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Preconditioning Neuroprotection in Global Cerebral Ischemia Involves NMDA Receptor-Mediated ERK-JNK3 Crosstalk

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Abstract

Previous work has demonstrated that ischemic preconditioning neuroprotection is associated with inhibition of JNK pathway activation. The present study was designed to examine the hypothesis that the suppression of JNK3 activation by preconditioning is mediated by NMDA receptors and crosstalk between ERK1/2 and JNK3. Preconditioning (3 min ischemia) 2 days before global cerebral ischemia (8-min) markedly decreased neuronal degeneration in hippocampus CA1, an effect abolished by pretreatment with the NMDA receptor antagonist, MK-801. Furthermore, preconditioning abolished cerebral ischemia-induced JNK3 activation and enhanced ERK1/2 activation, an effect reversed by MK-801. Due to the inverse relationship between ERK1/2 and JNK3 activation following preconditioning, we hypothesized that ERK1/2 may regulate JNK3 activation following preconditioning. In support of this contention, pretreatment with the MEK inhibitor, PD98059 significantly attenuated preconditioning-induced ERK1/2 phosphorylation, and strongly reversed preconditioning down-regulation of JNK3 phosphorylation. This finding suggests that ERK1/2 signaling is responsible for preconditioning-induced down-regulation of JNK3 activation. Western blot analysis and immunohistochemistry further demonstrated that preconditioning, in an NMDA-dependent manner, enhanced activation of the pro-survival factors, p-CREB and Bcl-2, while attenuating activation of putative pro-death factors, p-c-Jun and Fas-L in the hippocampus CA1. As a whole, the study demonstrates that preconditioning attenuation of pro-death JNK3 in the hippocampus CA1 following global cerebral ischemia is mediated by NMDA receptor-Induced crosstalk between ERK1/2 and JNK3. The ERK1/2-mediated reduction of JNK3 activation leads to enhanced prosurvival signaling (P-CREB and Bcl-2 induction) and attenuation of prodeath signaling (p-c-Jun and Fas-L), with subsequent induction of ischemic tolerance.

Keywords

Global cerebral ischemia; hippocampus; cell signaling

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INTRODUCTION

Sublethal preconditioning ischemia prevents delayed neuronal cell death in the rodent and gerbil hippocampus CA1 region after a severe ischemic insult, a phenomenon known as ischemic tolerance (Kitagawa et al., 1990, Kirino et al., 1991, Kirino, 2002, Obrenovitch, 2008). Despite intense study, the mechanisms underlying ischemic tolerance in the brain are not fully understood. A robust elevation of the excitatory neurotransmitter glutamate has been implicated to have a critical role in induction of delayed neuronal cell death following cerebral ischemia (Szatkowski and Attwell, 1994, Arundine and Tymianski, 2004, Hara and Snyder, 2007). The overactivation of N-methyl-D-aspartate (NMDA) receptors by the excess glutamate is widely considered to mediate the delayed neuronal death in the hippocampal CA1 region following global cerebral ischemia (Gee et al., 2006, Liu et al., 2007, Benquet et al., 2008). However, mild activation of NMDA receptors has been suggested to actually exert a potential beneficial neuroprotective action in the hippocampus and other brain regions (Kato et al., 1992, Bond et al., 1999, Grabb and Choi, 1999, Miao et al., 2005, Soriano et al., 2006). Along these lines, NMDA receptors have been shown to play a key role in preconditioning neuroprotection as evidenced by the finding that preconditioning with low doses of NMDA protect against both apoptotic and excitotoxic insults (Jiang et al., 2005, Soriano et al., 2006), and by the fact that administration of NMDA receptor antagonists abolishes ischemic preconditioning neuroprotection in both rat and gerbil global cerebral ischemia models (Kato et al., 1992, Bond et al., 1999, Miao et al., 2005).

Currently, the mechanism of how NMDA receptors mediate preconditioning neuroprotection and ischemic tolerance is poorly understood. A number of groups have shown that ischemic preconditioning regulates kinase activation and gene expression as key mechanisms to attenuate delayed neuronal cell death (Shimazaki et al., 1994, Meller et al., 2005, Choi et al., 2006). The regulation of gene expression is thought to be due, at least in part, to changes in activation of the transcription factors, cyclic adenosine monophosphate response element binding protein (CREB) and c-Jun, with ischemic preconditioning enhancing CREB activation and inhibiting c-Jun activation (Nakajima et al., 2002, Colangelo et al., 2004, Meller et al., 2005). With respect to regulation of kinases, studies have shown that several members of the mitogen-activated protein kinase (MAPK) family are strongly regulated by ischemic preconditioning and may play a key role in ischemic tolerance. For instance, extracellular signal regulated kinases-1/2 (ERK1/2) have been shown to be activated by ischemic preconditioning in the hippocampus CA1 (Choi et al., 2006), and based on inhibitor studies suggested to play an important pro-survival role in ischemic tolerance (Gu et al., 2001b, Jin et al., 2006). In contrast, ischemic preconditioning has been shown to attenuate activation of c-Jun-N-terminal kinase-3 (JNK3) (Zhang et al., 2006), which has been implicated to be an important pro-death signal mediating the demise of pyramidal CA1 neurons in response to cerebral ischemia (Ozawa et al., 1999, Nozaki et al., 2001, Irving and Bamford, 2002). The inverse relationship between ERK1/2 and JNK3 activation following ischemic preconditioning suggests a possible causal relationship between the two kinases, e.g. potential crosstalk whereby ERK1/2 activation mediates the attenuation of JNK3 activation.

The purpose of the present study was to test this hypothesis by inhibiting ERK1/2 activation and determining the effect upon activation of JNK3 following ischemic preconditioning. A second goal of the current study was to test the hypothesis that NMDA receptors mediate ischemic preconditioning modulation of the transcription factors, CREB and c-Jun, and that this leads to enhancement of the antiapoptotic factor, Bcl-2, with a concomitant attenuation of the proapoptotic factor, Fas-L – thereby facilitating the development of ischemic tolerance. The results of our study reveal that NMDA receptor activation is critical for ischemic preconditioning up-regulation of ERK1/2 activation and down-regulation of JNK3 activation and that prevention of ERK1/2 activation by use of a MEK inhibitor reinstates both JNK3

activation and delayed neuronal cell death. Our study further revealed a role for NMDA receptors in ischemic preconditioning up-regulation of CREB and Bcl-2, two key pro-survival factors, and in the down-regulation of c-Jun and its transcriptional target, Fas-L, a proapoptotic factor. As a whole, the studies enhances our understanding of the role of NMDA receptors in ischemic tolerance, and provide evidence that NMDA receptors induce a novel crosstalk between ERK1/2 and JNK3 that is critical for the protective and tolerance-inducing effects of ischemic preconditioning.

MATERIALS & METHODS

Materials

The source of the antibodies used in the study are as follows: Anti-active JNKs (recognizes JNK1, JNK2, and JNK3), anti-phospho-ERK1/2 (Thr 202/Tyr 204), anti-phospho-c-Jun (Ser 73), anti-Fas-L, and anti-Bcl-2 antibodies were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-JNK3 antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-phospho-CREB (Ser133) was from Cell Signaling Technology, Inc. (Beverly, MA, USA). Nitrocellulose filter was from Amersham Pharmacia (Little Chalfont, UK). BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) were from Promega (Madison, WI, USA). Vectastain ABC Elite kit and 3,3-diaminobenzidine peroxidase substrate kit was from Vector Laboratories (Burlingame, CA, USA). ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100) was purchased from Chemicon International (Temecula, CA, USA). MK-801 (1 mg/kg in 0.9% saline by intraperitoneal injection 15 min prior to preconditioning) and PD98059 were from Calbiochem (San Diego, CA, USA). All the other chemicals were from Sigma (St Louis, MO, USA) unless indicated otherwise.

Intracerebroventricular administration

PD98059 was prepared by dissolving in DMSO at a concentration of 10 mg/ml. Before in fusion, PD98059 was diluted in sterile saline yielding a 0.1 mg/ml solution in 1% DMSO. Drug infusion was performed using a microinjector through a preimplanted cannula in the left cerebral ventricle (from the bregma: anteroposterior, -0.8 mm; lateral, 1.5mm; depth, 3.5 mm). A volume of 2 µl was infused over 5 min. Infusion of 1% DMSO in saline served as vehicle control. PD98059 was given 2 days after the preconditioning ischemia and 15 min before the second ischemia.

Induction of forebrain ischemia

Adult male Sprague–Dawley rats weighing 250–300 g were used (all procedures were approved by the institutional animal care committee). Brain ischemia was induced by four-vessel occlusion, as described previously (Pulsinelli and Brierley, 1979, Gu et al., 2001a). Briefly, under anaesthesia with chloral hydrate (350 mg/kg, i.p.), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. Ischemia was induced by occluding the common arteries with aneurysm clips for 8 min. Rats which lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during ischemia were selected for the experiments. Rats with seizures were discarded. An EEG was monitored to ensure isoelectricity within 30 s after carotid artery occlusion. Carotid artery blood flow was restored by releasing the clips. Rectal temperature was maintained at about 37°C during and 2 h after ischemia. For preconditioning, animals under went 3 minute ischemia via bilateral occlusion of the common carotid arteries 2 days prior to 8 min global cerebral ischemia. Sham controls were performed using the same surgical exposure procedures, except that the arteries were not occluded.

Tissue preparation

For brain tissue preparation, rats were killed under anesthesia at the indicated time points after cerebral ischemia. The hippocampal CA1 regions were microdissected from both sides of the hippocampal fissure at 0°C and quickly frozen in liquid nitrogen. Tissues were homogenized in ice-cold homogenization medium consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 12 mM β -glycerophosphate, 3 mM dithiothreitol (DTT), 2 mM sodium orthovanadate (Na_3VO_4), 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and 5 $\mu\text{g}/\text{mL}$ each of leupeptin, pepstatin A and aprotinin. The homogenates were centrifuged at 15 000 g for 30 min at 4°C, supernatants were collected and stored at -80°C for use.

Immunoprecipitation and western blotting Western Blotting

To explore the phosphorylation of JNK3, homogenate samples were immunoprecipitated with anti-phospho-JNK antibody, and then western blotted with anti-JNK3 antibody, or directly western blotted with anti-JNK3 antibody to examine the expression. For immunoprecipitation, samples (each containing 400 μg of protein) were diluted four-fold with HEPES buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100 and 1 mM each of EGTA, EDTA, PMSF and Na_3VO_4 . Samples were pre-incubated for 1 h with 20 μL Protein A/G, and then centrifuged to remove any protein adhered non-specifically to the Protein A/G. The supernatant was incubated with 5 μg of anti-active JNKs antibody for 4 h at 4°C. After the addition of Protein A/G-sepharose, the mixture was incubated at 4°C for an additional 2 h. Samples were triple washed with HEPES buffer and eluted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled.

Western blot analysis was carried out on 10% SDS-PAGE according to the method previously described (Gu et al., 2001a). Proteins were electrotransferred onto nitrocellulose filter. After blocking for 2 h in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) and 3% BSA, the membranes were incubated overnight with primary antibody in PBST containing 3% BSA. Detection was carried out by the use of proper alkaline phosphatase conjugated IgG (1:20,000) and developed with NBT/BCIP assay kit. After immunoblotting, the density of the bands was scanned using an image analyzer (LabWorks Software, UVP Upland, CA, USA).

Immunohistochemistry

Rats were anesthetized with chloral hydrate and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed overnight in paraformaldehyde, processed and embedded in paraffin. Coronal brain sections (6 μm thick) were cut on a microtome (Leica RM2155, Nussloch, Germany). Postfixed brains, dehydrated with 30, 50, 70, 80, 90, 95, and 100% ethanol, respectively, were embedded with paraffin. Sections were de-paraffinized in xylene and rehydrated with ethanol at graded concentrations of 30–100% (v/v), and washing with water. High-temperature antigen retrieval was performed in 1 mM citrate buffer. To block endogenous peroxidase activity, sections were incubated for 2 h in a solution containing 0.01% sodium azide and 0.1% H_2O_2 in PBS. To reduce non-specific staining, sections were incubated for 2 h in a blocking solution containing 1% BSA, 2% normal goat serum, 0.3% Triton X-100 and 5% non-fat dry milk in PBS. The sections were then incubated in the primary antibodies (anti-p-ERK1/2, p-c-Jun, p-CREB, Bcl-2, 1:100 diluted in 0.1% BSA, 0.3% Triton X-100 and 1% normal goat serum in PBS), overnight at 4°C. Alternate sections from each brain were incubated without primary antibody as negative controls. After washes three times in PBS, the sections were incubated for 2 h in secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) made up in 0.1% BSA, 0.3% Triton X-100 and 1% normal goat serum in PBS. Sections were washed and incubated with avidin-conjugated horseradish peroxidase (diluted 1:200 in 0.3% Triton X-100) for 1 h at 37°C, followed by avidin-biotin-peroxidase

(ABC, Vector Laboratorie) at room temperature. The sections were examined with a light microscopy.

Histology

Rats were perfusion fixed with 10% paraformaldehyde 5 days after ischemia under anesthesia. Paraffin sections (6- μ m thick) were prepared and stained with 0.1% (w/v) Cresyl violet. The sections were examined with a light microscope and the neuronal density of the hippocampal CA1 pyramidal cells was expressed as the number of cells per 1-mm length of the CA1 pyramidal cells counted under a light microscope (x400).

TUNEL staining

TUNEL staining was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's protocol with minor modifications. The paraffin-embedded coronal sections were deparaffinized and rehydrated, and then treated with protease K at 20 mg/ml for 15 min at room temperature. Sections were incubated with reaction buffer containing TdT enzyme and at 37 °C for 1 h. After washing with stop/wash buffer, sections were treated with anti-digoxigenin conjugate for 30 min at room temperature and subsequently developed color in peroxidase substrate. The nuclei were lightly counterstained with hematoxylin.

Statistical evaluation

Four to five independent animals were sampled at each time point for western blotting study, immunostaining and histology examination. Semi-quantitative analysis of the Western blot bands was performed with the Image J analysis software (Version 1.30v; Wayne Rasband, NIH, USA). All values are expressed as the means $\pm$ SD. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test or Newman–Keuls test. Differences of $p < 0.05$ were considered significant.

RESULTS

Effect of MK-801 on Ischemic Preconditioning Neuroprotection in Hippocampus CA1 Following Global Cerebral Ischemia

To determine the role of NMDA receptors in ischemic preconditioning neuroprotection, the NMDA receptor antagonist, MK-801 was administered 15 min prior to preconditioning and cresyl violet staining was performed to examine the surviving cells of CA1 pyramidal neurons after 5 days of reperfusion. Normal CA1 pyramidal cells showed round and pale stained nuclei, the shrunken cells with pyknotic nuclei were counted as dead cells. As shown in Fig. 1A and 1B, 8-min global cerebral ischemia (R5d) induced severe cell death as compared to sham control (Sham), whereas preconditioning 3 min (P + R5d) 2 days before 8 min global cerebral ischemia markedly decreased the neuronal degeneration. MK-801 administration prior to preconditioning significantly attenuated the neuroprotective effect of preconditioning upon pyramidal neurons in the hippocampus CA1 (Fig. 1A and 1B).

To further investigate the potential protective mechanism, we performed TUNEL staining to assess the apoptotic-like neurons in hippocampal CA1 region at 3 days' reperfusion after 8 min global cerebral ischemia ischemic insult. After 3 days of reperfusion, rats were perfusion-fixed with paraformaldehyde. As shown in Fig. 1A, 8-min global cerebral ischemia (R3d) markedly increased the number of TUNEL-positive cells in hippocampus CA1 region as compared to Sham control (Sham) (Fig 1A). Ischemic preconditioning dramatically decreased the number of TUNEL-positive cells as compared to the 8-min global cerebral ischemia group (Fig. 1A).

Additionally, MK-801 treatment abolished the preconditioning effect on lowering the number of TUNEL-positive cells in the CA1 (Fig. 1A). Taken together, these results indicate that preconditioning can rescue neurons from apoptotic neuronal death in rat hippocampal CA1 region via a NMDA receptor-mediated mechanism.

NMDA Receptor-Mediated Activation of ERK1/2 and Inactivation of JNK3 in Hippocampus CA1 Following Preconditioning

The goal of these studies was to determine the role of NMDA receptors in preconditioning regulation of the prosurvival and prodeath kinases, ERK1/2 and JNK3, respectively in hippocampus CA1. As shown in Fig. 2A–D, Western blot analysis revealed that 8-min global cerebral ischemia significantly decreased ERK1/2 phosphorylation 3 days after reperfusion as compared to sham. In contrast, phosphorylation of JNK3 was markedly increased (over 3-fold increase) by 8-min global cerebral ischemia as compared to sham (Fig. 2B&D). Preconditioning reversed the down-regulation of ERK1/2 phosphorylation induced by 8-min global ischemia, and this effect by preconditioning was completely blocked by MK-801 pretreatment (Fig. 2A&C). Preconditioning also significantly attenuated the up-regulation of JNK3 activation observed following 8-min global ischemia, and the preconditioning effect was significantly reversed by pretreatment with MK-801 (Fig. 2B&D). Thus, preconditioning enhances ERK1/2 activation while attenuating JNK3 activation in hippocampus CA1.

Evidence that Preconditioning-Induced JNK3 Inactivation is Mediated by ERK1/2 Signaling in Hippocampus CA1 Region

Since ERK1/2 and JNK3 activation showed an inverse relationship following preconditioning, we hypothesized that ERK1/2 may regulate JNK3 activation in the hippocampus CA1 following preconditioning. To address this possibility, we administered a MEK inhibitor, PD98059 via icv injection 15 min before 8-min ischemia and determined the effect upon JNK3 phosphorylation. As shown in Fig. 3A&B, treatment with PD98059 significantly attenuated ERK1/2 phosphorylation as compared to preconditioning and vehicle groups (Fig. 3A&B). Intriguingly, PD98059 strongly reversed preconditioning down-regulation of JNK3 phosphorylation in hippocampus CA1. This finding suggests that ERK1/2 signaling is responsible for preconditioning-induced down-regulation of JNK3 activation.

NMDA Receptors Mediate Preconditioning-Induced Down-Regulation of c-Jun Activation and Fas-L and Up-Regulation of p-CREB and Bcl-2 in Hippocampus CA1

It is well known that the transcription factor, c-Jun is activated by JNK, and that c-Jun regulates induction of the proapoptotic factor, Fas-L. We thus examined the effect of preconditioning and MK-801 on c-Jun activation and Fas-L levels after 8-min global cerebral ischemia. As shown in Fig. 4A&B, 8-min global cerebral ischemia (R3d) significantly increased p-c-Jun and Fas-L levels in hippocampus CA1 at 3 days following reperfusion as compared to sham controls. In contrast, preconditioning (P+R3d) abolished the global ischemia-induced elevation of p-c-Jun and Fas-L in hippocampus CA1. The effect of preconditioning on p-c-Jun and Fas-L was significantly reversed by pretreatment with MK-801, while vehicle alone pretreatment had no effect. The results suggest that preconditioning inhibition of p-c-Jun and Fas-L was mediated by NMDA receptors.

Fig. 4C&D illustrates the effect of preconditioning and NMDA receptors in regulation of CREB and the antiapoptotic factor Bcl-2 in hippocampus CA1 3d after global cerebral ischemia. As shown in Fig. 4C&D, preconditioning (P+R3d) markedly increased p-CREB and Bcl-2 levels in hippocampus CA1 as compared to sham and R3d groups. MK-801 pretreatment significantly attenuated preconditioning induction of p-CREB and Bcl-2 levels as compared to vehicle control. The results suggest that NMDA receptors mediate preconditioning ability to enhance activation of the transcription factor, CREB and the antiapoptotic factor Bcl-2.

Immunohistochemical Analysis of p-ERK1/2, p-c-Jun, p-CREB and Bcl-2 Levels in the Hippocampus CA1 Following Preconditioning and MK-801 Treatment

To further confirm the Western blot results, we utilized immunohistochemistry in hippocampus CA1 at 3d after global cerebral ischemia using specific antibodies. As shown in Fig. 5, 8-min global cerebral ischemia (R3d) significantly decreased p-ERK1/2 levels in hippocampus CA1 at 3 days following reperfusion as compared to sham controls. In contrast, preconditioning (P + R3d) prevented the global ischemia-induced reduction of p-ERK1/2 in hippocampus CA1. The effect of preconditioning on p-ERK1/2 was significantly reversed by pretreatment with MK-801. The results confirm the results observed using Western blot analysis and further suggest that preconditioning induction of p-ERK1/2 is mediated by NMDA receptors.

Fig. 5 also demonstrates that preconditioning (P + R3d) abolishes cerebral ischemia induction of p-c-Jun in the hippocampus CA1, and that MK-801 treatment (P+R3d+MK801) reverses the preconditioning effect upon p-c-Jun levels. Furthermore, preconditioning markedly up-regulated p-CREB and Bcl-2 immunostaining levels in hippocampus CA1, and this effect was markedly attenuated by MK-801 treatment. These findings further confirm with the results observed used Western blot analysis, and further suggest that NMDA receptors mediate preconditioning regulation of p-c-Jun, p-CREB and Bcl-2.

DISCUSSION

Regulation of the transcription factor CREB has been implicated to be important in the induction of ischemic tolerance. Our study confirmed the enhanced phosphorylation of CREB in rat hippocampus CA1 following ischemic preconditioning, and demonstrated that the increased CREB phosphorylation is mediated by activation of NMDA receptors. Previous work has shown that CREB is a prosurvival target of ERK1/2 (Arany et al., 2005). Thus, the enhanced ERK1/2 activation observed by ischemic preconditioning in our study may suggest that CREB phosphorylation is achieved via an NMDA receptor-dependent activation of upstream ERK1/2. With respect to the possible downstream genes regulated by CREB activation, Meller et al (Meller et al., 2005) has reported that CREB activation after ischemic preconditioning enhances the expression of the antiapoptotic gene, Bcl-2 in the cerebral cortex of a rat MCAO model. Our study demonstrates that Bcl-2 protein is similarly elevated by ischemic preconditioning in the hippocampus CA1, which parallels CREB phosphorylation, and that the Bcl-2 elevation by ischemic preconditioning is mediated by NMDA receptor activation. Thus, our study provides evidence that NMDA receptors mediate activation of the prosurvival factors ERK1/2 and CREB in the hippocampus CA1 after ischemic preconditioning, which leads to enhanced Bcl-2 and protection against apoptosis.

In addition to activation of ERK1/2-CREB-Bcl-2 prosurvival signaling, our study suggests that NMDA receptors also mediate a *parallel* ischemic preconditioning-induced attenuation of JNK3-c-Jun proapoptotic signaling activation in the hippocampus CA1 after global cerebral ischemia. Previous work by members of our group had shown that JNK3 phosphorylation is attenuated in the hippocampus CA1 by ischemic preconditioning 3 days after global cerebral ischemia (Zhang et al., 2006). Our current study provides a mechanistic explanation for the regulatory effect of ischemic preconditioning by demonstrating that the attenuation of JNK3 phosphorylation is mediated by NMDA receptors via ERK1/2 crosstalk. Precisely how ERK1/2 can down-regulate JNK3 phosphorylation remains unclear. However, previous work by a number of groups has shown that the multi-domain scaffold protein, POSH (plenty of Sh3s) plays an important role in the regulation of JNK1-3 activation (Xu et al., 2003, Zhang et al., 2005). POSH creates a scaffold complex of Rac1, JNK and its upstream activator, MLK 1-3, leading to MLK1-3 activation and subsequent JNK phosphorylation (Xu et al., 2003). Preconditioning has been shown to induce disassembly of the POSH-Rac1-MLK3-JNK complex, which may explain the attenuation of JNK3 activation (Zhang et al., 2006).

Interestingly, examination of the POSH sequence reveals that it has putative ERK binding domains, raising the possibility that ERK1/2 may exert a direct inhibitory effect upon POSH complex assembly, thereby providing a potential mechanism for ERK1/2 attenuation of JNK3 phosphorylation observed after ischemic preconditioning. This interesting possibility requires further study.

Our study also showed that ischemic preconditioning attenuated Fas-L levels, a proapoptotic factor that is a known downstream target of c-Jun (Faris et al., 1998, Herdegen et al., 1998, Suhara et al., 2002). Fas-L binds to its receptor Fas, leading to activation of caspase-dependent apoptosis in neurons (Le-Niculescu et al., 1999). The attenuation of Fas-L (and c-Jun activation) by preconditioning ischemia in our study was abolished by pretreatment with MK801, suggesting that NMDA receptors are critical for ischemic preconditioning attenuation of proapoptotic JNK3-c-Jun-Fas-L signaling in the hippocampus CA1. The TUNEL staining results obtained in our study further confirms the importance of NMDA receptors in the antiapoptotic effect of ischemic preconditioning, as MK801 markedly reversed the attenuation of TUNEL staining induced by ischemic preconditioning in the hippocampus CA1.

It is important to know how much were the effects of the preconditioning stress itself on the changes in ERK1/2-CREB-Bcl-2 and JNK3-c-Jun-Fas-L signaling systems. Therefore, rats were subjected to 3 min ischemia for reperfusion several times or followed by sham operation (instead of 8-min ischemia). Results (Supplemental Fig. 1) showed that preconditioning stress itself is not sufficient to significantly modulate the proapoptotic factors, p-JNK3, p-c-Jun and Fas-L in the hippocampus CA1. However, p-ERK, p-CREB and Bcl-2 were elevated at 1 day and 3 days after reperfusion. This suggests that the prosurvival signaling already changed substantially before induction of 8-min ischemia in the preconditioned animals, and these changes probably underlies the protective mechanisms of mild preconditioning ischemia.

In conclusion, the current studies provide evidence that NMDA receptors mediate ischemic tolerance by inducing a prosurvival ERK1/2-CREB-Bcl-2 signaling pathway, with concomitant attenuation of proapoptotic JNK3-c-Jun-Fas-L signaling in the hippocampus CA1. The study further demonstrates a novel NMDA receptor-mediated crosstalk between ERK1/2 and JNK3 that leads to attenuation of JNK3 activation and is critical for induction of ischemic tolerance in the hippocampus CA1. As a whole, the studies emphasize the importance of NMDA receptors in ischemic tolerance and provide a mechanistic understanding to the vital role that these receptors play in mediating ischemic preconditioning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CREB	cyclic adenosine monophosphate response element binding protein
ERK1/2	extracellular signal-regulated kinase 1/2
JNK	

c-Jun-N-terminal kinase

NMDA

N-methyl-d-aspartate

MAPK

mitogen activated protein kinases

TUNEL

Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

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Fig. 1. Effect of the NMDA receptor antagonist, MK-801 on ischemic preconditioning attenuation of apoptosis and neuronal cell death in the hippocampus CA1. (A)

TUNEL or Cresyl violet staining was performed on the sections from the hippocampi in sham animals (Sham), rats subjected to 3 or 5 days of reperfusion after 8 min of ischemia (R3d and R5d, respectively), rats subjected to 3 min ischemic preconditioning 2 days prior to 8-min global cerebral ischemia (P+R3d or P+R5d), and rats pretreated with MK-801 15 min prior to ischemic preconditioning (MK-801). **(B)** The number of survival CA1 neurons per 1mm length of the medial CA1 pyramidal cell layer was counted as indicated. Note that ischemic preconditioning significantly increased the number of the survival neurons compared with control at 5 days of reperfusion in the CA1 region, an effect significantly attenuated by

MK-801. Data were obtained from four to five independent animals and a typical experiment is presented. * $p < 0.05$ vs. sham control; # $p < 0.05$ vs. P + 5d groups, magnification 400 $\times$ and the scale bar 10 μm . P: cerebral ischemia preconditioning.

Fig. 2. Western blotting analysis of the effects of MK-801 on ERK1/2 and JNK3 activation and expression in hippocampal CA1 after ischemic preconditioning

Rats were subjected to sham operation (sham), 8-min global cerebral ischemia + 3d reperfusion (R3d); ischemic preconditioning (P+R3d), ischemic preconditioning + saline + 3d reperfusion (saline), ischemic preconditioning + MK-801 pretreatment + 3d reperfusion (MK-801).

Hippocampal CA1 protein lysates were collected and Western blotting was performed. **(A)** Representative blots corresponding to phosphorylation and expression levels of ERK1/2 at 3 days after reperfusion in hippocampal CA1 region. **(B)** Representative blots corresponding to phosphorylation and expression levels of JNK3 at 3 days after reperfusion in hippocampal CA1 region. **(C & D)** Semiquantitative analysis of the levels of ERK1/2 (C) and JNK3 (D) levels and phosphorylation in hippocampal CA1 region. Data are mean $\pm$ SD (n = 4). *p < 0.05 vs. sham control; #p < 0.05 vs. vehicle groups.

Fig. 3. Western blotting analysis of the effects of the MEK inhibitor, PD98059 on ERK1/2 and JNK3 activation in hippocampal CA1 after ischemic preconditioning

Rats were subjected to sham operation (sham), ischemic preconditioning (P+R3d), ischemic preconditioning + saline + 3d reperfusion (saline), ischemic preconditioning + PD98059 pretreatment + 3d reperfusion (PD98059). Hippocampal CA1 protein lysates were collected and Western blotting was performed. **(A)** Representative blots corresponding to phosphorylation levels of ERK1/2 and JNK3 at 3 days after reperfusion in hippocampal CA1 region. **(B)** Semiquantitative analysis of the levels of p-ERK1/2 and p-JNK3 levels in hippocampal CA1 region from the different groups. Data are mean $\pm$ SD (n = 4). *p < 0.05 vs. sham control; #p < 0.05 vs. vehicle groups.

Fig. 4. Effect of MK-801 on activation of the transcription factors, c-Jun and CREB, and their transcriptional targets, Fas-L and Bcl-2, in the hippocampal CA1 region after ischemic preconditioning

Rats were subjected to sham operation (sham), 8-min global cerebral ischemia + 3d reperfusion (R3d); ischemic preconditioning (P+R3d), ischemic preconditioning + saline + 3d reperfusion (saline), ischemic preconditioning + MK-801 pretreatment + 3d reperfusion (MK-801).

Hippocampal CA1 protein lysates were collected and Western blotting was performed. **(A)** Representative blots corresponding to levels of p-c-Jun and fas-L at 3 days after reperfusion in hippocampal CA1 region. **(C)** Representative blots corresponding to levels of p-CREB and Bcl-2 at 3 days after reperfusion in hippocampal CA1 region. **(B & D)** Semiquantitative

analysis of the levels of indicated factors in hippocampal CA1 region. Data are mean $\pm$ SD (n = 4). *p < 0.05 vs. sham control; #p < 0.05 vs. vehicle groups.

Fig. 5. Immunohistochemical confirmation of ischemic preconditioning and MK-801 effects upon p-ERK1/2, p-c-Jun, p-CREB and Bcl-2 levels in hippocampal CA1 region at 3 days after reperfusion

Rats were subjected to sham operation (sham), 8-min global cerebral ischemia + 3d reperfusion (R3d); ischemic preconditioning (P+R3d), or ischemic preconditioning + MK-801 pretreatment + 3d reperfusion (MK-801) and hippocampal sections collected for immunohistochemical analysis. Results are representative from an $n = 4-5$ rats per group. Magnification 400 $\times$ and the scale bar 10 μ m. See text for description.