Inhibition of the Cerebral Ischemic Injury by Ethyl Pyruvate With a Wide Therapeutic Window

Young-Mi Yu; Jung-Bin Kim; Kang-Woo Lee, PhD; Seong Yun Kim, MD, PhD; Pyung-Lim Han, PhD; Ja-Kyeong Lee, PhD

Background and Purpose—Ethyl pyruvate (EP) is a pyruvate derivative that has been reported recently to prevent lethality in mice with established lethal sepsis and systemic inflammation. In this study, we examined the neuroprotective effect of EP in a rat cerebral ischemia model of middle cerebral artery occlusion (MCAO).

Methods—Male Sprague-Dawley rats were subjected to 1 hour of MCAO, and EP was administered at various time points before or after MCAO. The changes in the brain infarction, neurological deficits, microglia activation, and proinflammatory cytokine expression were evaluated. BV2 microglial cells were also used to access the anti-inflammatory effect of EP.

Results—The administration of EP intraperitoneally at 30 minutes before or at 4 or 12 hours after MCAO reduced the infarct volume to 10.3±3.4% (n=6; P<0.05), 21.5±2.7% (n=6; P<0.05), and 44.3±4.0% (n=6; P<0.05), respectively, of that of the control group. The significant reduction in infarct volume was accompanied by the suppression of the clinical manifestations associated with cerebral ischemia, including motor impairment and neurological deficits, microglial activation, and proinflammatory cytokine expression. The neuroprotective effect of EP was yet evident when it was administered as late as 24 hours after MCAO/reperfusion (76.5±4.70%; n=6; P<0.05). EP suppressed lipopolysaccharide induced activation of BV2 cells, as was evidenced by a reduction in NO release and the accompanying induction of proinflammatory cytokines.

Conclusions—These results suggest that EP affords the strong protection of the delayed cerebral ischemic injury with a wide therapeutic window. (Stroke. 2005;36:2238-2243.)

Key Words: middle cerebral artery occlusion■ microglia■ inflammation■ neuroprotection

Cerebral ischemia leads to brain injury through a complex series of pathophysiological events leading to neuronal death and subsequent neurological dysfunction. The major pathogenic mechanisms of this cascade may include glutamate excitotoxicity, Zn2+ toxicity, peri-infarct depolarization, inflammation, and apoptosis. Excitotoxicity and Zn2+ toxicity result in acute and massive neuronal death in the ischemic core.1 There is a wealth of evidence to suggest that this acute neuronal damage is followed by a second round of neuronal injury, called delayed neuronal death, in the neighboring areas of the ischemic core.2 Of the many pathophysiological events that may contribute to this delayed injury, the cell-mediated processes that are associated with postischemic inflammation and apoptosis have been studied extensively.3

Pyruvate is a key intermediate in the metabolism of glucose and a potent reactive oxygen species (ROS) scavenger. The protective effects of pyruvate against oxidative stress have been reported in various tissues including neurons.4,5 However, the usefulness of pyruvate as a therapeutic agent is abrogated by its poor stability in solution.6 Ethyl pyruvate (EP) is a stable and lipophilic derivative of pyruvate.7 EP inhibits the structural and functional damage in the intestinal mucosa induced by mesenteric ischemia/reperfusion in rats.8,9 EP also protects various tissue injuries related with lethal sepsis and systemic inflammation and increases the survival rate.10,11

In the current study, we explored the neuroprotective effect of EP in the posts ischemic brain using a rat model of middle cerebral artery occlusion (MCAO). The administration of EP intraperitoneally in the rat before or up to 24 hours after MCAO notably suppressed the clinical manifestations of cerebral ischemia, indicating a potent neuroprotective effect of EP against cerebral ischemic injury.

Materials and Methods

Surgical Procedures for MCAO

All experiments were performed in accordance with the guidelines for animal research at Inha University School of Medicine. Male Sprague-Dawley rats (250 to 300 g) were anesthetized with 5% isoflurane in a gas mixture of 30% oxygen and 70% nitrous oxide,
and MCA was occluded for 1 hour by the suture occlusion method as described previously.\textsuperscript{12} The left femoral artery was cannulated for monitoring of arterial blood pressure (blood pressure analyzer; Digi Med) and for blood sampling to analyze pH, Pao\textsubscript{2}, Paco\textsubscript{2}, and blood glucose concentration (I-STAT; Sensor Devises). The regional cerebral blood flow was monitored by laser Doppler flowmeter (Periflux System 5000; Perimed). A thermoregulated heating pad and over-brain blood flow was monitored by laser Doppler flowmeter (Periflux IL-4, and GAPDH were described previously.\textsuperscript{12,13} 

NO Measurement

BV2 cells were grown in DMEM supplemented with penicillin (20 U/mL), streptomycin (20 mg/mL), and 10\% heat-inactivated FBS (Gibco). The BV2 cells (2\times10\textsuperscript{5}) were plated on 6-well plates and treated with lipopolysaccharide (LPS; 0.1 \mu g/mL) for 24 hours. To measure the amount of NO produced by the BV2 cells, 100 \mu L of conditioned medium was mixed with an equal volume of Griess reagent (0.5\% sulfanilamide and 0.05\% \textit{N}-1-naphthylethylenediamine) and incubated for 10 minutes at room temperature. The absorbance of the mixture at 550 nm was measured on a microplate reader.

Reverse Transcription–Polymerase Chain Reaction

The 6-mm coronal brain slices, 6 to 12 mm apart starting from the frontal pole of the frontal cortex, were prepared by a brain matrix device (RBM-40000; ASI Instruments). Total RNA was isolated using TRIsol reagent (Invitrogen), and 1 \mu g of RNA was used for the cDNA synthesis and subsequent PCR analysis according to manufacturer instructions (RT-PCR kit; Roche). The primer sequences of the target genes were as follows: anti-p38 antibody (SC-535; Santa Cruz Biotechnology), anti–extracellular signal-regulated kinase (ERK) antibody, and anti-p-ERK antibody (Cell Signaling) were used as primary antibodies. The secondary antibody, HRP-conjugated goat anti-rabbit IgG (1:2000 dilution), was applied for 1 hour at room temperature. After 1 hour, the sections were immersed in developer (HRP/3,3′-diaminobenzidine) system. The number of microglia in 0.06 mm\textsuperscript{2} (0.2\times0.3 mm) was obtained by scoring the GSA I-B4–positive cells in 12 photographs taken from 3 independent experiments.

Evaluation of the Neurological Deficit

The assessment of the neurological deficits was performed 2 days after MCAO/reperfusion. A modified postural reflex and hemiparesis test\textsuperscript{14} and a forelimb-placing test\textsuperscript{15} were used to evaluate the motor function. Postural reflex was scored on a 4-point grade scale: 0, normal function; 1, flexion of the torso and contralateral forelimb on lifting the animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, reclination to the contralateral side at rest; and 4, absence of spontaneous motor activity. The placing of each limb was graded as described previously: 0, immediate and complete placing; 1, delayed or incomplete placing (>2 s); and 2, no placing. The scores for the postural reflex and forelimb placing tests were summed for each animal.

Rota-Rod Test

At 24 hours before the surgery for MCAO, rats were conditioned on the rota-rod, which was set at a constant speed of 3 rpm, until they achieved the goal of remaining on the rotating spindle for 180 s. At 24 hours after the 1-hour MCAO, each rat received a test trial on the rota-rod at a speed of 5 rpm. Subsequently, the duration for which each rat remained in the rota-rod operated at speeds of 10 and 15 rpm were tested at 1-hour intertrial intervals.

Statistical Analysis

Statistical analysis was performed by ANOVA followed by the Newman–Keuls test. All data were presented as average±SEM, and a statistical difference was accepted at the 5\% level.

Results

EP Inhibits the Cerebral Ischemic Injury With a Wide Therapeutic Window

To investigate a neuroprotective effect of EP in cerebral ischemia, EP was administered intraperitoneally at the dose of 1, 4, 20, or 40 mg/kg at 4 hours after 1-hour MCAO, and the size of infarct volume was assessed after 2 days of reperfusion. The administration of 20 mg/kg or 40 mg/kg EP reduced the infarct volume down to 50.7±10.5\% (n=5; P<0.05) or 29.6±13.8\% (n=5; P<0.05) of that of the control animals, respectively (Figure 1A). Pretreatment with 40 mg/kg of EP at 30 minutes before the ischemic insult reduced the infarct volume to 10.1±3.4\% (n=6; P<0.05) of that of the control animals (Figure 1B and 1C). The administration of EP (40 mg/kg) at 4 hours or 12 hours after MCAO/reperfusion reduced the infarct volume to 21.5±2.7\% (n=6; P<0.05) and 44.3±4.0\% (n=6; P<0.05), respectively. Interestingly, the administration of EP even after 24 hours of reperfusion also suppressed the infarct volume to 76.5±4.7\% (n=6; P<0.05; Figure 1B and 1C). The physiological parameters mean arterial blood pressure, pH, Pao\textsubscript{2}, Paco\textsubscript{2}, and blood glucose were not significantly different between the control and EP-treated animals (Table). The comparable levels of reduction in infarct volume were observed in 30-minute preadministration and 4-hour postadministration groups when examined 14 days after MCAO (data not shown).

EP Improves Motor Impairment and Neurological Deficits of Rats With MCAO

The motor activity of rats insulted with MCAO was assessed using the rota-rod test. We confirmed that the normal and
sham-operated groups showed no difference in performance on the rota-rod at 5 rpm (179.0±1.5 and 175.4±3.5 s, respectively; Figure 2A). The rota-rod performance of vehicle-treated animals after 1-hour MCAO was reduced to 31.2±12.5 s (n=8; P<0.05; Figure 2A). EP treatment at 4 hours after MCAO/reperfusion conferred a notable recovery on the motor activity in the rota-rod (122.0±25 s; n=8; P<0.05; Figure 2A). The scores obtained from the repeated examinations used at 10 rpm or 15 rpm with a 1-hour interval also indicated a profound recovery from the motor impairment after the EP administration (Figure 2A). Consistent with these results, the neurological deficits, which were assessed by the modified postural reflex and hemiparesis14 and forelimb-placing tests15, were significantly reduced in the EP-administered groups (2.5±0.3 for 30-minute pretreatment group and 2.7±0.3 for 4-hour postadministered group; n=12; P<0.05) compared with the paired control group (4.9±0.4; n=12; Figure 2B).

![Figure 1](image1.png)  
**Figure 1.** Neuroprotective effects of EP in postischemic brain. A, EP (1, 4, 20, and 40 mg/kg) was administered intraperitoneally at 4 hours after the 1-hour MCAO. The TTC-stained infarction area generated at 2 days after MCAO in the coronal brain sections, corresponding to 8 mm apart starting from the frontal pole of the brain, was presented. B, EP (40 mg/kg IP) was administered 30 minutes before or 4, 12, or 24 hours after MCAO, and the infarctions on a series of coronal sections observed at 2 days after MCAO were visualized by TTC staining. The values above the figure represent the distance from the frontal pole of the brain. C, Data were presented as average±SEM (n=6; *P<0.05).

![Figure 2](image2.png)  
**Figure 2.** Recovery of the motor deficits by postadministration of EP. A, At 24 hours before the 1-hour MCAO, animals were pretrained on the rota-rod until they could stay on it for 3 minutes at 3 rpm. EP (40 mg/kg) was administered 4 hours after MCAO, and the rota-rod test was performed at constant speeds indicated. Con indicates control; EP, EP treatment at 4 hours after MCAO. B, Recovery of the neurological deficits induced by EP was evaluated by a modified postural reflex (filled box) and forelimb-placing tests (open box). EP (40 mg/kg) was administered 30 minutes before (pre 30) or 4 hours after (post 4 hour) MCAO, and the neurological deficits were examined at 2 days after MCAO. The data (A and B) are presented as average±SEM (n=8 to 12; *P<0.05).

<table>
<thead>
<tr>
<th>Physiological Parameters</th>
<th>Vehicle-treated Group (n=8)</th>
<th>EP-treated Group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base</td>
<td>During Ischemia</td>
</tr>
<tr>
<td>Rectal temperature, °C</td>
<td>37.4±0.5</td>
<td>37.0±0.4</td>
</tr>
<tr>
<td>pH</td>
<td>7.43±0.06</td>
<td>7.49±0.08</td>
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<tr>
<td>P02, mm Hg</td>
<td>173.8±20.3</td>
<td>174.0±11.3</td>
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<tr>
<td>Pco2, mm Hg</td>
<td>36.4±2.8</td>
<td>36.0±7.1</td>
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<tr>
<td>Hb, g/dL</td>
<td>14.4±1.1</td>
<td>13.3±1.3</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>102±9</td>
<td>119±8</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>96.9±8.4</td>
<td>97.2±5.3</td>
</tr>
</tbody>
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Values are means±SD.  
One-way ANOVA revealed no significant intergroup difference for any variable.  
MABP indicates mean arterial blood pressure; Hb, hemoglobin.
To investigate the effect of EP on the inflammatory process in the postischemic brain, the activation of microglia was examined using immunostaining with isolectin-B4 (GSA I-B4), a microglia marker. In the sham-operated animals, GSA I-B4-positive cells were barely detected in the brain (Figure 3A). One day after MCAO/reperfusion, activated microglia were detected in the penumbra area in the ischemic hemisphere (Figure 3B). Two days after MCAO/reperfusion, the activation of the microglia progressed further, revealing the morphology of phagocytic microglia, which were enriched in the infarction core of the ischemic hemisphere (Figure 3C). In contrast, activated microglia were barely detectable in the brains of the EP-administered rats, wherein a few ramified microglia were detected along with resting ones (Figure 3D and 3E).

In addition to the suppression of microglia activation, the induction of proinflammatory cytokines was also inhibited. The administration of EP, either 30 minutes before or 4 hours after reperfusion, suppressed the induction of TNF-α (36.6±11.3% and 66.6±9.3%, respectively; n=4), IL-1β (24.0±4.2% and 25.5±4.3%, respectively; n=4), COX-2 (44.1±4.2% and 69.6±5.7%, respectively; n=4), and iNOS (16.1±5.7% and 29.2±5.3%, respectively; n=4) in the ischemic hemisphere (Figure 4A). In contrast, the levels of IL-13 and IL-4, anti-inflammatory cytokines that were reduced in postischemic brain, recovered after the treatment with EP at 30 minutes before (98.4±7.5% and 191.8±10.5%, respectively) or 4 hours after (287.7±10.3% and 398.2±12.6%, respectively) MCAO/reperfusion (Figure 4A). Furthermore, the induction of p38 mitogen-activated protein kinase (p38 MAPK), which is known to mediate microglia activation and be involved in the inflammatory process in postischemic brain, was also suppressed by either 30 minutes before (28.6±14.3%; n=4; P<0.05) or 4 hours after (30.6±15.6%; n=4; P<0.05) treatment of EP (Figure 4B). However, there was no change in the level of phosphorylated ERK by 30 minutes before (95.2±13.2%; n=4) or 4 hours after (93.5±15.1%; n=4) treatment of EP (Figure 4C), or in the total amount of p38 MAPK or the level of p-ERK in the presence of EP was...
Figure 5. Suppression of LPS-induced activation of BV2 cells by EP. BV2 microglial cells (2×10⁵ cells) were incubated with 0.1 μg/mL of LPS along with various concentrations of EP (1, 5, 10, or 20 mmol/L) for 24 hours. The production of NO and the iNOS expression level were determined by measuring nitrite (A) and RT-PCR (B), respectively. The changes in the amount of nitrite are presented as average±SEM (n=4; A). Changes in the RNA level of proinflammatory cytokines TNF-α, COX-2, and IL-1β were examined by RT-PCR in the presence of an increasing amount of EP or with 5 mmol/L pyruvate (C; *P<0.05).

Discussion

In this study, we demonstrated that EP can be used as a potent neuroprotectant in the postischemic brain. EP afforded observable neuroprotection even when it was administered 24 hours after reperfusion. It is in contrast to the limited protective time window of pyruvate, which was only effective when it was administered within 0 to 1 hour after the onset of reperfusion. The extended therapeutic window implies that EP affords neuroprotection via a mechanism that modulates the delayed damaging processes in the postischemic brain.

There is a wealth of evidence to suggest that the inflammatory reactions contribute to the late stages of ischemic injury and result in a worsening of the neurological outcome. Postischemic inflammation might contribute to ischemic damage probably through multiple mechanisms. In the active state, microglia produce cytotoxic molecules, such as NO, oxygen radicals, arachidonic acid derivatives, and cytokines. The EP-dependent inhibition of LPS-induced activation of BV2 cells (Figure 5) and microglial activation in the postischemic brain (Figures 3 and 4) suggest that the inhibition of microglia activation might be a mechanism by which EP exerts its neuroprotective effect. In fact, the anti-inflammatory effect of EP has been reported in hemorrhagic shock and sepsis. In contrast, pyruvate showed almost no suppressive effect on microglia activation, proinflammatory cytokine expression (Figure 1), and p38 MAPK activation (J.-B. Kim and J.-K. Lee, unpublished observation, 2005), indicating again that EP functions in a way that is quite distinct from that of pyruvate in the postischemic brain.

It was observed consistently that pretreatment with EP exerted better protection than post-treatment except for the regulation of the anti-inflammatory cytokines (Figures 1, 2, and 4). This result suggests that in addition to the anti-inflammatory effect at delayed time points, EP may also be involved in the protection of the acute damaging process. First, EP may function as a metabolic substrate to decrease the cytosolic NADH/NAD⁺ ratio and maintain the cellular phosphorylation potential ATP/ADP-Pi; this mechanism has been proposed to explain some of the beneficial effects of the parent compound pyruvate. Second, EP may function as an effective scavenger of ROS, as does pyruvate. Regarding this issue, we found that EP exerts a protective effect on a primary cortical culture when it is exposed to oxidative stress (Kim et al, unpublished data, 2005). Third, EP may act as a Zn²⁺ chelator, thus suppressing the Zn²⁺ toxicity in the ischemic brain. We observed that EP could reduce Zn²⁺ dependent cell death in a primary cortical culture (Kim et al, unpublished data, 2005). Therefore, we speculate that the neuroprotective mechanism of EP in vivo might be produced through multiple mechanisms, one of which is the suppression of microglial activation, as demonstrated in the current study. Although the specific target of EP that produces neuroprotection remains to be elucidated, our results suggest a value of EP as a therapeutic means to suppress cerebral ischemic injury with a wide therapeutic window.

Acknowledgments

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References


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