Neuroprotective Effects of Preconditioning Ischemia on Ischemic Brain Injury through Down-regulating Activation of JNK1/2 via N-Methyl-D-aspartate Receptor-mediated Akt1 Activation*

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Our previous studies have demonstrated that the JNK signaling pathway plays an important role in ischemic brain injury and is mediated via glutamate receptor 6. Others studies have shown that N-methyl-D-aspartate (NMDA) receptor is involved in the neuroprotection of ischemic preconditioning. Here we examined whether ischemic preconditioning down-regulates activation of the mixed lineage kinase-JNK signaling pathway via NMDA receptor-mediated Akt1 activation. In our present results, ischemic preconditioning could not only inhibit activations of mixed lineage kinase 3, JNK1/2, and c-Jun but also enhanced activation of Akt1. In addition, both NMDA (an agonist of NMDA receptor) and preconditioning showed neuroprotective effects. In contrast, ketamine, an antagonist of NMDA receptor, prevented the above effects of preconditioning. Further studies indicated that LY294002, a 5-methyl-isoxasazole-4-propionic acid, and kainate receptors (1, 2) are further divided into NMDA, N-methyl-d-aspartate receptor (NMDA) receptor channel activity, and NR2 subunits determine the specificity of receptor function (1). The overactivation of NMDA receptor has been widely believed to be the main signal resulting in cell injury in response to ischemic insults (6). However, the mild activation of NMDA receptor is supposed to induce neuroprotection against excitotoxicity in cultured cerebellar granule neurons in vitro (7). Some previous studies have shown a neuroprotective effect of NMDA on kainate-induced neuronal cell death in mouse hippocampus in vivo (8). The bulk of studies have shown that the hippocampal CA1 pyramidal cell is the most vulnerable neuron to ischemic injury (9–11). However, after a relative mild brain ischemia, cells will become more resistant to severe ischemic insult. This phenomenon has been called ischemic tolerance (12) or ischemic preconditioning. Ischemic preconditioning has drawn considerable attention as it provides a way to study the mechanisms of endogenous neuroprotection. Although the overactivation of NMDA receptor has been widely believed to be the main signal resulting in cell injury in ischemic insults, the mild activation of NMDA receptor has been proposed to be involved in the induction of brain ischemic tolerance (13–15).

However, the subsequent cellular signaling pathways leading to neuronal protective effects are largely unknown. MAPK signaling pathway is believed to be an important mediator in the signal transduction from cell surface to the nucleus playing a pivotal role in the regulation of neuronal survival and apoptosis (16, 17). This pathway consists of MAPK kinase kinases, MAPK kinases, and MAPKs. Every member is activated through phosphorylation by its upstream kinases (18). Mixed lineage kinase (MLK) phosphorylates mitogen-activated protein kinase kinase 4/7, which in turn phosphorylates JNK at its dual specific residues and activates it (19). JNK, also named stress-activated protein kinase, has already been widely investigated for its active actions in response to various stimuli, such as heat shock, inhibition of protein glycosylation, exposure to inflammatory cytokines, ultraviolet irradiation, and ischemia (20). Our previous studies have shown that JNK is significantly activated with two peaks occurring at 30 min and 3 days after ischemia/reperfusion (I/R) (21). The latter activation peak is considered to account for apoptosis. Other studies have documented that JNK is activated by MLK3, which is activated through GluR6-PSD95-MLK3 module in brain ischemia and reperfusion (22). As apoptosis is an active form of cell death, it should be associated with alterations of gene expression. Gene expression is regulated by immediate early genes such as c-jun and c-fos. Therefore, these genes are considered to be involved in apoptotic neuronal cell death. In fact, some studies have revealed that c-Jun plays important roles in neuronal cell death in vitro and in vivo (23, 24). That c-Jun

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system. Based on studies of pharmacology, electrophysiology, and molecular biology, the ionotropic receptors are further divided into NMDA, α-amino-3-hydroxy-5-methyl-isoxasazole-4-propionic acid, and kainate receptors (1, 2). The ionotropic glutamate receptor NMDA subtype is a kind of prominent ligand-gated ionic channel in excitatory synapses that is involved in ischemic neuronal cell damage events. Functional NMDA receptor (NR) is composed of three types of sub-units: NR1, NR2 (2A–2D), and NR3 (3A/3B) (3–5). NR1 is the principal subunit for NMDA receptor channel activity, and NR2 subunits determine the specificity of receptor function (1). The overactivation of NMDA receptor has been widely believed to be the main signal resulting in cell injury in response to ischemic insults (6). However, the mild activation of NMDA receptor is supposed to induce neuroprotection against excitotoxicity in cultured cerebellar granule neurons in vitro (7). Some previous studies have shown a neuroprotective effect of NMDA on kainate-induced neuronal cell death in mouse hippocampus in vivo (8). The bulk of studies have shown that the hippocampal CA1 pyramidal cell is the most vulnerable neuron to ischemic injury (9–11). However, after a relative mild brain ischemia, cells will become more resistant to severe ischemic insult. This phenomenon has been called ischemic tolerance (12) or ischemic preconditioning. Ischemic preconditioning has drawn considerable attention as it provides a way to study the mechanisms of endogenous neuroprotection. Although the overactivation of NMDA receptor has been widely believed to be the main signal resulting in cell injury in ischemic insults, the mild activation of NMDA receptor has been proposed to be involved in the induction of brain ischemic tolerance (13–15). However, the subsequent cellular signaling pathways leading to neuronal protective effects are largely unknown. MAPK signaling pathway is believed to be an important mediator in the signal transduction from cell surface to the nucleus playing a pivotal role in the regulation of neuronal survival and apoptosis (16, 17). This pathway consists of MAPK kinase kinases, MAPK kinases, and MAPKs. Every member is activated through phosphorylation by its upstream kinases (18). Mixed lineage kinase (MLK) phosphorylates mitogen-activated protein kinase kinase 4/7, which in turn phosphorylates JNK at its dual specific residues and activates it (19). JNK, also named stress-activated protein kinase, has already been widely investigated for its active actions in response to various stimuli, such as heat shock, inhibition of protein glycosylation, exposure to inflammatory cytokines, ultraviolet irradiation, and ischemia (20). Our previous studies have shown that JNK is significantly activated with two peaks occurring at 30 min and 3 days after ischemia/reperfusion (I/R) (21). The latter activation peak is considered to account for apoptosis. Other studies have documented that JNK is activated by MLK3, which is activated through GluR6-PSD95-MLK3 module in brain ischemia and reperfusion (22). As apoptosis is an active form of cell death, it should be associated with alterations of gene expression. Gene expression is regulated by immediate early genes such as c-jun and c-fos. Therefore, these genes are considered to be involved in apoptotic neuronal cell death. In fact, some studies have revealed that c-Jun plays important roles in neuronal cell death in vitro and in vivo (23, 24). That c-Jun

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activates gene transcription is dependent on its phosphorylation state. Furthermore, activated JNK specifically phosphorylates the N-terminal activation domain of transcription factor c-Jun at serines 63 and 73, thereby increasing transcriptional activity of c-Jun (24).

Akt, also known as protein kinase B, is one of the downstream kinases of phosphoinositide 3-kinase (PI3K) that is involved in survival signals in growth factor-mediated signaling cascades. The activation of Akt has been shown to depend on the phosphorylation of Thr-308 and Ser-473 (25, 26). Studies show that NMDA receptor 2B subunit binds to the Src homology 2 domain of PI3K during global ischemia (27, 28), suggesting the possibility that Akt can be activated by NMDA receptor upon the participation of PI3K. In addition, Yano et al. (29) show that NMDA receptor stimulation induces Akt activation through Ca2+/calmodulin-dependent protein kinase kinase in NG108-15 cells, leading to antiapoptosis. In many cases, active Akt phosphorylates Bad (30, 31), caspase-9 (32), and forkhead-related transcription factors (33) and thereby induces antiapoptotic effects. Furthermore, Akt also phosphorylates MLK3 Thr-674 and negatively regulates MLK3. Akt is a survival protein, and previous studies have shown that Akt plays a pivotal role in ischemic tolerance (34).

Therefore, based upon the evidence mentioned above, we postulated that ischemic preconditioning might inhibit the activation of JNK via Akt activation induced by NMDA receptor. In the present studies, we examined the effects of preconditioning, ketamine, or NMDA treatment on the activation of JNK1/2, c-Jun, and Akt1 in hippocampal CA1 region. We also examined functional interaction between Akt1 and the JNK pathway to identify the mechanism underlying ischemic tolerance.

EXPERIMENTAL PROCEDURES

Materials—Anti-JNK1/2 antibody, anti-p-MLK3, anti-MLK3, NMDA, ketamine, KN62, alkaline phosphatase-conjugated goat anti-rabbit IgG, and goat anti-mouse IgG were obtained from Sigma. Anti-c-Jun antibody, anti-p-c-Jun, and anti-Akt1 were from Santa Cruz Biotechnology. Anti-p-JNK1/2, nitroblue tetrazolium, alkaline phosphatase-conjugated donkey anti-rabbit IgG, and 5-bromo-4-chloro-3-indoly phosphate/nitroblue tetrazolium color substrate. After immunoblotting, the bands on the filters were scanned and analyzed with an image analyzer (LabWorks Software, UVP Inc., Upland, CA). The optical density of the band in each lane was expressed as -fold versus that in sham control in the same filter.

Histology—Five days after ischemia, rats were deeply anesthetized and perfused intracardially with 0.9% NaCl followed by 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed quickly and further fixed with the same fixation solution at 4 °C overnight. Postfixed brains, dehydrated with 30, 50, 70, 80, 90, 95, and 100% ethanol, respectively, were embedded with paraffin. The coronal sections were prepared 5 μm thick using a microtome followed by deparaffinizing with xylene, dehydrating with ethanol at graded concentrations of 30–100% (v/v), and washing with water. Sections were stained with 0.1% (w/v) cresyl violet for assessment of neuronal damage in the hippocampal CA1 region. The numbers of viable neurons were quantitatively analyzed within rectangular areas of 0.02 mm2.

Data Analysis—Five and eight independent animals were sampled for each treatment for Western blotting study and histology examination, respectively. Data of those results were evaluated statistically using analysis of variance (ANOVA) with Newman-Keuls test. In all cases, differences were considered significant at p < 0.05.

RESULTS

Preconditioning Inhibited Phosphorylation of MLK3, JNK1/2, and c-Jun and Enhanced That of Akt1 in Hippocampal CA1 Region—First, to investigate the role of JNK pathway in ischemic preconditioning, we examined the phosphorylation levels of MLK3 and JNK1/2 in hippocampal CA1 region. Our previous studies indicated that activation of MLK3 in the hippocampal CA1 subfield peaks at 30 min and 6 h after I/R, and that of JNK1/2 peaks at 30 min and 3 days (17). The latter peaks are considered to account for delayed neuronal death because JNK signaling activation alone is not sufficient to lead to neuronal death. In addition, prolonged JNK activation above a threshold might be required for brain cell death. Thus, we investigated the effect of preconditioning on the activation of MLK3 and JNK1/2 at 6 h or 3 days after reperfusion, respectively. As shown in Fig. 1, A and B, the activations of MLK3 and JNK1/2 were significantly decreased in preconditioned groups compared with nontreated ischemic groups. In addition, to examine the potential role of Akt in the preconditioning-induced inhibition on the activation of MLK3 and JNK1/2, we tested the effect of Akt1 inhibitor on the phosphorylation of MLK3 and JNK1/2. As shown in Fig. 1, C and D, the activations of MLK3 and JNK1/2 were significantly increased by Akt1 inhibitor compared with control groups.

Drug Administration—All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo technique. Drug administration was invariably carried out between 10.00 and 15.00 h to avoid possible diurnal rhythm effects as described previously (37). The rats were injected intraperitoneally with ketamine (30 mg/kg) dissolved in 0.9% NaCl 20 min before preconditioning ischemia. NMDA dissolved in 1% phosphate-buffered saline (pH 7.4) was administrated at a dose of 100 mg/kg three times with an interval of 2 h. 24 h later, ischemia was induced (9). LY294002 and KN62, dissolved in Me2SO at a concentration of 5 μM/l, were administrated intracerebroventricularly (5 μl; bregma: 1.5 mm lateral, 0.8 mm posterior, 3.5 mm deep).

Western Blotting—Rats were killed by decapitation at the specified time. Hippocampi were dissected into CA1 and CA3/DG subfields from hippocampal fissure. Tissues were homogenized in 1:10 (w/v) ice-cold homogenization buffer consisting of 50 mM MOPS (pH 7.4), 50 mM NaF, 20 mM Na3PO4, 20 mM p-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprontin, and 10 μg/ml peptatin A with a Teflon-glass homogenizer followed by centrifugation at 800 × g for 15 min at 4 °C, and the supernatant was used. The nuclear pellets were extracted with extraction buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 0.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and enzyme inhibitors for 30 min at 4 °C with constant agitation. After being centrifuged at 12,000 × g for 15 min at 4 °C, supernatants as nuclear parts were collected. Samples were stored at −70 °C and thawed only once. After determination of the protein concentration, 50 μg of protein of each sample was heated at 100 °C for 5 min with loading buffer containing 0.125 m Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol, and 0.002% bromphenol blue and then separated by SDS-PAGE using 10% acrylamide gels (38). The proteins were electro-transferred onto nitrocellulose filter (pore size, 0.45 μm) according to the method of Towbin et al. (39). Blotting filters were incubated with 3% bovine serum albumin in TBST (10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20) at 4 °C for 6 h and probed with anti-JNK1/2 antibody (1:5000), anti-active (diphosphorylated) JNK1/2 antibody (1:3000), anti-c-Jun antibody (1:2000), anti-active (diphosphorylated) c-Jun (1:100), anti-p-MLK3 (1:1000), anti-MLK3 (1:200), anti-Akt1 antibody (1:5000), or anti-active (diphosphorylated) Akt1 (1:3000) at 4 °C overnight. Detection was carried out using alkaline phosphatase-conjugated goat anti-rabbit IgG (1:20,000), goat anti-mouse IgG (1:40,000), or donkey anti-goat IgG (1:5000) and developed using 5-bromo-4-chloro-3-indoly phosphate/nitroblue tetrazolium color substrate. After immunoblotting, the optical density of the band in each lane was expressed as -fold versus that in sham control of the same filter.

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and JNK1/2 after brain ischemia were inhibited markedly by preconditioning in hippocampal CA1 region. Second, we explored the effect of preconditioning on phosphorylation of c-Jun, a specific substrate of JNK. Because the activation of c-Jun has already been reported to peak at 3 h after lethal ischemia, we investigated c-Jun at 3 h after reperfusion. As shown in Fig. 1C, the phosphorylations of c-Jun induced by brain ischemia and reperfusion were inhibited markedly by preconditioning in hippocampal CA1 region. Third, some studies have demonstrated that Akt negatively regulates MLK3, which is an upstream of JNK (40), and that MLK3 activation peaks at 6 h after reperfusion (41). Furthermore, our primary experiments demonstrated that Akt activation is enhanced at 10 min after reperfusion but attenuated by preconditioning. Therefore, we selected 10 min after reperfusion to investigate Akt1 activation based on the consideration that the phosphorylation state of MLK3, which was induced by an increase of Akt1 activation at 10 min, is maintained for at least 6 h and leads to the inhibition of MLK3 activation at 6 h after reperfusion. As shown in Fig. 1D, activation of Akt1 was increased markedly by preconditioning. No significant changes occurred concerning the expressions of JNK1/2 and Akt1. The protein levels of c-Jun were not altered between ischemic and ischemic preconditioning. Finally, we examined whether preconditioning is associated with cell survival in hippocampal CA1 region. Normal CA1 pyramidal cells showed round and pale stained nuclei after cresyl violet staining. Five days after lethal ischemia, most CA1 pyramidal cells were shrunken with pyknotic nuclei. As shown in Fig. 2, A–C, a single ischemic insult of 6 h, Q. H. Guan, D. S. Pei, and Q. G. Zhang, submitted.

FIG. 1. Western blotting analysis of MLK3, JNK1/2, c-Jun, and Akt1 activations and expressions in hippocampal CA1 subfields in brain ischemic tolerance. Before 6 min of ischemia, rats were subjected to 3 min of ischemia as preconditioning and were decapitated at 10 min, 3 h, 6 h, or 3 days after reperfusion. Extracts from the hippocampi of the rats and sham controls were subjected to Western blotting analysis with anti-p-MLK3 antibody, anti-MLK3 antibody, anti-p-JNK1/2 antibody, anti-JNK1/2 antibody, anti-p-c-Jun antibody, anti-c-Jun antibody, anti-p-Akt1 antibody, and anti-Akt1 antibody. A, representative blots corresponding to phosphorylation and expression levels of MLK3 at 6 h after reperfusion in hippocampal CA1 subfields. B, representative blots corresponding to phosphorylation and expression levels of JNK1/2 at 3 days after reperfusion in hippocampal CA1 subfields. C, representative blots corresponding to phosphorylation and expression levels of c-Jun at 3 h after reperfusion in hippocampal CA1 subfields. D, representative blots corresponding to phosphorylation and expression levels of Akt1 at 10 min after reperfusion in hippocampal CA1 subfields. E, semi-quantitative analysis of the levels of MLK3, JNK1/2, c-Jun, and Akt1 phosphorylation in hippocampal CA1 regions. Data are mean ± S.D. (n = 4). *, p < 0.05, indicates statistical difference versus sham group. #, p < 0.05, indicates statistical difference versus reperfusion (ANOVA followed by Newman-Keuls test). R, reperfusion; d, days; m, minutes; P, preconditioning.

FIG. 2. Effect of pretreatment with NMDA on ischemia-induced neuronal loss and LY294002 on preconditioning-induced neuronal survival in the pyramidal cell layer of the hippocampal CA1 subfield. Rats were pretreated with NMDA (100 mg/kg, intraperitoneal) or phosphate-buffered saline (PBS) 1 day before 6 min of ischemia and reperfusion. Another group of rats were pretreated with LY294002 (25 µg/rat) or DMSO (DMSO) 20 min before preconditioning. Five days later, brains were fixed with paraformaldehyde followed by preparation of coronal sections from paraffin-embedded brains and subsequent staining with cresyl violet to determine cell survival in neuronal layers of the hippocampi (n = 4). The boxed areas of CA1 subfield are shown at higher magnification, and the number of viable neurons in these areas was counted. A–G, images of hippocampi at lower magnification (×400). H–N, images of hippocampal CA1 at higher magnification (×400). Scale bar, 30 µm. P, preconditioning; LY, LY294002.
min induced severe cell death. Neuronal density was significantly increased by preconditioning ischemia compared with neuronal density after a single ischemic insult. The numbers of surviving pyramidal cells in hippocampal CA1 region after a single ischemic insult and after preconditioning treated rats were 12/11006 and 82/11006% of those in the sham operation (Fig. 2, H–J), respectively. These data indicated that preconditioning inhibited the phosphorylations of both JNK1/2 and c-Jun but enhanced activations of Akt1, inducing neuroprotection in hippocampal CA1 region.

Ketamine Prevented the Effect of Preconditioning on the Inhibitions of JNK1/2 and c-Jun and Activation of Akt1—Studies from others (13, 15) have demonstrated a critical role of NMDA receptor in the induction of ischemic tolerance. Here we determined whether NMDA receptor mediates the effects of preconditioning-induced inhibitions of JNK1/2 and c-Jun and an increase of Akt1 activation. Rats were pretreated with ketamine 20 min before preconditioning followed by 6 min of ischemia. Phosphorylation levels of JNK1/2, c-Jun, and Akt1 in CA1 regions were examined at 3 days, 3 h, and 10 min after reperfusion, respectively. As shown in Fig. 3, A–C, ketamine alone
did not affect the activation levels of JNK1/2 and c-Jun in normal rats and the I/R-insulted rats. However, the attenuating effect of preconditioning on JNK1/2 and c-Jun activation and the increase in Akt1 activation in CA1 regions were prevented by ketamine, an antagonist of NMDA receptor. Rats pretreated with vehicle did not achieve the same results. At the same time, there was no change in the expression of JNK1/2 and Akt1. These data suggested that the inhibitions of JNK1/2 and c-Jun as well as Akt1 activation by preconditioning might be mediated by NMDA receptor activation.

**NMDA Treatment Inhibited Activation of JNK1/2 and c-Jun, Enhanced Activation of Akt1, and Resulted in Neuronal Survival**—To further confirm whether NMDA receptor plays a pivotal role in ischemic tolerance and moderates activation of NMDA receptor leading to cell survival, we treated rats with NMDA 1 day before 6 min of ischemia. The phosphorylations and expressions of JNK1/2, c-Jun, and Akt1 in CA1 regions were examined in contrast with those in sham operation and vehicle control at 3 days, 3 h, and 10 min, respectively. As shown in Fig. 4A–C, the activations of JNK1/2 and c-Jun were significantly decreased, whereas Akt1 activation was increased. Vehicle controls had no change in the activations of the three proteins compared with the rats without pretreatment. The expressions of JNK1/2 and Akt1 remained unchanged. The protein level of c-Jun was not affected by NMDA or vehicle. Next we also examined whether NMDA is associated with cell survival in CA1 region. Rats were subjected to 6 min of ischemia with administration of NMDA 1 day before and

**Fig. 5. Western blotting analysis of the effects of LY294002 on MLK3, JNK1/2, c-Jun, and Akt1 activations and expressions in hippocampal CA1 subfields.** Rats were administered with LY294002 (25 μg/kg, intracerebroventricular) or 0.9% Me₂SO (DMSO) 20 min prior to preconditioning and were decapitated after 6 min of ischemia. Extracts obtained from the rats with sham operation, LY294002 alone, reperfusion only, reperfusion plus ketamine, preconditioning, and vehicle, and LY294002 treatment before preconditioning were subjected to Western blotting. A, representative blots corresponding to phosphorylation and expression levels of MLK3 at 6 h after reperfusion in hippocampal CA1 subfields. B, representative blots corresponding to phosphorylation and expression levels of JNK1/2 at 3 days after reperfusion in hippocampal CA1 subfields. C, representative blots corresponding to phosphorylation and expression levels of c-Jun at 3 h after reperfusion in hippocampal CA1 subfields. D, representative blots corresponding to phosphorylation and expression levels of Akt1 at 10 min after reperfusion in hippocampal CA1 subfields. E, semiquantitative analysis of the levels of JNK1/2, c-Jun, and Akt1 phosphorylation in hippocampal CA1 regions. Data are mean ± S.D. (n = 4). *, p < 0.05, indicates statistical difference versus sham group. ∧, p < 0.05, indicates statistical difference versus reperfusion. #, p < 0.05, indicates statistical difference versus reperfusion with preconditioning (ANOVA followed by Newman-Keuls test). LY, LY294002; R, reperfusion; P, preconditioning; d, days; m, minutes.

**Preconditioning Inhibits JNK1/2 Activation via Akt1**
were perfusion-fixed with paraformaldehyde 5 days later. Coronal sections were subjected to cresyl violet staining for examining the neurons. In hippocampal CA1 regions, neurons subjected to single ischemia or phosphate-buffered saline administration were severely damaged in contrast to that of sham control (Fig. 2E). CA1 region of NMDA-treated rats showed significant neuroprotection (Fig. 2D). The numbers of surviving pyramidal cells in CA1 region of phosphate-buffered saline-treated and NMDA-treated rats were 12 ± 6 and 80 ± 5% of those in sham operation, respectively (Fig. 2, K and L). These results further indicated the possibility that NMDA receptor might mediate the inhibition of JNK1/2 and c-Jun as well as Akt1 activation in ischemic tolerance. Furthermore, NMDA receptor might mediate the neuroprotection induced by preconditioning.

PI3K and CaMK Are Involved in the Signaling Pathway Induced by Preconditioning—It is well known that Akt is one of the downstream kinases of PI3K, and Akt1 negatively regulates MLK3, which is upstream of the JNK pathway (40). Therefore, the functional interaction between Akt1 and MLK3 will explain the mechanism underlying the inhibition of the activations of JNK and c-Jun by preconditioning. To determine whether Akt is involved in neuroprotection induced by preconditioning, we first administered rats with LY294002 20 min before preconditioning and examined the alteration of the phosphorylation of Akt1, MLK3, JNK1/2, and c-Jun. The results showed that the activations of MLK3, JNK1/2, and c-Jun were significantly enhanced and activation of Akt1 was inhibited by LY294002 compared with preconditioned only or vehicle control group (Fig. 5, A–D). No change was observed regarding expression of any of the proteins. To further determine whether Akt is involved in neuroprotection induced by preconditioning, we also administrated rats with LY294002 20 min before preconditioning and perfusion-fixed them on the 5th day of reperfusion. Results from cresyl violet staining showed that neurons in hippocampi subjected to preconditioning were protected significantly compared with those without preconditioning. However, hippocampal CA1 regions pretreated with LY294002 showed sever neuron loss (Fig. 2G). The viable pyramidal cells counted within the 0.02-mm² rectangular areas were 81 ± 7, 85 ± 9, and 15 ± 6% of those in sham control, corresponding to hippocampi with preconditioning and with vehicle or LY294002 pretreatment, respectively (Fig. 2G). It has been reported previously that Akt is activated by CaMK (29). To further investigate the mechanism underlying the induction of ischemic tolerance, we administered KN62 20 min before preconditioning and examined the phosphorylation of Akt1. As shown in Fig. 6A, the activation of Akt1 was inhibited by KN62 compared with the preconditioned and vehicle groups. No change was observed regarding expression of Akt1. Taken together, the above data indicated that both PI3K and CaMK were involved in the activation of Akt1 and the neuroprotection induced by preconditioning.

DISCUSSION

In the present studies, we showed that preconditioning inhibited the activations of JNK1/2 and c-Jun during reperfusion after lethal ischemia through NMDA receptor-mediated activation of Akt1. These findings might provide some clues to understanding the mechanism underlying ischemia tolerance and to finding clinical therapies for stroke using the endogenous neuroprotection.

JNK, an important signaling protein in cell apoptosis, is activated significantly in I/R with two peaks at 30 min and 3 days after reperfusion (21). c-Jun, which is directly downstream of JNK and initiates transcription and expression of apoptosis proteins, is also involved in ischemic neuronal death.

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CaMK and PI3K pathways participate in the mechanism of Akt activation induced by NMDA receptor stimulation. Furthermore, Akt activation in glutamate-treated striatal neurons is Ca\(^{2+}\)-dependent and PI3K-dependent (43). Consistent with the above facts, both LY294002 and KN62 prevented the activation of Akt, which was induced by preconditioning in our studies. This confirms that preconditioning activates Akt1 through both CaMK and PI3K. In the present studies, we showed that Akt1 activation was increased by preconditioning, whereas Akt1 activation could be blocked by ketamine pretreatment. Akt1 activation was also significantly increased upon NMDA treatment 1 day before lethal ischemia. Therefore, it is clear that Akt1 plays a role closely associated with NMDA receptor in ischemic tolerance.

Akt1 negatively regulates MLK3 through phosphorylating MLK3 Thr-674 (40); thus it can be postulated that Akt1 activation inhibits JNK (a downstream kinase of MLK3) and c-Jun (a specific substrate of JNK) in ischemic tolerance. Additionally we found that LY294002 treatment prior to preconditioning decreased Akt1 activation and increased the activation of MLK3, JNK, and c-Jun, thus blocking neuroprotection of preconditioning. Thus, Akt1 functions as a critical mediator in the signaling pathway in which NMDA receptor mediated inhibition of the activations of MLK3, JNK1/2, and c-Jun. In previous studies, we have demonstrated that neither of the two activation peaks of JNK in I/R was due to NMDA receptor (44). The 30-min peak could be eliminated by N-acetylcysteine, an antioxidant, suggesting that the early activation of JNK was likely induced by reactive oxygen species (44), whereas the 3-day peak could be significantly inhibited by 6,7-dinitroquinoxaline-2,3-(1H,4H)-dione, a non-NMDA receptor antagonist (41). Thus, in I/R, the latter activation of JNK was mediated by stimulation of non-NMDA receptor, which could be explained by GluR6-PSD95-MLK3 module in rat hippocampus (22). Although NMDA receptor did not mediate the activation of the JNK pathway in I/R, it mediated the inhibition of JNK in ischemic tolerance. This reflects the cross-talk between the two types of glutamate receptors. In this cross-talk, Akt also acts as a pivotal mediator. Therefore, we can conclude that preconditioning-induced down-regulations of JNK and c-Jun and neuronal survival were mediated by NMDA receptor via Akt1 activation.

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