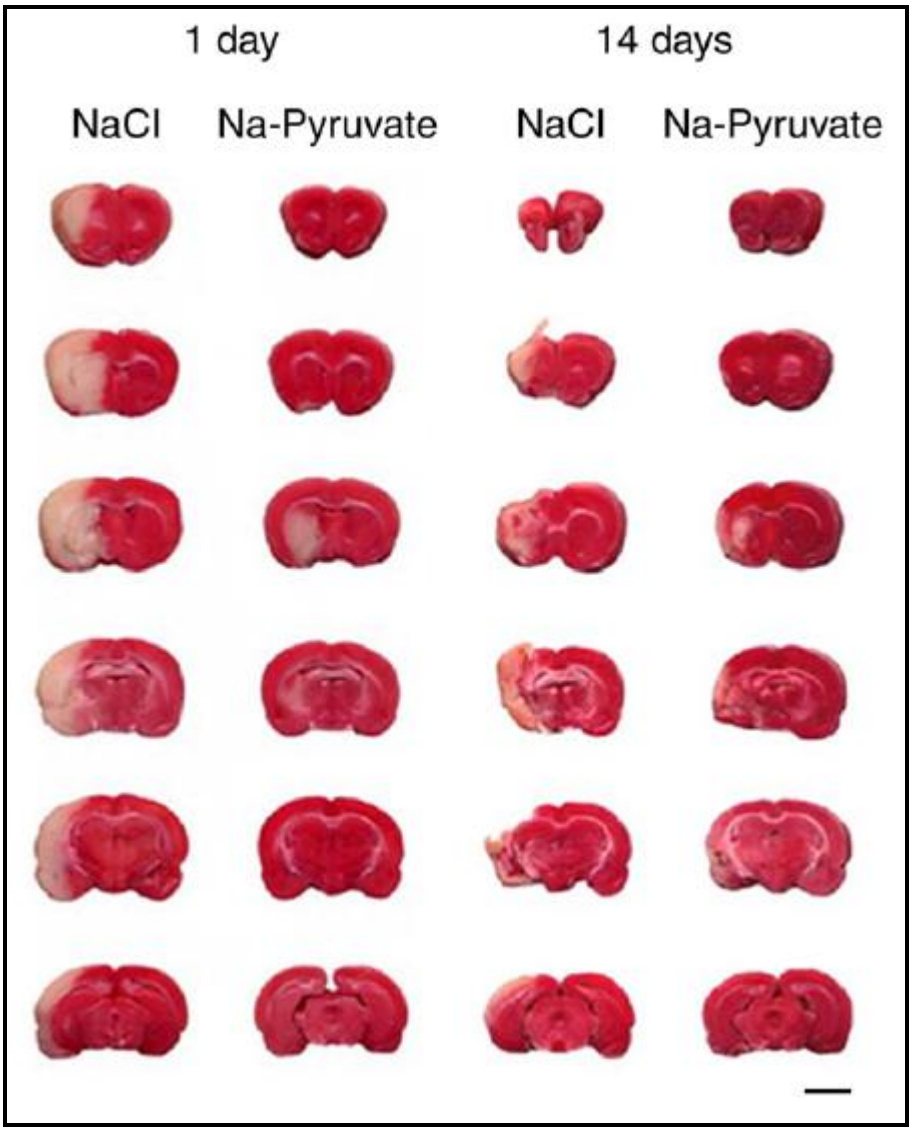
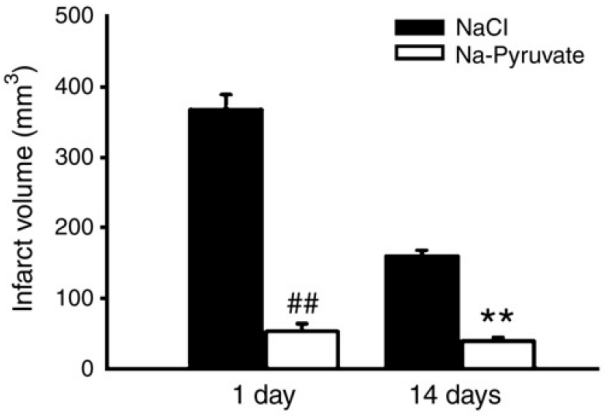


Fig. 8. Sustainability of protection against permanent focal cerebral ischemia. Thirty minutes after MCA occlusion, rats (n=5−11) were intraperitoneally injected with Na-Pyruvate (125 mg/kg) or saline. Twenty-four hours or 14 days later, rat brains were removed and 2-mm-thick coronal sections stained with TTC. Sections were photographed. Scale bar, 5 mm.

A.



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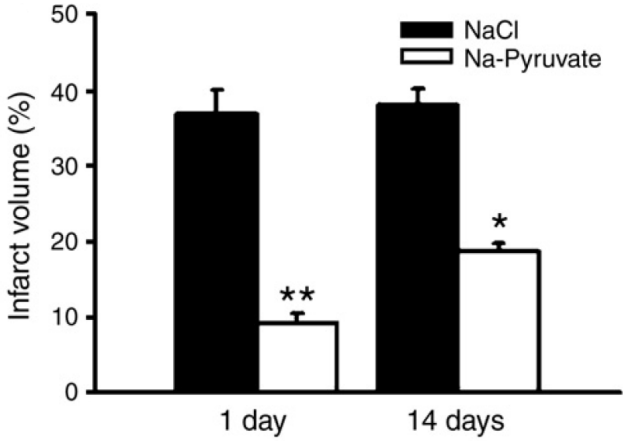
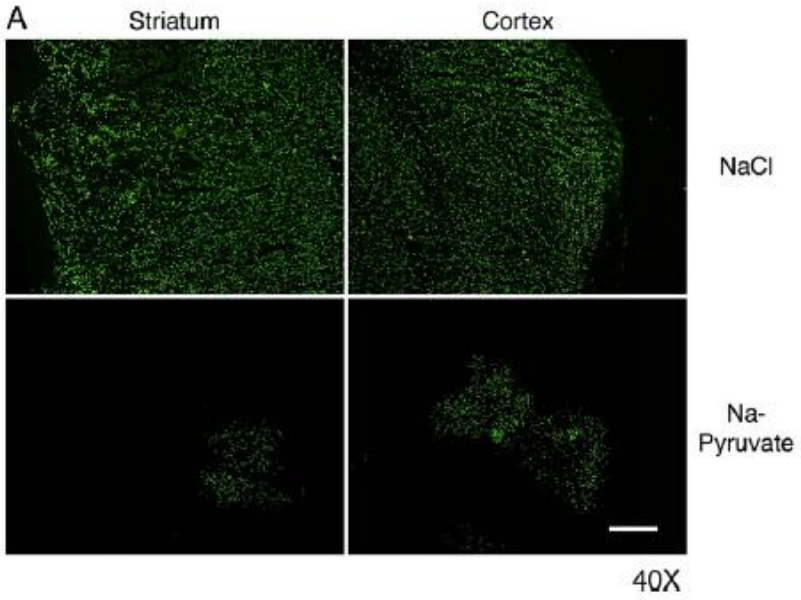


Fig. 9. Sustainability of protection against permanent focal cerebral ischemia. Thirty minutes after MCA occlusion, rats (n=5–11) were intraperitoneally injected with Na-Pyruvate (125 mg/kg) or saline. Twenty-four hours or 14 days later, rat brains were removed and 2-mm-thick coronal sections stained with TTC. Infarct volume was measured with the direct (mm^3, A) and indirect (% of contralateral side, B) * denotes difference from saline control (*$P < 0.05$, **$P < 0.001$, ## $P < 0.0001$, two-tailed t test with Bonferroni correction for two comparisons).

To confirm the protective effect at the single cell level, sections were stained according to the TUNEL method. In both the cortex and striatum, numerous TUNEL (+) neurons were identified in the saline controls (Figs. 10). By comparison, the pyruvate-treated rats showed a markedly reduced number of TUNEL (+) neurons in both the cortex and striatum (by 89% in the cortex and by 65% in the striatum). This degree of protection correlated closely with the degree of infarct reduction in either area (Fig. 9).

In addition to histological protection, pyruvate treatment reduced weight loss, a functional parameter of ischemic stroke in rats, 24 h after MCA occlusion (Fig. 11A). The pyruvate group gained more than 20 g over the baseline level 14 days after MCA occlusion while the saline group failed to regain weight. The rota-rod was used to assess overall motor function. The initial score on the day of the operation, measured as the length of time the rats held onto the rotarod, was 92±9.7 (SEM) s (Fig. 11B). The score increased to 155± 13.3 s 14 days after the sham operation, probably reflecting a learning effect. In saline-treated rats that underwent MCAocclusion, the score 14 days after the operation was significantly lower than the initial score or the score 14 days after the sham operation. In contrast, in pyruvate-treated rats that underwent MCA occlusion, the score 14 days after the operation was significantly greater than the initial score and almost the same as the score 14 days after the sham operation. These results show that scores for the saline and pyruvate groups 14 days after MCA occlusion are highly significant.



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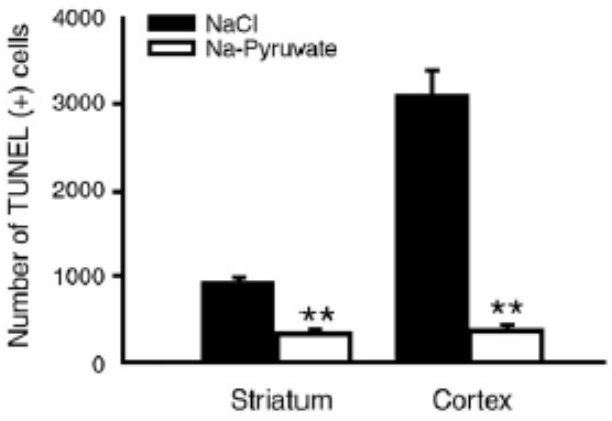
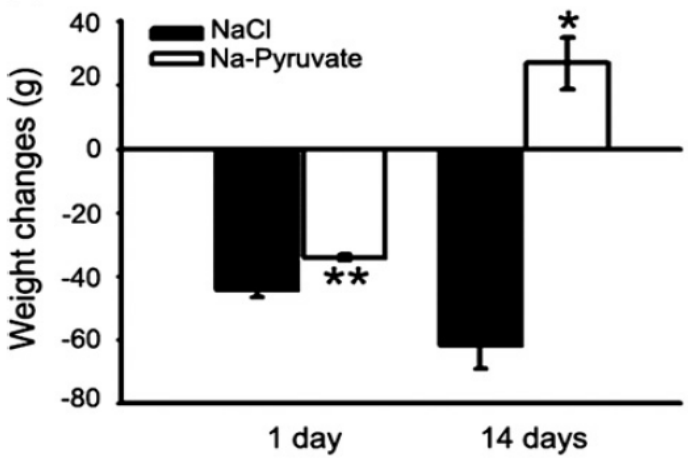


Fig. 10. Neuronal death. (A) Thirty minutes after MCA occlusion, rats were intraperitoneally injected with Na-Pyruvate (125 mg/kg) or saline. Twenty-four hours later, rat brains were removed and stained with the TUNEL method in cortex and striatum. (B) Bars denote the number of TUNEL-positive neurons in cortex and striatum (n=3−4). Scale bar, 500 μm.

A.



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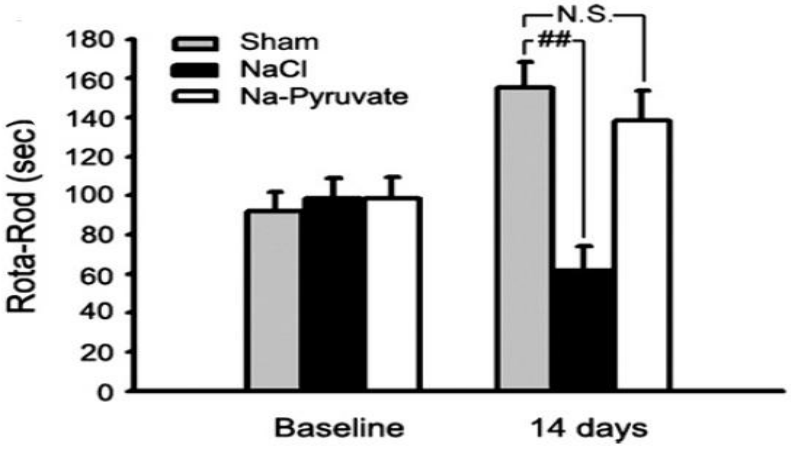


Fig. 11. Functional parameters. (A) Bars represent percent change of body weight (n=5−11) at 1 and 14 days after MCA occlusion, from values obtained immediately prior to MCA occlusion. (B) Bars denote rota-rod scores (seconds) before ischemia or 14 days after sham operation (n=11) or 14 days after MCA occlusion in saline (n=9) and pyruvate groups (n=8). * denotes difference from saline control (*P < 0.05, **P < 0.01, two-tailed t test with Bonferroni correction for two comparisons).

MR spectroscopy in living rats was performed to assess metabolic changes within the ischemic areas. At 1, 2, 6 and 24 h after MCA occlusion, ischemic and non-ischemic contralateral brain regions [3×3 mm^2 square (box, Fig. 15) with 2.5 mm thickness] were subjected to 1H-MRS. Pyruvate (125 mg/kg) was given ip 30 min after the occlusion of the MCA. The lactate doublet peak, the marker for ischemic injury, began to rise in the ischemic area 1 to 2 h after MCA occlusion (not shown), and continued to rise after 6 h (Figs. 12, 13). The NAA peak, a marker for neurons, decreased concurrently after 6 h. Consistent with the protective effect of pyruvate, the average height of the lactate peak (normalized to creatine) was substantially lower in the pyruvate group than in the saline controls after 6 h of ischemia (Figs. 12, 14A). As shown above, a high dose (1000 mg/kg) of pyruvate (ip) did not protect against focal ischemia. This result is consistent with the lack of reduction of the lactate peak by high dose pyruvate treatment (Figs. 13, 14B).

Finally, diffusion-weighted MR imaging was performed to assess infarct formation in living rats. Results from these experiments also demonstrated substantial protection by 125 mg/kg pyruvate, but not by 1000 mg/kg pyruvate at 6 and 24 h after the onset of permanent MCA occlusion (Figs. 15). Subsequent postmortem brain examination with TTC staining confirmed the dosedependent protective effect of pyruvate in these rats.

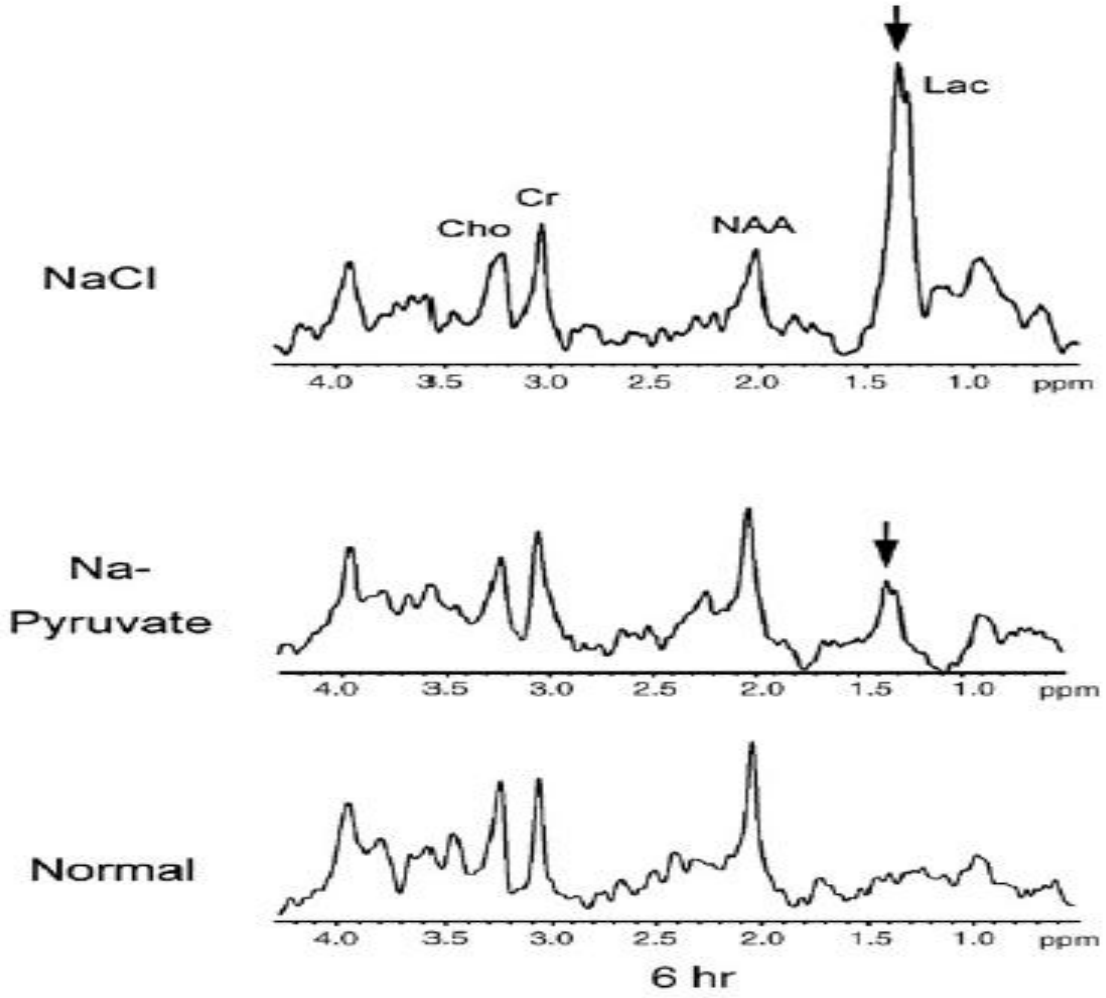


Fig. 12. Magnetic resonance spectroscopy (MRS). MRS data were obtained from ischemic brain regions 6 h after MCA occlusion in rats injected ip with saline (upper) or 125 mg/kg Na-Pyruvate (middle). For reference, MRS data from a non-ischemic brain region are also presented (bottom). Compared with the size of the lactate doublet peak (arrows) in saline control, which in the Na-Pyruvate-treated rat was significantly smaller.

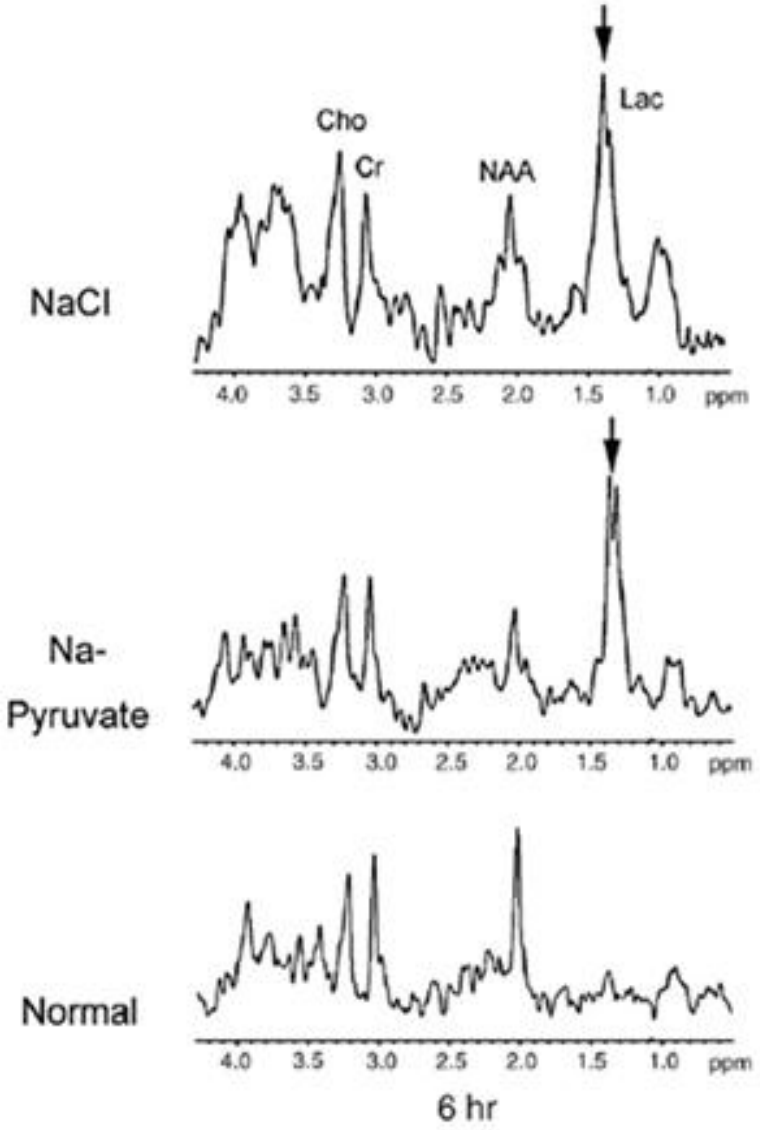
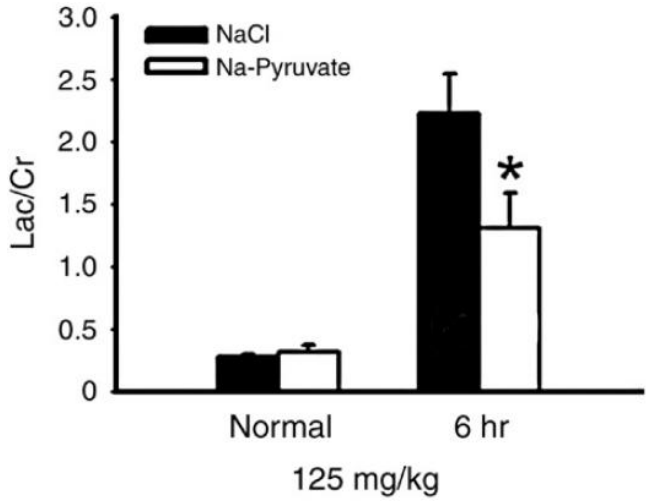


Fig. 13. Magnetic resonance spectroscopy (MRS). MRS data from ischemic brain regions 6 h after MCA occlusion in rats injected ip with saline (upper) or 1000 mg/kg Na-Pyruvate (lower). Again, MRS data from a non-ischemic brain region are also presented (bottom). The lactate peaks (arrows) appear similar in size in both cases.

A.



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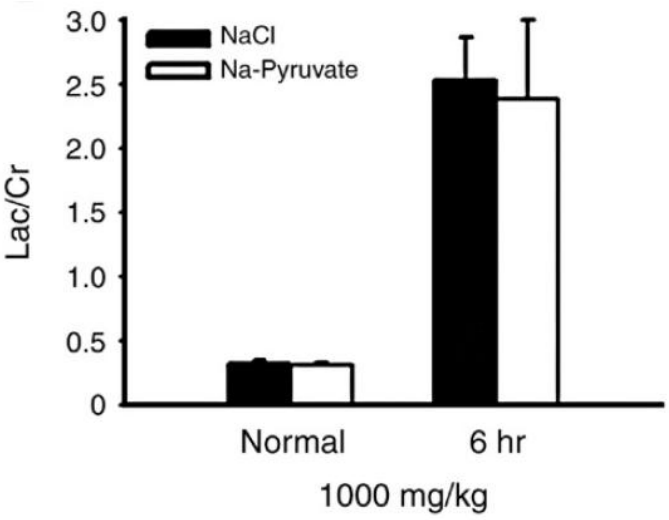

Fig. 14. Magnetic resonance spectroscopy (MRS). (A) Bars denote the lactate/creatine ratio (mean+SEM, n=7−10) before or 6 h after MCA occlusion in rats treated with saline or 125 mg/kg Na-Pyruvate. The lactate/Cr ratio increased after ischemia in both the saline control and the Na- Pyruvate-treated groups. However, compared with the saline control,

the lactate/Cr ratio in the Na-Pyruvate group (125 mg/kg) was significantly smaller.**).** (B) Bars denote the lactate/creatine ratio (mean+SEM, n=4−8) before or 6 h after MCA occlusion in rats treated with saline or 1000 mg/kg Na-Pyruvate. In this nonprotective dose, the lactate/Cr ratio in the Na-Pyruvate group rose to the same level as in the saline control.

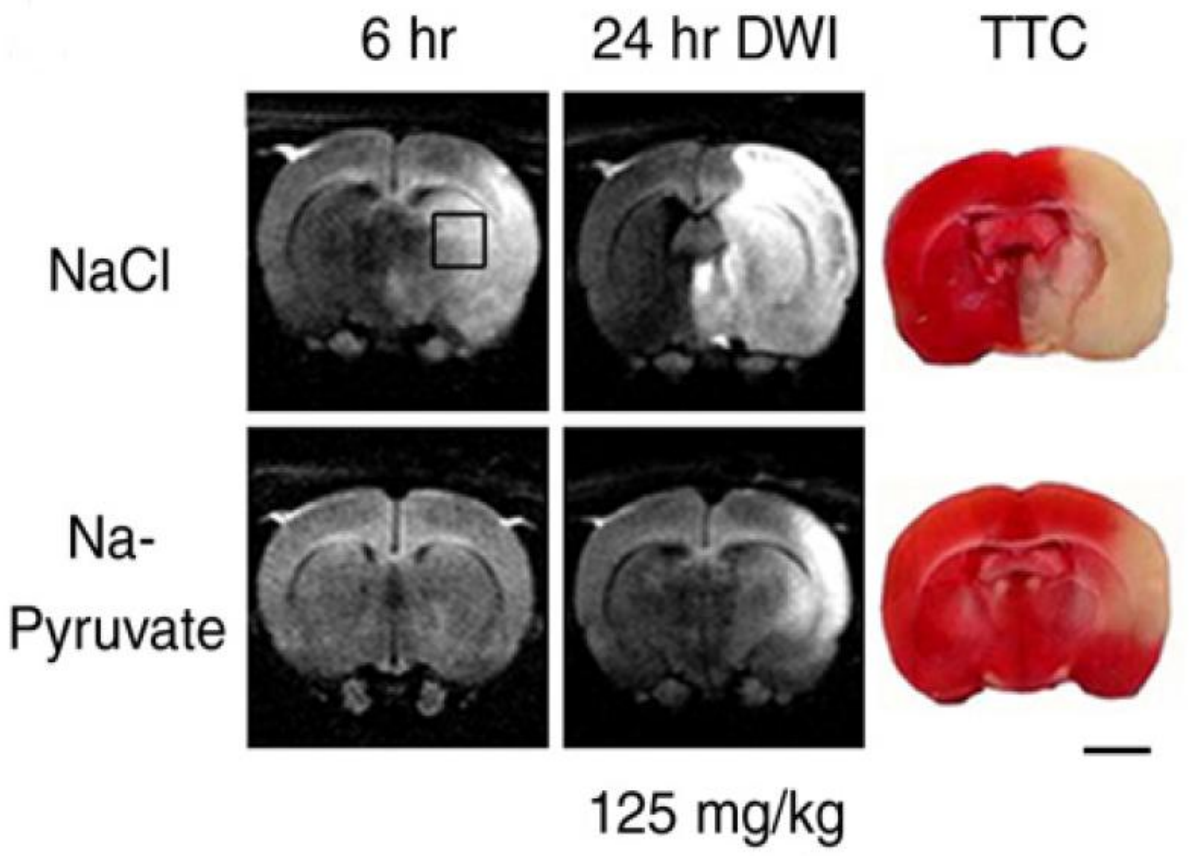

Fig. 15. Diffusion-weighted MR imaging of Na-Pyruvate (125 mg/kg) treated. Diffusion-weighted MR images (DWI) of rat brains 6 and 24 h after MCA occlusion, saline- or Na-Pyruvate (125 mg/kg)-treated. AfterMRimaging, brains were harvested and stained with TTC. Box, 3×3 mm2; scale bar, 5 mm.

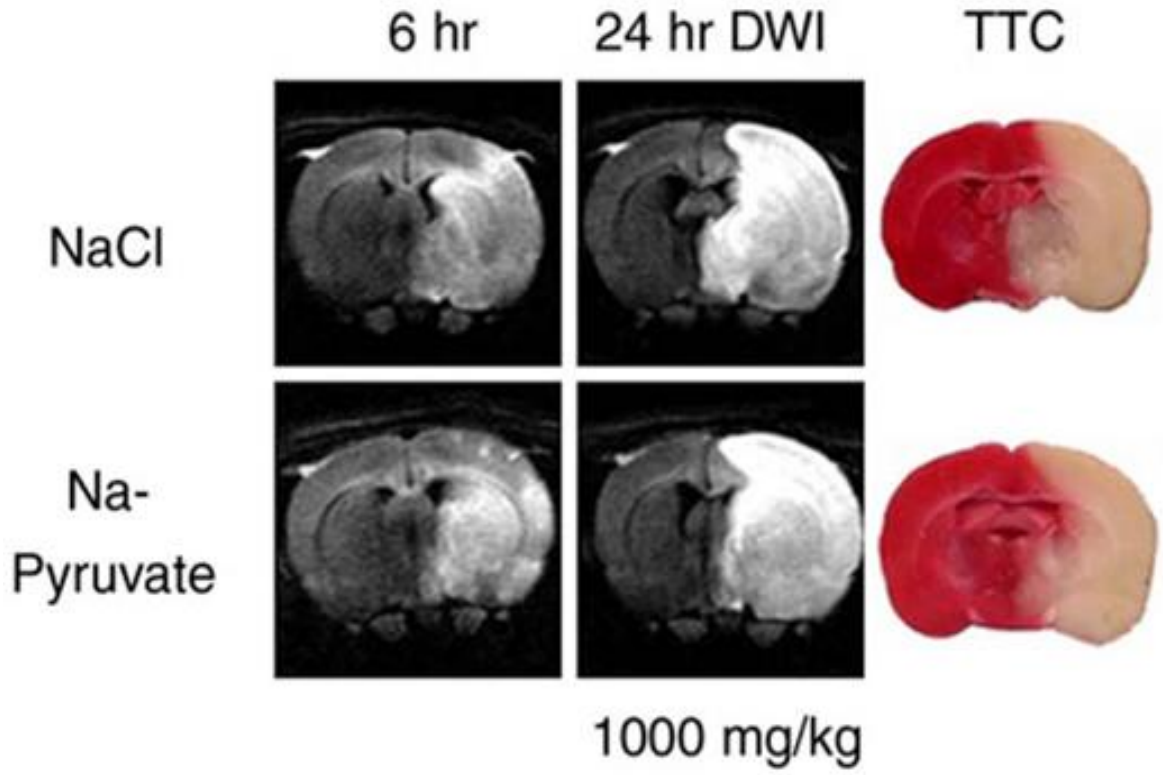

Fig. 16. Diffusion-weighted MR imaging of Na-Pyruvate (1000 mg/kg) treated. Diffusion-weightedMR images in saline- or Na-Pyruvate (1000 mg/kg)-treated rats. White areas represent areas that are destined to form infarcts. After MR imaging, brains were harvested and stained with TTC. At this high dose, no protection was noted in either diffusion-weighted images or images stained with TTC. Box, 3×3 mm2; scale bar, 5 mm.

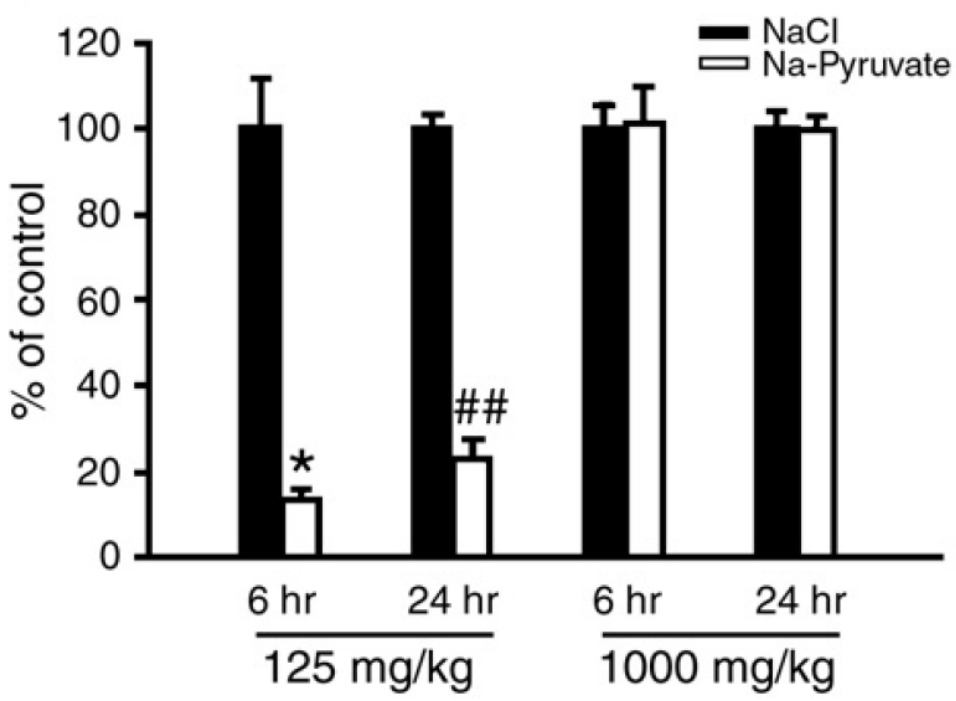

Fig. 17. Diffusion-weighted MR changing. Bars denote % areas of diffusion weighted MR changes (saline control as 100%, n=3−4) at 6 and 24 h after the onset of permanent MCA occlusion in rats treated with 125 mg/kg or 1000 mg/kg Na-Pyruvate.

5. Discussion

The present study demonstrates that pyruvate, an endogenous metabolite of the glycolytic pathway, is remarkably effective in both transient and permanent models of focal cerebral ischemia. No difference in infarct size or in pyruvate protective effects was noted between transient and permanent MCA occlusions. The infarct volumes ranged between 350 and 450 mm^3 in both cases. At doses of 62.5–250 mg/kg, pyruvate reduced the infarct size by 50–80%. In the permanent MCA occlusion model, the protective effect of pyruvate lasted up to 14 days. Since little difference in the protective efficacy of pyruvate was noted between ip and iv administration, both routes may result in similar blood and brain levels.

The infarct reduction by pyruvate seems well-correlated with improvements in functional parameters such as body weight and motor ability. Of various behavioral tests, we performed the rota-rod test to functionally assess the overall motor function, as motor areas like striatum were included in the area significantly protected by pyruvate.

In a previous study, we showed that 500–1000 mg/kg pyruvate offered significant protection against selective neuronal death induced by transient global cerebral ischemia. These findings are not consistent with results from the present study, which showed that the same high dose of pyruvate failed to reduce infarct size in models of focal brain ischemia. The reason for the U shaped dose response curve of pryuvate protection in focal ischemia is not known. One possibility is that at high doses of pyruvate the accumulated lactate may

contribute to neuronal injury (Kalimo et al., 1981; Wagner et al., 1992; Siesjo et al., 1996) and negate the neuroprotective effect of pyruvate in focal ischemia. The reason why high doses of pyruvate lose protective effects only in focal ischemia, may be because anaerobic metabolism is more prolonged in focal ischemia than in global ischemia. Consistently, the increase of lactate peaks lasts only 1 h in global ischemia (Nagamoto et al., 1995) but longer than 24 h in focal ischemia. If so, the pyruvate→lactate conversion may be greater and more detrimental in focal ischemia.

As most pyruvate is converted to lactate in complete ischemia, the observation of smaller lactate peaks in ischemic areas of pyruvate-treated rats compared to those in saline controls by 1HMRS implies that significant penumbral areas with borderline ischemia may be present even in the permanent occlusion model (Garcia et al., 1993; Zhang et al., 1994; Lipton, 1999). In penumbral areas, neurons may still have some capacity for oxidative phosphorylation, and the addition of protective doses of pyruvate may help restore energy balance through the TCA cycle.

In contrast, with high doses of pyruvate, the greater rate of lactate formation may overwhelm the TCA cycle-mediated pyruvate protection, resulting in diminished protection. Another possibility, although seems less likely, is that the U shaped dose response curve is produced by lactate-unrelated mechanisms (e.g. changes in perfusion, BBB permeability, inflammation, etc.). In this case, the high lactate peaks in ischemic areas of rats treated with high dose pyruvate may

be not the cause but a result of more extensive neuronal injury. Although our results are consistent with the possibility that higher doses of pyruvate lose its protective effects by increasing the lactate production, more systematic studies may be needed to address the precise mechanism for the U shaped dose response.

The marked protective effect of pyruvate in rat models of focal cerebral ischemia observed in the present study, at first glance, does not seem to agree with negative results obtained with 250–1000 mg/ kg of pyruvate in the permanent MCA occlusion model (Gonzalez- Falcon et al., 2003). Although Gonzalez-Falcon et al. (2003) tested 250 mg/kg that did not reduce infarcts, doses lower than 250 mg/kg, which showed marked infarct-reducing effects, were not examined. On the other hand, the results of the present study are in agreement with those of Gonzalez-Falcon et al. (2003) with respect to the lack of protective effect of high doses of pyruvate against focal cerebral ischemia. Difference in results at 250 mg/kg pyruvate, may be due to differences in anesthesia, temperature control and/or operative procedures.

Although the cytoprotective effect of pyruvate is reported in a number of cell systems including hypoxia, ischemia, oxidative stress and metal toxicity, the mechanism underlying the protective effect of pyruvate is largely unknown (Wang and Cynader, 2001; Alvarez et al., 2003; Chang et al., 2003; Mazzio and Soliman, 2003; Sharma et al., 2003; Chung et al., 2004; Yoo et al., 2004; Frenzel et al., 2005). There is some evidence that pyruvate is a direct antioxidant (O'Donnell-Tormey et al., 1987; Desagher et al., 1997). In addition, Sheline et al.

(2002) suggest that pyruvate functions to normalize NAD+ levels in zinc-injured cells. We also found that pyruvate is a highly effective protectant against zinc toxicity in cultured cortical cells, but had no effect against calcium-overload excitotoxicity (Lee et al., 2001). The role of endogenous zinc in neuronal death has been well established in transient global ischemia. Although not as clear in the MCAocclusion model of focal ischemia, zinc accumulation in degenerating neurons was observed. Together, these results suggest that zinc contributes to ischemic neuronal death in both focal and global cerebral ischemia (Koh et al., 1996; Sorensen et al., 1998; Lee et al., 2001; Lee et al., 2002; Calderone et al., 2004; Yi et al., 2004; Bonanni et al., 2006). It is possible that the neuroprotective capacity of pyruvate observed in the present study involves protection against zinc toxicity. As in the case of zinc toxicity, NAD+ levels decreased in the infarct core, and pyruvate inhibited it. Hence, this effect may contribute to pyruvate protection. However, nicotinamide, a PARP inhibitor that was previously reported to be modestly effective in focal ischemia (Ayoub et al., 1999; Maynard et al., 2001), showed no protective effects in our study. Hence, additional mechanisms such as direct antioxidative effects (O'Donnell-Tormey et al., 1987; Desagher et al., 1997) may also contribute. Further investigations are required into the role of pyruvate in protection against zinc toxicity and to determine the mechanism underlying the neuroprotective capacity of pyruvate.

Pyruvate is a natural metabolite of the glycolytic pathway and hence should not lead to serious side effects. One potential concern is the possibility that

excess pyruvate in the ischemic area may lead to accumulation of lactate, which may aggravate acidosis and cell injury associated with focal cerebral ischemia (Rehncrona et al. 1981, 1985; Kalimo et al., 1981; Siemkowicz, 1985). In the present study, we showed that at the high non-protective doses of pyruvate, the level of lactate increases, whereas at the lower protective doses, there was no increase in the level of lactate. This U-shaped dose response may be a significant problem in the development of pyruvate as a neuroprotective drug for ischemic stroke. Despite losing efficacy at higher doses, the likely lack of serious side effects and the rather remarkable degree of protection of neurons at lower doses (62.5–250 mg/kg) make pyruvate an attractive neuroprotectant candidate in ischemic stroke.

Chapter 4. Conclusion

The present study demonstrated that protease-defective S478A of tissue plasminogen activator and pyruvate provided therapeutic effects in rat brain ischemia model.

At first we demonstrated that intraventricular administration of tPA or its non-protease mutant, S478-tPA, 15 min after MCA occlusion, provided substantial brain protection in rats. The fact that tPA completely loses its protective efficacy after heat denaturation, indicates that the protective effect of tPA or S478- tPA is entirely dependent upon the native protein conformation. Both tPA and S478A-tPA reduce the infarct volume more than 60% and the protective effects of tPA and S478A-tPA persisted 7 days after ischemia induction. The functional improvement in motor deficit scores and the degree of weight reduction 7 days after ischemia was also notable.

At second we showed that pyruvate, an endogenous metabolite of the glycolytic pathway, was remarkably effective in both transient and permanent models of focal cerebral ischemia. Distinct protective effects of pyruvate were demonstrated on both transient and permanent MCA occlusions. The infarct volumes were ranged between 350 and 450 mm^3 in both transient and permanent MCA occlusions. However, pyruvate treatment (62.5–250 mg/kg) reduced the infarct sizes significantly (50-80%). In the permanent MCA occlusion model, the protective effect of pyruvate lasted up to 14 days. Since little difference in the protective efficacy of pyruvate was noted between ip and iv administration, both

routes may result in therapeutic effects.

The infarct reduction by pyruvate seems well-correlated with improvements in functional parameters such as body weight and motor ability. Of various behavioral tests, we performed the rota-rod test to functionally assess the overall motor function because motor areas like striatum were significantly protected by pyruvate. Magnetic resonance (MR) spectroscopy revealed that protective doses of pyruvate, but not the non-protective doses, were associated with a reduction in the level of lactate compared with saline controls. Diffusion-weighted MR images further confirmed infarct reduction in pyruvate-treated rats.

Since S478A-tPA lacks protease activity, which has been implicated in causing cerebral hemorrhage or aggravating excitotoxicity, its parenchymal neuroprotective effect may be useful in treatment of ischemic stroke. In addition, pyruvate is an endogenous metabolite glycolysis, and hence is unlikely to have serious side effects. The combined use of a neuroprotective agent would enhance neuron survival and thereby extend the window of opportunity for thrombolytic treatment to potentially benefit more patients.

Chapter 5. References

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국문초록

랫드 뇌 허혈성 모델에서 프로테아제 결손 조직플라스미노겐 활성인자와 피루브산의 치료효과

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수세기 동안 허혈로 인해 야기되는 뇌졸중 치료 약물에 대해 많은 연구가 진행 되었으나 아직까지 표준 치료제를 찾지 못하고 있는 실정이다. 본 연구는 뇌졸중 치료 가능성 약물을 찾아 랫드를 이용한 뇌허혈 모델에 투여 후 치료 효과를 증명하는 데에 목적을 두고 실행하였다.

첫번째 뇌졸중 치료 가능 약물로 재조합 조직 플라스미노겐 활성제(tPA)와 프로테아제 결손 조직플라스미노겐 활성인자(S478-tPA)를 선택하여 효소 비의존성으로 뇌실질 보호 효과를 확인하였다. 뇌조직 세포 배양 조건에서는 tPA와 S478A-tPA는 아연에 의한 세포독성에 대해서는 뇌세포 보호 효과가 있었다. 랫드 영구적인 뇌허혈 모델에 중뇌동맥 폐쇄 15분 후 플라스민이 적은 뇌실로 tPA와 S478A-tPA를 투여시 대조군에 비해 65-70% 뇌경색이 감소 되었다. 뇌허혈 일주일 이후에도 대조군에 비해 뇌경색 감소 효과와 운동결손이 개선되는 뇌졸중 치료

효과는 지속되었다.

두번째로 피루브산을 선택하여 뇌졸중 치료효과를 관찰하였다. 피루브산을 랫드의 일시적 (1시간) 또는 영구적 뇌허혈 모델에 복강 또는 정맥주사로 62.5-250 mg/kg 농도로 투여시 투여방법과 농도에 상관없이 뇌경색이 대조군에 비해 50%이상 줄어드는 것을 확인 할 수 있었다. 영구적인 뇌허혈 모델에서는 투여 14일 이후에도 뇌경색과 운동결손을 감소시키는 뇌졸중 치료효과를 보였다. 흥미롭게도 높은 농도의 피루브산투여는 뇌경색을 감소시키지는 않았다. 이는 수소자기공명분광분석을 통한 젖산 농도 측정시 치료농도에 비해 높은 농도를 보여주는 것으로 확인할 수 있었다.

S478A-tPA는 뇌출혈과 흥분세포독성을 증가시키는데 관여하는 단백질 효소분해활성 기능은 없으면서 뇌조직실질에 신경보호효과와 뇌경색치료에 효과적이라고 할 수 있다. 피루브산나트륨는 인체 내생의 해당작용 대사산물로서 알려진 부작용이 거의 없는 무해한 당이며 저가의 약물이다. 본 실험의 결과로 이 두가지 약물은 인체 부작용이 없는 뇌졸중 치료 후보 약물로 사용가능성을 제시할 수 있다.

주요어: 뇌졸중, 랫드 뇌 허혈, 조직 플라스미노겐 활성제, 피루브산, 신경보호효과, 수소자기공명분광분석

학번: 2008-30472