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ACTIVATION OF GABA RECEPTORS ATTENUATES NEURONAL APOPTOSIS THROUGH INHIBITING THE TYROSINE PHOSPHORYLATION OF NR2A BY Src AFTER CEREBRAL ISCHEMIA AND REPERFUSION

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Abstract—Cerebral ischemia can induce both the increase of excitation and the decrease of inhibition, which leads to neuronal excitotoxicity. Since glutamatergic and GABAergic transmissions work by each counterbalancing the function of the other, enhancing GABAergic activity should balance excessive glutamatergic excitation. But the potential mechanisms underlying these effects are obscure. Here, we used two GABA agonists, muscimol and baclofen, and performed immunoblotting, immunoprecipitation and histology analysis to evaluate the neuroprotective effects by stimulating GABA receptors in rat four-vessel occlusion (4-VO) ischemic model, and to investigate the potential mechanism. Our results indicate that whether in global cerebral ischemia *in vivo*, or in oxygen glucose deprivation (OGD) *in vitro*, coapplication of muscimol with baclofen can protect neurons from neuronal death through down-regulating the function of *N*-methyl-D-aspartic acid (NMDA) receptors via attenuating the tyrosine phosphorylation of NR2A subunit. We further elucidate that the phosphorylation level of Src kinase and the interaction among Src, post-synaptic density protein 95 and NR2A were also suppressed by coapplication of muscimol with baclofen. Both MK-801, a specific antagonist of NMDA receptors, and chelerythrine, an inhibitor of protein kinase C (PKC), could down-regulate the phosphorylation of NR2A via inhibiting the activation of Src and PKC respectively. These results suggest that the modified pattern of dynamic balance between excitation and inhibition by coactivation of the GABA receptors in cerebral ischemia can attenuate the excitatory NMDAR via inhibiting a novel postsynaptic NMDAR/Src-mediated signal amplification, the 'NMDAR-Ca^{2+} → PKC → Src → NMDAR-Ca^{2+}' cycle.

Key words: cerebral ischemia, GABA receptor, NMDA receptor, muscimol, baclofen, Src.

It has been shown that the balance between neuronal excitation and inhibition in brain is critical for maintaining normal brain function. Cerebral ischemia can induce the imbalance of excitation and inhibition. Glutamate, the major excitatory neurotransmitter in the CNS, is studied most often in relation to the development of cerebral ischemia-induced cell death. Glutamate receptors are classified into two groups: metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). Ionotropic glutamate receptors have been pharmacologically divided into three classes: *N*-methyl-D-aspartic acid (NMDA), AMPA, and kainate (KA) receptors. The NMDA receptor is a ligand-gated calcium channel that plays an essential role in neuronal development, addiction, and learning and memory (Kumari and Ticku, 2000). Excessive release of glutamate causes over-activation of glutamate receptors, mainly NMDA receptors, and subsequent calcium overloading, which is known as excitotoxicity, and then activates calcium-dependent signaling cascade reactions of postsynaptic neurons that eventually lead to neuronal death (Lee et al., 1999). The NMDA receptors are formed by assembly of two classes of subunits, a principal NR1 subunit and modulatory NR2 subunits (NR2A–NR2D) (Kutsuwada et al., 1992). The NMDA receptors are phosphorylated on tyrosine residues of NR2 subunits, particularly NR2A and NR2B (Lau and Huganir, 1995). Evidence has accumulated that tyrosine phosphorylation of NMDA receptors is mediated principally by Src family protein tyrosine kinases (PTK), which up-regulates the function of NMDA receptors (Yu et al., 1997; Lu et al., 1999). However, the exact signaling pathway of NR2A tyrosine phosphorylation induced by ischemia and reperfusion is still not sure. Previous studies indicate that several molecules including proline-rich tyrosine kinase 2 (Pyk2) and protein kinase C (PKC) are involved in this process (Siciliano et al., 1996; Lu et al., 1999). In cerebral ischemia, our previous studies showed the activity of Src and Pyk2, and the interactions among Src, NR2A and post-synaptic density protein 95 (PSD-95) critically related to the phosphorylation of NR2A (Hou et al., 2003; Liu et al., 2005).

While glutamate neurotransmission has received widespread attention in cerebral ischemia, relatively few investigators have focused on the ischemia-induced alterations in inhibitory neurotransmission. GABA is the primary inhib-

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Abbreviations: CAMK II, CaM-dependent protein kinase II; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMSO, dimethyl sulfoxide; EBSS, Earl's balanced salt solution; h-DMEM, high-glucose Dulbecco's modified Eagle medium; I/R, ischemia/reperfusion; MK-801, hydrogen maleate (M107); NMDA, *N*-methyl-D-aspartic acid; OGD, oxygen-glucose deprivation; PBS, phosphate buffer; PBST, phosphate buffer with 0.1% Tween 20; PKC, protein kinase C; PMSF, phenylmethylsulfonyl; PSD-95, post-synaptic density protein 95; PTK, protein tyrosine kinase; PY, phosphotyrosine; Pyk2, proline-rich tyrosine kinase 2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, Src-homology; 4-VO, four-vessel occlusion.

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itory neurotransmitter in the CNS. GABA may be of particular importance because it functions in opposition to glutamate, thus enhanced GABAergic activity should balance excessive glutamatergic excitation, the pivotal event leading to cell death (Allen et al., 2004). GABA produces its physiological effects by acting on three different receptor subtypes: $GABA_A$, $GABA_B$ and $GABA_C$ (Bormann 2000). At present, little is known about $GABA_C$ receptor-mediated events. $GABA_B$ receptors, which exist at both pre- and post-synaptic locales, are metabotropic G-protein-coupled and mediate the slow inhibitory neurotransmission of GABA signaling via several second messenger pathways and Ca^{2+} and K^+ ion channels (Bormann, 2000). Ionotropic $GABA_A$ receptors, in contrast, produce fast inhibitory synaptic transmission via an intrinsic chloride channel. Activation of $GABA_A$ receptors increases Cl^- conductance, inducing hyperpolarization and reducing cell excitability (Oja et al., 1990). Previous studies suggest that the pharmacologically increased level of GABA activity is correlated with lessened degree of brain ischemic injury (Johansen and Diemer, 1991). Conversely, other studies failed to show the benefit of activating GABA receptors in ischemia, particularly those agents relatively specific for the $GABA_B$ receptor (Rosenbaum et al., 1990). Furthermore, whether stimulation of GABA receptors has neuroprotective effects is not certain. Therefore, the potential neuroprotective mechanism of GABA receptor activation in cerebral ischemia remains to be elucidated.

Previous studies showed that stimulation of NMDA receptors induced by cerebral ischemia leads to an influx of Ca^{2+}, which activates Pyk2 via Ca^{2+}/CaM or PKC (Lev et al., 1995; Siciliano et al., 1996). Our recent studies also confirmed that Src and Pyk2 bound to NR2A earlier than the increase in tyrosine phosphorylation of NR2A after cerebral ischemia (Liu et al., 2001; Ma and Zhang, 2003). Activated Pyk2 may bind to Src and activate it, and then increase the association of PSD-95 with Src and NR2A, subsequently promoting tyrosine phosphorylation of NR2A and amplifying the up-regulation of NMDA receptor function. The positive feedback leads to intracellular Ca^{2+} overload and contributes to ischemia neuronal death. Thus, the present study was aimed to identify whether stimulation of the inhibitory GABA receptors has neuroprotective effects through inhibiting the phosphorylation of NR2A via disassembly of Src-PSD95-NR2A signaling module in cerebral ischemic-reperfusion, so as to find a novel therapeutic approach to cerebral ischemia.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

The following primary antibodies were used: Mouse monoclonal anti-PSD-95 (P246-100UL), rabbit polyclonal anti-NMDAR2A (G9038), mouse monoclonal anti-phosphotyrosine (PY) (P3300) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-Src (#05-184), mouse monoclonal anti-phospho-Src (Tyr416) (#16-249) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-NMDAR2A (AB1555) was purchased from Chemicon (Temecula, CA, USA). Mouse monoclonal anti-phospho-Src (PY416) (SA314) was purchased from Biomol (Plymouth Meeting, PA, USA). The secondary antibodies used in our experiment were goat anti-rabbit IgG and goat anti-mouse IgG. They were from Sigma. ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100) was purchased from Chemicon. Muscimol (EA-126) was obtained from Biomol. Baclofen (B5399) and chelerythrine chloride (C2932) were purchased from Sigma. PP2 (#529573) and PP3 (#529574) were obtained from Calbiochem. MK-801 was purchased from Sigma.

Animal model of ischemia

Adult male SD rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 250–300 g were used. The experiments using rats were approved by the animal use committee in the institution and the experiments were conducted accordingly. 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. Four-vessel occlusion (4-VO) cerebral ischemia was induced as described before (Zhang et al., 2003). Briefly, under anesthesia 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. 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 controlled at 36.5–37.5 °C before and after ischemia–reperfusion and after treatment with drugs via a temperature-regulated heating pad. Sham control animals received the same surgical procedures except that carotid arteries were not occluded.

Administration of drugs

Muscimol and baclofen were dissolved in saline respectively. Muscimol (1 mg/kg) (Costa et al., 2004) and baclofen (20 mg/kg) (Jackson-Friedman et al., 1997) were coapplied or given in isolation to the rats 30 min before ischemia through i.p. injection. Rats were given MK-801 (3 mg/kg dissolved in saline) (Mabuchi et al., 2001) or vehicle (saline) by i.p. administration 60 min prior to ischemia. Fifteen micrograms of PP2 or PP3 (Hanke et al., 1996) or chelerythrine chloride dissolved in 10 μl dimethyl sulfoxide (DMSO) was given 30 min before ischemia. An equal volume of DMSO infusion in the rats served as vehicle control. Drug infusion was performed, using a microinjector through both cerebral ventricles (from the bregma: anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) at a rate of 1 μl/min.

Tissue preparation

For brain tissue preparation, rats were killed under anesthesia at several time points of reperfusion after 15 min of global cerebral ischemia. Whole brains were removed for dissections and the hippocampal CA1 regions were microdissected from both sides of the hippocampal fissure and immediately frozen in liquid nitrogen. Cytosol fractions were extracted as previously described procedure (Ogita and Yoneda, 1994). Briefly, tissue samples were homogenized in 1.5 ml of 10 mM Hepes, pH 7.9, 0.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 5 mM DTT, 10 mM β-phosphoglycerol, 1 mM Na_3VO_4, 1% NP-40, 1 mM benzamidine and enzyme inhibitors: 5 mg/ml phenylmethylsulfonyl (PMSF), 5 mg/ml each of pepstatin A, leupeptin, aprotinin, and then were centrifuged for 10 min at 800×g. The supernatant was removed and stored at −80 °C until use. The protein concentra-