ONLINE SUPPLEMENT

Supplemental Methods

Antibodies
Rabbit polyclonal anti-Akt, anti-p-Akt (Ser473), anti-MKK7, anti-p-MKK7 (Ser171, Thr275), anti-p-MLK3 (Thr277, Ser281), monoclonal anti-JNK3 (55A8) and monoclonal anti-β-actin (13E5) antibodies were purchased from Cell Signaling Biotechnology (Boston, MA). Rabbit polyclonal anti-GluK2, anti-MLK3, and mouse monoclonal anti-p-JNKs (Thr 183, Tyr 185, G-7) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal anti-PSD-95 (clone 7E3-1B8) antibody was obtained from Sigma (Saint Louis, MO).

Drug Administration
A selective PI3K inhibitor LY294002 (BioMol, Plymouth Meeting, PA), a specific Akt inhibitor Akt inhibitor IV (Calbiochem) and a potent MEK1 inhibitor PD98059 (Cell Signaling Biotechnology) were used to detect the role of PI3K-Akt and MEK1 signaling in the postconditioning-mediated neuroprotection respectively. LY294002 (100 nmol) or PD98059 (20 nmol) in 10μl DMSO was infused into the rat cerebral ventricle (from the bregma: posterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) through a stepper-motorized microsyringe (Stoelting, Wood Dale, IL) 20 minutes before ischemia. Akt inhibitor IV (100 nmol) in 10μl DMSO was infusied into the rat cerebral ventricle 2 hours after ischemia.

Histological Assessment
Rats were perfusion-fixed with 4% paraformaldehyde under anesthesia after 5 days of reperfusion. Brains were removed and further fixed with the same fixation solution at 4°C overnight. Post-fixed brains were embedded by paraffin and then coronal sections (6μm thick) were prepared using a microtome. The paraffin embedded brain sections were deparaffinized with xylene and rehydrated in a gradient of ethanol, followed by washing with distilled water. The sections were stained with cresyl violet for the assessment of neuronal survival in the hippocampus. The number of surviving hippocampal CA1 neurons per 1 mm length was counted as the neuronal density.

Immunoprecipitation
The hippocampal CA1 regions were isolated after the indicated times of reperfusion and rapidly frozen in liquid nitrogen. Samples were homogenized in ice-old homogenization buffer. ¹ The homogenates were centrifuged at 800g/4°C for 10 minutes and the supernatants were collected. Sample proteins were incubated overnight at 4°C with appropriate antibodies diluted in immunoprecipitation buffer. ¹ After the addition of protein A/G, the mixture was incubated at 4°C for an additional 2 hours. The bound proteins were collected from Protein A/G by boiling for 5 minutes in Laemmli sample buffer.
Immunoblot
Protein samples were separated by SDS-PAGE and then electrotransferred onto a nitrocellulose membrane. After blocking, the membranes were probed with primary antibodies overnight at 4°C. Detection was carried out by appropriate alkaline phosphatase-conjugated IgG (Sigma) and developed with NBT/BCIP assay kit (Promega).

Statistical Analysis
The results are expressed as means±standard deviation (SD). For each type of experiment, data were obtained from at least three independent measurements. Statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) followed by the least significant difference test or Newman-Keul’s test. Differences were considered significant at \( P<0.05 \).

Supplemental Figure

**Figure S1.** LY294002 (LY) or Akt inhibitor IV (AI) has no effect on the neuronal survival in the rat hippocampal CA1 subregion. Nissl staining on neuronal survival after 5 days following drug administration. (a-c) Low-power views of hippocampus sectors. Scale bars = 500 μm. (d-f) High-power views of hippocampal CA1 pyramidal cell layer of (a-c). Scale bars = 50 μm. Neuronal density was counted as numbers of surviving pyramidal neurons per 1mm length. Data are mean ± SD (n=5).

Supplemental Discussion
The role of MEK1-ERK1/2 pathway remains controversial, since both activation and inhibition of ERK1/2 are reported to mediate neuronal survival in conditions associated with cerebral ischemia. 2,3 Although different changes in ERK1/2 phosphorylation after postconditioning have been reported, 4,5 Pignataro and colleagues found that ERK1/2 may be unrelated to the protective effect of postconditioning after focal ischemia. 4 In this work, our result showed that inhibiting MEK1-ERK1/2 signal by PD98059 didn’t affect protective effect of postconditioning after global ischemia, which is consistent with the study by Pignataro and colleagues.
Considering dual effects of ERK1/2 in ischemic brain damage and controversial data for ERK1/2 phosphorylation in postconditioning, more studies are needed to clarify the role of MEK1-ERK1/2 in the postconditioning neuroprotection.

Supplemental References