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N-Acetylcysteine inhibit the translocation of mixed lineage kinase-3 from cytosol to plasma membrane during transient brain ischemia in rat hippocampus

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Abstract

Mixed lineage kinase-3 (MLK3) is a recently described member of the MLK subfamily of Ser/Thr protein kinases that interacts with mitogen-activated protein kinase (MAPK) pathways. In this study, we investigated the translocation of MLK3 during transient cerebral ischemia in rat hippocampus. Transient brain ischemia was induced by the four-vessel occlusion in Sprague–Dawley rats. Our data show that MLK3 can translocate from cytosolic fraction to the membrane fraction during ischemia and the increased MLK3 in the membrane fraction bind to postsynaptic density protein 95 (PSD-95). The antioxidant *N*-acetylcysteine (NAC) could inhibit the translocation of MLK3 from cytosolic fraction to the membrane fraction and decrease the interactions of MLK3 and PSD-95 in the membrane fraction. Consequently, these results indicate that reactive oxygen species (ROS) was closely associated with MLK3 translocation induced by transient global ischemia in rat hippocampus.

Keywords: Cerebral ischemia; Mixed lineage kinase-3 (MLK3); Postsynaptic density protein 95 (PSD-95); *N*-Acetylcysteine (NAC)

Mitogen-activated protein kinase (MAPK) pathways are one of the most important intracellular signal transduction pathways transmitting extracellular stimuli to the nuclei of cells and respond to a variety of stimuli including oxidative stress, cytokines, and initiators of cell death [8,9,12]. Recently, mixed lineage kinase (MLK) family members have been shown to function as mitogen activated protein kinase kinase kinases (MAPKKKs), which primarily activate the JNK pathway, also called as stress activated protein kinase pathway (SAPK) [16]. Members of MLK family comprise MLK1 [2], MLK2/MST [3] MLK3/SPRK/PTK1 [4], dual leucine zipper kinase (DLK/MUK/ZPK) [5] and leucine zipper bearing kinase (LZK) [6]. It is generally accepted that MLKs are potent activators of JNK pathway, however, some members of mixed lineage kinase can activate p38 and ERK pathway in some instance.

Mixed lineage kinase-3 (MLK3) [7], also called *Src* homology 3 domain (SH3)-containing proline-rich protein kinase (SPRK) [4], or protein-tyrosine kinase-1 is a member of the mixed-lineage kinases and is reported to be the first example of a protein that contains an SH3 domain and possess serine/threonine kinase activity. The structure of MLK3 contains an N-terminal Src-homology 3 (SH3) domain followed by a kinase domain, dual leucine zipper domain and a Cdc42/Rac interactive binding (CRIB) domain. MLK3 can phosphorylate and activate MAPKKs including MKK4 and MKK7, which in turn, activate JNKs on both Thr-183 and Tyr-185 residues. A number of studies have proved that JNK activation is involved in the hippocampal neuron apoptosis after ischemia injury. MLK3, as an upstream kinase of JNK, also has been shown to be associated with neuronal apoptosis [20]. Our previous study indicated that MLK3 was involved in the neuronal cell death in response to ischemia. Meanwhile, K252a, the inhibitor of MLK3, showed a significant neuroprotective action against brain ischemia [13]. In the present study, we investigated the subcellular distribution of MLK3 using western blotting and immunoprecipitation (IP) analysis during transient cerebral ischemia. The antioxidant *N*-acetylcysteine (NAC) was used to explore the mechanism underlying the subcellular localization of MLK3.

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Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 200–250 g were fed with water and food ad libitum. The experimental procedures were approved by the local legislation for ethics of experiments on animals. Transient brain ischemia was induced by four-vessel occlusion (4-VO) as described before [15]. Briefly, rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and both vertebral arteries were occluded permanently by electrocautery. On the following day, both carotid arteries were occluded with aneurysm clips to induce cerebral ischemia. During ischemia, animals were elected to match the following criteria: completely flat electroencephalographs, maintenance of dilated pupils, absence of a cornea reflex when exposed to strong light stimulation, and maintenance of rectal temperature at 36.5–37.5 °C. Those not matching these criteria were excluded. Sham control were performed using the same surgical procedures except that the carotid arteries were not occluded.

At a certain time during ischemia, rats were decapitated immediately and then the hippocampi were removed and quickly frozen in liquid nitrogen. The hippocampi were homogenized in ice-cold homogenization buffer containing 50 mM 3-(Nmorpholino) propanesulfonic acid (MOPS) (Sigma; pH 7.4), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM $MgCl_2$, 0.2 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphogrycerol, 1 mM *p*-nitrophenyl phosphate (PNPP), 1 mM benzamidine, 1 mM phenylmethylsulfonyl (PMSF) and 5 μg/ml each of leupeptin, aprotinin, pepstatin A. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C. Supernatants (whole tissue) were collected and centrifuged at 100,000 × *g* for 30 min at 4 °C. The supernatants were collected as cytolsolic fraction (s) and then added 500 μl homogenization buffer containing 1% Triton X-100 to the pellets, which then were dealt with supersonic and collected as crude membranes fraction (p). Protein concentration was determined by the methods of Lowry et al. Samples were stored at −80 °C until use.

Tissue homogenates (400 μg of protein) were diluted fourfold with 50 mM HEPES buffer, (pH 7.4), containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and 1 mM each of EDTA, EGTA, PMSF and Na_3VO_4. Samples were preincubated for 1 h with 20 μl protein A sepharose CL-4B (Amersham, Uppsala, Sweden) at 4 °C, and then centrifuged to remove proteins adhered nonspecifically to protein A. The supernatants were incubated with 1–2 μg primary antibodies for 4 h or overnight at 4 °C. Protein A were added to the tube for another 2 h incubation. Samples were centrifuged at 10,000 × *g* for 2 min at 4 °C and the pellets were washed with immunoprecipitation buffer for three times. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS–PAGE loading buffer and then isolated by centrifuge. The supernatants were used for immunoblot analysis.

For immunoblot, proteins were separated on 10% polyacrylamide gels and then electrotransferd onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocked for 3 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies in TBST for 2 h and developed using NBT/BCIP color substrate (Promega, Madison, USA). The density of the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP Upland, CA, USA).

The following primary antibodies were used: rabbit polyclonal anti-MLK3 (sc-13072), were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-PSD-95 (CP35-100UL) was bought from Oncogene. The secondary antibodies used in our experiment were goat anti-mouse IgG and goat anti-rabbit IgG. They were from Sigma.

Values were expressed as mean ± S.D. and obtained from four independent rats. Statistical analysis of the results was carried out by one-way analysis of the variance (ANOVA) followed by the Duncan's new multiple range method or Newman–Keuls test. *P*-values < 0.05 were considered significant.

We firstly examined the subcellular distribution of MLK3 during transient cerebral ischemia in rat hippocampus with the methods of immunoblotting (IB). As shown in Fig. 1A upper panel, the levels of MLK3 in the membrane fraction increased at 10 min ischemia and remained high at 30 min ischemia. Correspondingly, the middle panel of Fig. 1A showed that the levels of MLK3 in the cytosolic fraction decreased from 10 min ischemia.

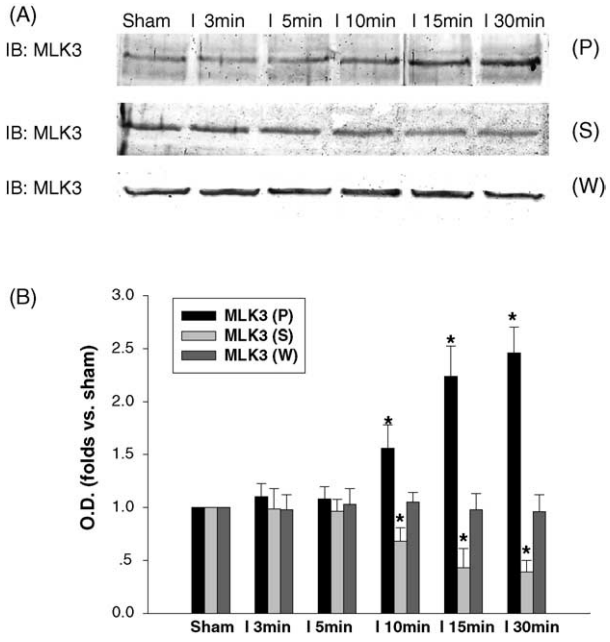

Fig. 1. Western blotting analysis of the translocation of MLK3 from cytosolic fractions to crude membrane fractions during transient brain ischemia. Samples were from rats subjected to sham operation, 3, 5, 10, 15 and 30 min ischemia without reperfusion. (A) Immunoblotting analysis of the protein levels of MLK3 in the crude membrane fractions fraction, cytosolic fraction and whole tissue. Transient brain ischemia induced an increase of MLK3 in the crude membrane fractions and a decrease of MLK3 in the cytosolic fraction. Total protein levels of MLK3 in the hippocampus were not changed. (B) Quantitative representation of the protein levels of MLK3 in the crude membrane fractions cytosolic fraction and whole tissue. Data are the mean ± S.D. and are expressed as folds vs. sham. * $P < 0.05$ vs. sham ($n = 4$ rats). P, crude membranes fraction; S, cytosolic fraction; W, whole tissue.