N-methyl-D-aspartate receptors mediate diphosphorylation of extracellular signal-regulated kinases through Src family tyrosine kinases and Ca\(^{2+}\)/calmodulin-dependent protein kinase II in rat hippocampus after cerebral ischemia

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Abstract: Objective: Extracellular signal-regulated kinases (ERKs) can be activated by calcium signals. In this study, we investigated whether calcium-dependent kinases were involved in ERKs cascade activation after global cerebral ischemia.

Methods: Cerebral ischemia was induced by four-vessel occlusion, and the calcium-dependent proteins were detected by immunoblot.

Results: Lethal-simulated ischemia significantly resulted in ERKs activation in N-methyl-D-aspartate (NMDA) receptor-dependent manner, accompanying with differential upregulation of Src kinase and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) activities. With the inhibition of Src family tyrosine kinases or CaMKII by administration of PP2 or KN62, the phosphorylation of ERKs was impaired dramatically during post-ischemia recovery. However, ischemic challenge also repressed ERKs activity when Src kinase was excessively activated.

Conclusions: Src family tyrosine kinases and CaMKII might be involved in the activation of ERKs mediated by NMDA receptor in response to acute ischemic stimuli in vivo, but the intense activation of Src kinase resulted from ischemia may play a reverse role in the ERKs cascade.

Keywords: cerebral ischemia; extracellular signal-regulated kinases; NMDA receptors; Src family tyrosine kinases; CaMKII

1 Introduction

Global ischemia has been shown to induce neuronal apoptosis or necrosis in rat hippocampus. N-methyl-D-aspartate (NMDA) receptor-dependent calcium influx plays a pivotal role in neuronal damage resulted from ischemia, which also triggers the complex intracellular cascades\(^1\). Extracellular signal-regulated kinases (ERKs), the representative members of mitogen-activated protein kinase (MAPK) family, have been demonstrated to be activated by Ca\(^{2+}\) influx in vitro\(^2,3\) and occasionally in vivo\(^4\). ERKs immediately participate in modulating the expression of early genes like c-fos and Egr-1, and control cellular proliferation, differentiation, survival, and even apoptosis. The activation of ERK appears to result from their diphosphorylation at both threonine and tyrosine residues in the regulatory region. Previous studies indicate that Src family tyrosine kinases are involved in the activation of Ras (mitogen activated protein kinase kinase kinase, MAPKKK) elicited by hypoxia in vitro, and so contribute to ERK phosphorylation through the MAPKKK/MAPKK/MAPK cascade\(^5\).

Recent in vitro studies suggest that Src family tyrosine kinases are also associated with the activation of Akt and ERK elicited by glutamate\(^6\).

Src family tyrosine kinases have nine members including Src, Fyn, Lck, Hck, Blk, Lyn, Fgr, Yes, and Yrk. Src kinase is largely expressed in the brain. The cellular Src protein in the vertebrates can usually be maintained on a stable baseline by two kinds of intramolecular binding of Src homology 2 (SH2) to the Src homology 3 (SH3) and to the tyrosine residue 527 (Tyr-527). Dephosphorylation at Tyr-527 and the following autophosphorylation at Tyr-416 result in the activation of Src in response to various stimuli\(^7\). Src kinase can be activated by the elevation of cytoplasmic calcium, and involved in ERK activity through a Src-Ras
cassette\(^{[3]}\).

Furthermore, calmodulin-dependent protein kinase II (CaMKII), a well-known calcium/calmodulin sensor with broad substrate specificity, has also been found involve in ERK cascades by phosphorylating SynGAP in neurons\(^{[8,10]}\), and some correlative phenomena have been observed in glutamate-induced neuronal injury\(^{[11]}\). CaMKII is enriched in postsynaptic densities and is easily activated by calcium ion. The autophosphorylation on Thr-286 in the CaMKII regulatory domain may render the kinase calcium/phospho- and calmodulin independently activated\(^{[12]}\).

In the present report, the temporal effects of ischemia alone and of post-ischemia recovery on the Src, CaMKII and ERKs proteins were examined separately with phospho- and non-phospho-antibodies in the ischemia-sensitive region of rat hippocampus; and then the effect of NMDA receptor antagonist, Src family tyrosine kinases or CaMKII inhibitor on the ischemia-induced activation of ERKs was assessed. We found that Src family tyrosine kinases and CaMKII were closely related to the NMDA receptor-induced ERK activation stimulated by ischemia. But the intense activation of Src kinase did not participate in the upregulation of ERK activity after brain ischemia. The different and complex mechanism remains to be further elucidated.

## 2 Materials and methods

### 2.1 Surgical procedures

Adult male Sprague–Dawley rats (250–300 g, from Experimental Animal Center, Nanjing Medical University) were selected for animal model. All procedures involving the care and use of rat were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Four-vessel occlusion method was used to induce forebrain ischemia as previously described\(^{[13]}\). Namely, rats were deeply anesthetized with chloral hydrate (300 mg/kg), and both vertebral arteries were occluded permanently by electrocoagulation, then they were recovered and fasted overnight. The next day, both carotid arteries were occluded with aneurysm clips for 5, 10, 15, or 30 min. The rectal temperature of the animals was kept at 37.0 °C. Only the rats losing righting reflex and unresponsive to light with dilated pupils were used in the following study. The electroencephalogram was monitored to ensure isoelectricity. The sham operation animals were prepared following the same surgical procedures except that the arteries were not occluded.

### 2.2 Administration of biochemical reagents

To evaluate the effect of NMDA receptor blocking and inhibiting Src family tyrosine kinases and CaMKII on the ischemia-induced ERK activation, the selective NMDA receptor inhibitor Ketamine or the same volume of the vehicle (saline) was administered to the rats (50 mg/kg, i.p.). After that, the CaMKII inhibitor 1-[N,O-bis(5-isouquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62), the selective Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP2), or the same volume of the vehicle (Me\(_2\)SO) was administered (25 μg / 5 μL, i.c.v.) separatively over 5 min by using a microinjector through the left cerebral ventricle (from the Bregma: anteroposterior, –0.8 mm; lateral, 1.5 mm; depth, 3.5 mm). All reagents were injected 30 min before occlusion.

### 2.3 Samples preparation and immunoblot

Rats were killed by decapitation at 0 (sham), 5, 10, 15 and 30 min of ischemia or at 15 min, 1 h, 6 h and 24 h of recovery after 15 min of ischemia. The whole hippocampus of each rat was rapidly separated and frozen in liquid nitrogen. Then tissues were homogenized in 1:10 (W/V) ice-cold homogenization buffer containing (in mmol/L) HEPES 50, KCl 100, MgCl\(_2\) 0.5, DTT 0.2, Na\(_2\)VO\(_4\) 5, NaF 50, EDTA 1, EGTA 1, PMSF 1, pH 7.4, and 1% mammalian protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO, USA). Cytoplasm protein was extracted by centrifuging the sample at 800 g for 10 min at 4 °C. Protein concentration was determined by the Bradford method\(^{[14]}\). Followingly, the extracted proteins were denatured in sodium dodecyl sulfate (SDS) sample buffer at 100 °C for 5 min, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocked for 3 h in phosphate-buffered saline with 3% bovine serum albumin (BSA), membranes were probed with anti-ERK antibody (polyclonal, 1:4 000 dilution; Cell Signaling Technology, Beverly, MA, USA), anti-active diphosphorylated ERK antibody (monoclonal, 1:2 000; Cell Signaling Thechnology), anti-Src antibody (polyclonal, 1:1 000, Cell Signaling Thechnology), anti-phospho-Src (monoclonal, Tyr-416, 1:1 000, Cell Signaling Thechnology), anti-CaMKII antibody (polyclonal, 1:3 000; Sigma, St. Louis, MO, USA), or anti-active-CaMKII antibody (polyclonal, Thr-286; 1:5 000, Promega) at 4 °C overnight. Detections were performed with alkaline phosphatase conjugated goat anti- rabbit IgG (1:5 000; Santa Cruz, CA, USA) and goat anti-mouse IgG (1:5 000; Zhongshan Golden Bridge Biotechnology, Beijing,