Neuroprotection of Ischemic Postconditioning by Downregulating the Postsynaptic Signaling Mediated by Kainate Receptors

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Background and Purpose—Ischemic postconditioning, a brief episode of ischemia after a prolonged ischemic insult, has been found to reduce the delayed neuronal loss after stroke. However, the mechanisms underlying such endogenous neuroprotective strategy remain obscure. In this study, we try to explore the excitatory postsynaptic signal events associated with neuroprotective effect of ischemic postconditioning.

Methods—Global cerebral ischemia was induced for 15 minutes by the 4-vessel occlusion method in male Sprague–Dawley rats. Ischemic postconditioning was conducted 10 minutes later by a single reocclusion for 3 minutes.

Results—A severe global cerebral ischemia after 5 days of reperfusion destroyed almost all hippocampal CA1 pyramidal neurons. A brief ischemic postconditioning robustly reduced the neuronal loss after ischemia. Preadministration of phosphoinositide 3-kinase inhibitor LY294002 blocked the neuroprotection of postconditioning, whereas mitogen-activated protein kinase kinase 1 inhibitor PD98059 had no effect. Ischemic postconditioning significantly increased the Akt phosphorylation (Ser473). In addition, postconditioning not only perturbed the binding of postsynaptic density protein-95 with glutamatergic kainate receptor subunit 2 and mixed lineage kinase 3 but also suppressed the downstream activation of mixed lineage kinase 3, mitogen-activated protein kinase kinase 7, and c-Jun N-terminal kinase 3. LY294002, but not PD98059, abolished the postconditioning-induced decreases in the assembly of glutamatergic kainate receptor subunit 2–postsynaptic density protein-95–mixed lineage kinase 3 complex and in the mixed lineage kinase 3–c-Jun N-terminal kinase 3 signaling. Akt inhibitor IV, a specific Akt inhibitor, showed the same effects as LY294002.

Conclusions—Ischemic postconditioning protects neurons against stroke by attenuating the postsynaptic glutamatergic kainate receptor subunit 2–postsynaptic density protein-95–mixed lineage kinase 3 signal cascade via phosphoinositide 3-kinase–Akt pathway. (Stroke. 2013;44:2031-2035.)

Key Words: cerebral ischemia ■ ischemic postconditioning ■ kainate receptors ■ mixed lineage kinase 3 ■ neuroprotection ■ postsynaptic density protein-95

Ischemic postconditioning, which refers to a single or a series of brief interference in the cerebral blood supply performed after a prolonged severe form of ischemic insult, has been established as a novel neuroprotective strategy against ischemic stroke. However, the molecular mechanisms responsible for such intravenous neuroprotection largely remain elusive. Excitatory postsynaptic signal events serve as the major mechanism responsible for ischemic neuronal loss. Recently, it has been revealed that postsynaptic density protein-95 (PSD-95) integrates the kainate receptors-evoked postsynaptic signaling by interacting with glutamatergic kainate receptor subunit 2 (GluK2) and downstream signal molecules, such as mixed lineage kinase 3 (MLK3). The formation of GluK2–PSD-95–MLK3 signal complex is consistent with the activation of MLK3, mitogen-activated protein kinase kinase 7 (MKK7), and c-Jun N-terminal kinase 3 (JNK3) signal cascade, which contributes to ischemic neuronal death. Here, we provide the first evidence that ischemic postconditioning prevents the kainate receptors-induced postsynaptic signaling. Phosphoinositide 3-kinase (PI3K)–Akt and mitogen-activated protein kinase kinase 1 (MEK1)–extracellular signal-regulated kinases 1/2 (ERK1/2) are well documented as survival pathways in regulating neuronal fate. Ischemic postconditioning increases the level of phosphorylated Akt (p-Akt Ser473), and PI3K inhibitors suppress the beneficial effect of postconditioning, implicating a role of PI3K–Akt pathway in the neuroprotective effect of postconditioning. Nevertheless, signal events downstream of the PI3K–Akt pathway associated...
with neuroprotection of the postconditioning are rarely known. Recently, ischemic postconditioning has been found to alter the level of p-ERK1/2 in the brain, yet the contribution of ERK1/2 to neuronal survival is still highly debatable. In this study, we tested whether both PI3K–Akt and MEK1–ERK1/2 signal are involved in the neuroprotection of postconditioning and further investigated their roles in the inhibition of postsynaptic signal events evoked by postconditioning.

Materials and Methods

All experiments were performed in accordance with the guidelines of the local Animal Care Committee. Adult male Sprague–Dawley rats weighing 250 to 300 g were given free access to food and water before surgery. Global cerebral ischemia was induced for 15 minutes by the 4-vessel occlusion method, as previously described. The sham operation was performed using the same surgical procedures except for occlusion of carotid arteries. Ten minutes after the ischemia, both carotid arteries were reoccluded for 3 minutes as the ischemic postconditioning.

Figure 1. Phosphoinositide 3-kinase (PI3K) pathway, but not mitogen-activated protein kinase kinase 1 (MEK1) pathway, associates with the neuroprotection of the postconditioning against global ischemia in the rat hippocampal CA1 subregion. LY294002 (LY), PD98059 (PD), or DMSO was treated 20 minutes before ischemia. A, Nissl staining on neuronal survival after 5 days of reperfusion after global ischemia (I/R5d). In panel A, subpanels a through f, low-power views of hippocampus sectors. Scale bars=500 μm. g through l, High-power views of CA1 pyramidal cell layer of (a–f). Scale bars=50 μm. Neuronal density was counted as numbers of surviving pyramidal neurons per 1 mm length. Data are mean±SD (n=5). *P<0.05 versus I/R5d; #P<0.05 versus I/R5d with postconditioning. B, Immunoblot analysis of the level of p-Akt and Akt. Relative levels of p-Akt were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus sham; #P<0.05 no postconditioning versus postconditioning.

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Results

Nissl (cresyl violet) staining showed that global cerebral ischemia followed by 5 days of reperfusion destroyed ≈95% of pyramidal neurons in the vulnerable hippocampal CA1 subfield. Postconditioning, 3-minute reocclusion performed 10 minutes after ischemia, significantly decreased the delayed neuronal loss. Pretreatment of PI3K inhibitor LY294002 reduced the postconditioning-mediated neuronal survival, whereas MEK1 inhibitor PD98059 did not affect the number of surviving neurons (Figure 1A; Figure I in the online-only content).

Figure 2. Phosphoinositide 3-kinase (PI3K) pathway, but not mitogen-activated protein kinase kinase 1 (MEK1) pathway, is involved in the postconditioning-induced inhibition of both glutamatergic kainate receptor subunit 2 (GluK2)—postsynaptic density protein-95 (PSD-95)—mixed lineage kinase 3 (MLK3) assembly and MLK3—mitogen-activated protein kinase kinase 7 (MKK7)–c-Jun N-terminal kinase 3 (JNK3) signaling. LY294002 (LY), PD98059 (PD), or DMSO was administered 20 minutes before ischemia/reperfusion (I/R). A, Immunoblot analysis of the level of p-MLK3 and MLK3. Relative levels of p-MLK3 were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus sham; †P<0.05 no postconditioning versus postconditioning. B, The binding of GluK2 and MLK3 with PSD-95 was measured by immunoprecipitation with an anti-GluK2 or anti-MLK3 antibody followed by blot with an anti-PSD-95 antibody. Relative levels of GluK2–PSD-95 and MLK3–PSD-95 binding were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus I/R6h; †P<0.05 versus I/R6h with postconditioning. C, Immunoblot analysis of the level of p-MLK3, p-MKK7, MLK3, MKK7, and JNK3. The level of p-JNK3 was measured by immunoprecipitation with an anti-p-JNKs antibody and immunoblot with an anti-JNK3 antibody. Relative levels of p-MLK3, p-MKK7, and p-JNK3 were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus I/R6h; †P<0.05 versus I/R6h with postconditioning.
Akt pathway is responsible for the postconditioning-induced neuroprotection and downregulation of glutamatergic kainate receptor subunit 2 (GluK2)–postsynaptic density protein-95 (PSD-95)–mixed lineage kinase 3 (MLK3)–c-Jun N-terminal kinase 3 (JNK3) signaling. Akt inhibitor IV (AI) or DMSO was treated 2 hours after ischemia. A, Nissl staining on neuronal survival after 5 days of reperfusion following global ischemia (I/R5d). In panel A, subpanels a through e, Low-power views of hippocampus sectors. Scale bars=500 μm. f through j, High-power views of CA1 pyramidal cell layer of (a–e). Scale bars=50 μm. Neuronal density was counted as numbers of surviving pyramidal neurons per 1 mm length. Data are mean±SD (n=5). *P<0.05 versus I/R5d; #P<0.05 versus I/R5d with postconditioning.

B, The binding of GluK2 and MLK3 with PSD-95 was measured by immunoprecipitation with an anti-GluK2 or anti-MLK3 antibody followed by blot with an anti-PSD-95 antibody. Relative levels of GluK2–PSD-95 and MLK3–PSD-95 binding were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus I/R6h; #P<0.05 versus I/R6h with postconditioning.

C, Immunoblot analysis of the level of p-MLK3, mitogen-activated protein kinase kinase 7 (p-MKK7), MLK3, MKK7, and JNK3. The level of p-JNK3 was measured by immunoprecipitation with an anti-p-JNKs antibody and immunoblot with an anti-JNK3 antibody. Relative levels of p-MLK3, p-MKK7, and p-JNK3 were normalized to respective sham. Data are mean±SD (n=3). *P<0.05 versus I/R6h; #P<0.05 versus I/R6h with postconditioning.
Data Supplement). Immunoblot analysis showed a similar change pattern of p-Akt at different times of reperfusion after ischemia with or without postconditioning, except a significant increase of p-Akt at 6 hours of reperfusion after postconditioning (Figure 1B). The data suggest that level of p-Akt up to a threshold value after reperfusion after ischemia may be required for the neuroprotection of postconditioning.

As shown in Figure 2A, postconditioning resulted in the decrease of p-MLK3 to the basal level at 6 and 24 hours of reperfusion. The data imply that the decline of MLK3 activity associates with the benefit of postconditioning. Next, postconditioning was found to disassociate the ischemia/reperfusion-induced binding of GluK2 and MLK3 with PSD-95, and to decrease the phosphorylation of MLK3, MKK7, and JNK3 (Figure 2B and 2C), suggesting a negative effect of postconditioning on the postsynaptic GluK2–PSD-95–MLK3 signal assembly and its downstream MLK3–JNK3 signal. LY294002 reversed the suppression of postconditioning in the association of GluK2 and MLK3 with PSD-95 and in the phosphorylation of MLK3, MKK7, and JNK3, whereas PD98059 did not play any effect (Figure 2B and 2C), which reveals a novel crosstalk between PI3K pathway and GluK2–PSD-95–MLK3–JNK3 signal induced by postconditioning.

Although Akt activity has been reported to be involved in the protection of postconditioning, the exact role of Akt signal has not been confirmed. Here, we found that Akt inhibitor IV abolished the postconditioning-mediated neuronal survival in the hippocampal CA1 subfield after global ischemia (Figure 3A). The binding of GluK2 and MLK3 with PSD-95 and the phosphorylation of MLK3, MKK7, and JNK3 stepped up in Akt inhibitor IV–treated postconditioning group (Figure 3B and 3C). These data indicate that the postconditioning-conferred inhibition of GluK2–PSD-95–MLK3–JNK3 signal cascade is PI3K–Akt pathway dependent.

**Discussion**

Ischemic stroke is one of the leading causes of death and disability worldwide. Rapid reperfusion, the only widely approved clinical treatment, mostly leads to further damage to the ischemic brain. It has been recently reported that ischemic postconditioning represents a novel neuroprotective strategy against ischemic brain damage. Exploring the signal events evoked by such endogenous neuroprotective approach may offer promising therapeutic targets for ischemic stroke. In this study, we confirm the efficacy of a single postconditioning ischemia in a global ischemic model and provide the first evidence that ischemic postconditioning prevents the excessive postsynaptic signaling.

PSD-95 acts as a postsynaptic organizer of excitatory signaling cascades. PSD-95 binds to NMDA and kainate receptor subunits, as well as to downstream signal molecules, such as MLK3, which facilitates the specificity and efficiency of signal transduction. Here, we found that ischemic postconditioning attenuates postsynaptic kainate receptors signaling by dissociating the GluK2–PSD-95–MLK3 signal complex. Whether postconditioning affects the NMDA receptors-triggered postsynaptic signaling still remains to be elucidated.

Our data further support a critical role of PI3K–Akt pathway in postconditioning neuroprotection. Also, we extend these data by establishing a novel association between PI3K–Akt pathway and GluK2–PSD-95–MLK3–JNK3 signal cascade. Previously, Gao et al showed a reduction of p-JNK level by postconditioning, although there is no reason to be mentioned. Our data suggest an important position of GluK2–PSD-95–MLK3 signal complex in the reverse correlation between pro-survival PI3K–Akt and prodeath MLK3–MKK7–JNK3 pathways. To date, we do not know how the activated PI3K–Akt signal acts on GluK2–PSD-95–MLK3 complex. It should be interesting to identify several Akt substrates in postsynaptic site.

In brief, postconditioning resets the balance between pro-survival PI3K–Akt and prodeath GluK2–PSD-95–MLK3–JNK3 pathways. Further work will be required to elucidate the molecular mechanisms involved in the correlation between PI3K–Akt pathway and excitatory postsynaptic signaling. Pharmacological approaches targeting postsynaptic signal events may provide a great protection when used after cerebral ischemia, which is more feasible clinically.

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**Disclosures**

None.

**References**

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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 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 infused 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. 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. 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. Although different changes in ERK1/2 phosphorylation after postconditioning have been reported, Pignataro and colleagues found that ERK1/2 may be unrelated to the protective effect of postconditioning after focal ischemia. 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**