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# NEUROPROTECTION OF PRECONDITIONING AGAINST ISCHEMIC BRAIN INJURY IN RAT HIPPOCAMPUS THROUGH INHIBITION OF THE ASSEMBLY OF GluR6—PSD95—MIXED LINEAGE KINASE 3 SIGNALING MODULE VIA NUCLEAR AND NON-NUCLEAR PATHWAYS

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**Abstract—Our previous studies showed that the assembly of the GluR6–PSD95–mixed lineage kinase 3 (MLK3) signaling module played an important role in rat ischemic brain injury. In this study, we aimed to elucidate whether ischemic preconditioning could downregulate the assembly of the GluR6–PSD95–MLK3 signaling module and suppress the activation of MLK3, MKK4/7, and c-Jun *N*-terminal kinase (JNK). As a result, ischemic preconditioning could not only inhibit the assembly of the GluR6–PSD95–MLK3 signaling module, diminish the phosphorylation of the transcription factor c-Jun, downregulate Fas ligand expression, attenuate the phosphorylation of 14-3-3 and Bcl-2 and the translocation of Bax to mitochondria, but also increase the release of cytochrome *c* and the activation of caspase-3. In contrast, both GluR6 antisense ODNs (oligodeoxynucleotides) and 6,7,8,9-tetrahydro-5-nitro-1 H-benz[g]indole-2,3-dione-3-oxime (NS102), an antagonist of GluR6 receptor, prevented the above effects of preconditioning, which shows that suppressing the expression of GluR6 or inhibiting GluR6 activity contributes negatively to preconditioning-induced ischemia tolerance. Taken together, our results indicate that preconditioning can inhibit the over-assembly of the GluR6–PSD95–MLK3 signaling module and the JNK3 activation. GluR6 subunit-containing kainite receptors play an important role in the preconditioning-induced neuronal survival and provide new insight into stroke therapy.** **© 2009 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: cerebral ischemia, GluR6, c-Jun, preconditioning, JNK3, PSD-95.**

Excitatory neurotransmitter is one of the main mechanisms of neuronal death in stroke. Ionotropic glutamates as the major excitatory neurotransmitter include *N*-methyl-D-aspartate (NMDA), AMPA and kainite (KA) (Seeburg, 1993; Hollmann and Heinemann, 1994). Although the bulk of the evidence suggests that NMDA receptors may play a pivotal role in ischemia-induced neuronal death, the role of KA receptors in ischemia brain injury was paid more and more attention. Garcia's and Mehta's studies (Garcia et al., 1998; Mehta et al., 2001) showed that the RLPGKETMA motif of the C terminus of GluR6, a subunit of KA receptors, could bind to the PDZ1 domain of the postsynaptic density protein PSD95/SAP90 through specific interaction. A recent study has also shown that MLK3 (mixed lineage kinase 3), an upstream kinase of c-Jun *N*-terminal kinase (JNK) (Tibbles et al., 1996), could interact with the SH3 (SRC homology) domain of PSD95 (Guan et al., 2005). Our previous studies demonstrated that the KA receptor subunit GluR6 mediated JNK activation was involved in ischemic brain injury (Pei et al., 2006).

Ischemic tolerance, also known as preconditioning ischemia, is induced by a sublethal transient ischemia, and then the stressed, but not injured, cells resist subsequent severe ischemia insult (Kitagawa et al., 1990; Kirino et al., 1991; Liu et al., 1992; Weih et al., 1999). Several signaling proteins were reported to contribute to the induction of cerebral ischemic tolerance, such as nNOS (Wiggins et al., 2003), Akt (Yano et al., 2001), extracellular signal-regulated kinase 1/2 (Gu et al., 2000; Garcia et al., 2004), and p38 (Nishimura et al., 2003). We have previously shown that preconditioning attenuated the ischemic cerebral injury induced by ischemia/reperfusion (I/R) and furthermore this process was mediated by NMDA receptor (Miao et al., 2005).

Our previous studies on the mechanism of neuronal cell death induced by global ischemia via GluR6 mediated signaling have focused on two different possible pathways: nuclear pathway and non-nuclear pathway (Guan et al., 2005). Activated JNK3 translocates into the nucleus and phosphorylates transcription factor c-Jun, which leads to increase of activator-protein 1 (AP-1) transcription activity and cell apoptosis. Activated JNK3 may enhance the expression of the Fas ligand (FasL), which could ultimately contribute to Fas receptor-mediated apoptosis. On the other hand, part of activated JNK remains in the cytosol, regulates the activation of non-nuclear substrates, including Bcl-2 family members such as the pro-apoptotic protein Bax, Bim and Bid, and promotes ischemic cell death by including the release of apoptogenic factor cytochrome *c* (Cao et al., 2001; Plesnila et al., 2001), which could ultimately contribute to mitochondrion-mediated apoptosis. Based on the above evidence, we wanted to elucidate whether preconditioning-induced neuronal survival was mediated by the GluR6–PSD95–MLK3 signaling module

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*Abbreviations:* AP-1, activator-protein 1; DMSO, dimethyl sulfoxide; FasL, Fas ligand; I/R, ischemia/reperfusion; JNK, c-Jun *N*-terminal kinase; KA, kainite; MLK3, mixed lineage kinase 3; NMDA, *N*-methyl-D-aspartate; NS102, 6,7,8,9-tetrahydro-5-nitro-1 H-benz[g]indole-2,3-dione-3-oxime; PMSF, phenylmethylsulfonyl fluoride; PNPP, p-nitrophenyl phosphate; TBST, Tris-buffered saline with 0.1% Tween 20.

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via the nuclear and non-nuclear pathways of JNK3, which occurs during ischemic injury.

In the present study, we demonstrated that ischemic preconditioning could inhibit the assembly of the G1uR6–PSD95–MLK3 signaling module. Furthermore, ischemic preconditioning decreased neuronal death induced by global ischemia via the nuclear and non-nuclear pathway of JNK3.

## EXPERIMENTAL PROCEDURES

### Antibody and reagents

The following primary antibodies were used: from Santa Cruz Biotechnology (Santa Cruz, CA, USA), goat polyclonal anti-G1uR6 (sc-7618), mouse monoclonal anti-phospho-JNKs (sc-6254), rabbit polyclonal anti-MLK3 (sc-13072), rabbit polyclonal anti-Bcl-2 (sc-492), rabbit polyclonal anti-Fas (sc-716), rabbit polyclonal anti-14-3-3 (sc-1019), rabbit polyclonal anti-Bcl-2 (sc-492), rabbit polyclonal anti-p-Bcl-2 (sc-16323-R) and rabbit polyclonal anti-c-Jun (sc-1694). Rabbit polyclonal anti-FasL (# 4273), rabbit polyclonal anti-Bax (# 2772), rabbit polyclonal anti-cytochrome c (# 4272), rabbit polyclonal anti-caspase-3 (# 9661), and rabbit polyclonal anti-phospho-p-MLK3 antibodies were obtained from Cell Signaling Biotechnology, Inc (Boston, MA, USA). The secondary antibodies were goat antimouse IgG, goat antirabbit IgG, and donkey anti-goat IgG (Sigma, St. Louis, MO, USA). Anti-G1uR6 antibody (catalog no. 06-309) was obtained from Upstate Biotechnology, Inc (Lake Placid, NY, USA). Mouse monoclonal anti- PSD95 (CP35-100UL) antibody was bought from Oncogene (Cambridge, MA, USA). Monoclonal antibody against cytochrome-*c* oxidase is from Molecular Probes (Eugene, OR, USA). Nonspecific mouse or rabbit IgG was also purchased from Sigma.

### Ischemic model

Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center of the Chinese Academy of Science) weighing 200–250 g were used. Experiments conformed to the local (Regulations for the administration of affairs concerning experimental animals) and international (Dolan K. 2000 Laboratory Animal Law. Blackwell Science, London, UK) guidelines on the ethical use of animals. Efforts were made to minimize the number of animals used in the experiments and their suffering. Anesthesia was induced with 20% choral hydrate [350 mg/kg i.p.], then vertebral arteries were electrocauterized and common carotid arteries were dissected free. Rats were allowed to recover for 24 h while food was withheld for 8 h overnight. To achieve global ischemia, four-vessel occlusion was included by occluding the common arteries with aneurysm clips. The rats which lost righting reflex within 15 s with their pupils dilated were selected for experiments. Rectal temperature was maintained between 37 and 38 °C throughout the procedure. The clips were released to restore carotid artery blood flow (Pulsinelli and Brierley, 1979). As preconditioning ischemia was desired, the occlusion was set for 3 min. Three days after, 6 min of ischemia was induced (Liu et al., 1992). Sham controls were obtained using the same surgical procedures mentioned above, excepting those used to obtain occlusion. All the rats were kept in a room maintained at a constant temperature of 23–25 °C.

### Administration of drugs

6,7,8,9-Tetrahydro-5-nitro-1 H-benz[g]indole-2,3-dione-3-oxime (NS102) (10 mM dissolved in 5 μl dimethyl sulfoxide [DMSO, N-179, Sigma]) was infused into the left cerebral ventricle (anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma) 20 min before preconditioning ischemia over 5 min by using a microinjector. Vehicle control rats received dissolvent DMSO. One hundred micrograms of specific antisense ODNs against GluR6 in 10 μl TE buffer (Nishimura et al., 2003) was administrated to the

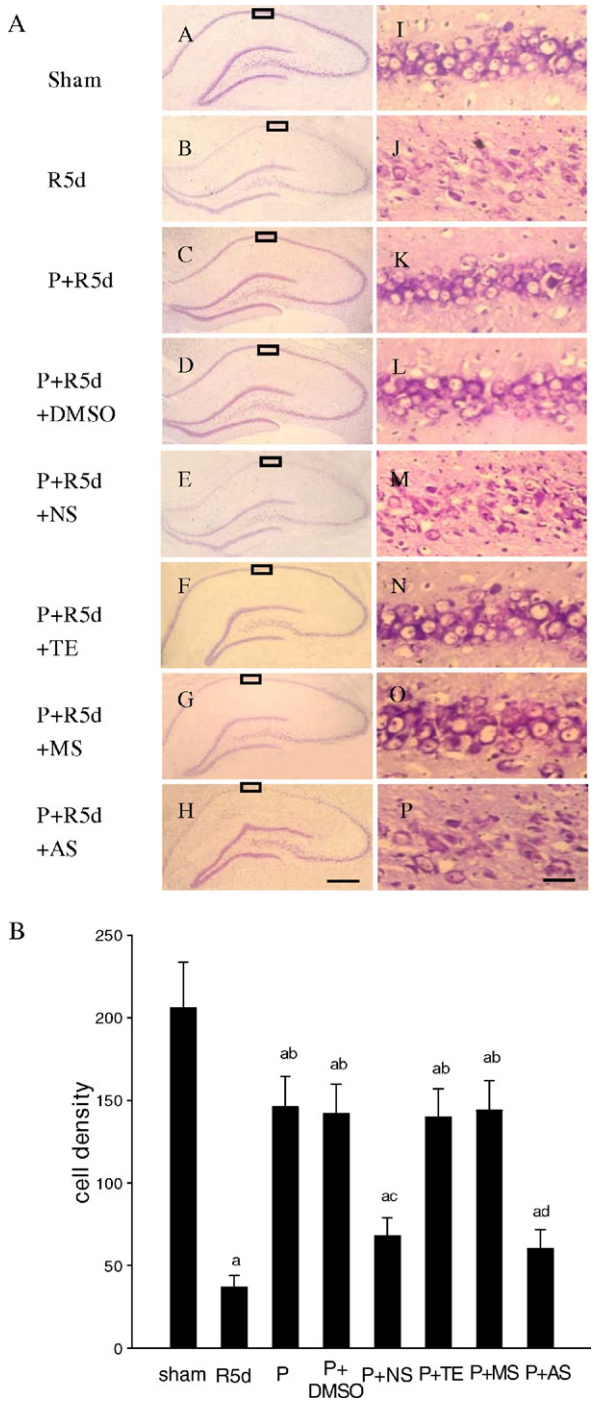


**Fig. 1.** Effects of preconditioning and NS102, GluR6 antisense ONDs on hippocampal CA1 subfield. Example of Cresyl Violet–stained sections of the hippocampi of sham-operated rats (A, I) and rats subjected to 6 min of ischemia followed by 5 days of reperfusion (B, J), and rats subjected to 6 min of ischemia followed by 5 days of reperfusion with preconditioning (C, K), the other groups of rats was pretreated with DMSO (D, L) or NS102 (10 mmol) (E, M) or GluR6 antisense (AS) or missense (MS) oligodeoxynucleotides or vehicle (TE) (F–P) 20 min before preconditioning. Data were obtained from six independent animals and results of a typical experiment are presented. Boxed areas in left column are shown at higher magnification in right column. A–H: ×40; I–P: ×400. Scale bar=200 μm in H; scale bar=10 μm in P.

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