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Tyrosine phosphorylation of HPK1 by activated Src promotes ischemic brain injury in rat hippocampal CA1 region

Ting Li[a,b], Xiu-Ju Yu[a], Guang-Yi Zhang[a,b,*]

[a] *Research Center for Biochemistry and Molecular Biology, Jiangsu Key Laboratory of Brain Disease Bioinformation, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou, Jiangsu 221002, China*
[b] *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200231, China*

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Abstract Hematopoietic progenitor kinase 1 (HPK1) is a hematopoietic cell-restricted member of the Ste20 serine/threonine kinase super family. We recently reported that HPK1 is involved in c-Jun NH2-terminal kinase (JNK) signaling pathway by sequential activation of MLK3–MKK7–JNK3 after cerebral ischemia. Here, we used 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine (PP2) and MK801 to investigate the events upstream of HPK1 in ischemic brain injury. Immunoprecipitation and immunoblot results showed that PP2 and MK801 significantly decreased the activation of Src, HPK1, MLK3, JNK3 and c-Jun, respectively, during ischemia/reperfusion. Histology and TUNEL staining showed PP2 or MK801 protects against neuron death after brain ischemia. We speculate that this unique signaling pathway through the tyrosine phosphorylation of HPK1 promotes ischemic brain injury by activated Src via *N*-methyl-D-aspartate receptor and, ultimately, the activation of the MLK3–MKK7–JNK3 pathway after cerebral ischemia.
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Keywords: HPK1; Cerebral ischemia; Src; *N*-methyl-D-aspartate receptor (NMDAR); Neuroprotection; c-Jun NH2-terminal kinase (JNK)

1. Introduction

Hematopoietic progenitor kinase 1 (HPK1) is a member of the germinal center kinase (GCK) sub-family of the Ste20 kinases [1]. The GCK/HPK1 family kinases are a group of mammalian serine/threonine protein related to the yeast protein kinase STE20. HPK1 is mainly expressed in hematopoietic organs and cells [2]. HPK1 is involved in many cellular signaling cascades including mitogen-activated protein kinase (MAPK) signaling, antigen receptor signaling, apoptosis, growth factor signaling, and cytokine signaling [1–3]. The GCK/HPK1 family kinases act as mediators that link surface receptors to the core c-Jun NH2-terminal kinase (JNK) signaling cascades.

The role of HPK1 in biological processes is studied best in T cells. HPK1, a 97-kDa serine/threonine kinase, contains a STE20-like kinase domain in its N-terminus, four proline-rich motifs, a caspase cleavage site, and a distal C-terminal Citron homology domain. The studies in T cells reveal that HPK1 is capable of interacting with a multitude of adaptor proteins including members of the Grb2 family, Nck family, Crk family, SLP-76 family, and actin-binding adaptors like HIP-55 [4–10]; the function of each adaptor protein is very different in terms of kinetics and signaling. HPK1 tyrosine phosphorylation and kinase activation depend on the presence of adaptor proteins. In addition to T cell receptor (TCR) and B cell antigen receptor engagement, ligand binding to transforming growth factor-β receptor (TGF-βR) [11,12], the erythropoietin receptor [13], Fas [14], and E prostanoid receptors [15] can also induce HPK1 kinase activity. With the exception of TCR-mediated signal transduction in which some mechanisms controlling HPK1 activation have been delineated, the exact biochemical mechanisms utilized by these receptors to activate HPK1 remain poorly understood. Although HPK1 is selectively expressed in hematopoietic tissues and cells, some breast and prostate tumor cell lines also express HPK1. Therefore, it is possible that HPK1 may play a role in other cells.

Our earlier studies showed that cerebral ischemia induced robust HPK1 kinase activity through tyrosine phosphorylation. Knockdown of HPK1 expression provided neuroprotection by reducing activation of the MLK3–MKK7–JNK3 pathway following cerebral ischemia in the rat hippocampus CA1 subfield [25]. The studies above delineated the 'downstream' cellular events of HPK1 signaling pathways in ischemic brain injury. To gain more insight into the function of HPK1 in cerebral ischemia, we used PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine), a selective Src family kinase inhibitor, and MK801, a selective NMDA receptor antagonist, before ischemia. Our results suggest a previously unknown mechanism of HPK1 activation in cerebral ischemia: activated Src via *N*-methyl-D-aspartate receptor (NMDAR) promotes HPK1 tyrosine phosphorylation and,

*Corresponding author. Address: Research Center for Biochemistry and Molecular Biology, Jiangsu Key Laboratory of Brain Disease Bioinformation, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou, Jiangsu 221002, China. Fax: +86 516 8574 8486.
E-mail address: gyzhang@xzmc.edu.cn (G.-Y. Zhang).

Abbreviations: GCK, germinal center kinase; HPK1, Hematopoietic progenitor kinase 1; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MKK7, MAPK kinase; NMDAR, N-methyl-D-aspartate receptor; PCR, polymerase chain reaction; PMSF, phenylmethylsulfluoride; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine; PTK, protein-tyrosine kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH2 and SH3, Src homology 2 and 3 domain, respectively; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; TCR, T cell receptor

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ultimately, results in the brain injury by the activation of MLK3–MKK7–JNK3 pathway after cerebral ischemia.

2. Materials and methods

2.1. Animals and treatments

Adult male SD rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 200–250 g were provided with water and food ad libitum. The experimental procedures were approved by the local legislation for ethics when carrying out experiments on animals. Rats were given MK801 (3 mg/kg dissolved in saline) [16] or vehicle (saline) by intraperitoneally (i.p.) injection 60 min prior to ischemia. Fifteen micrograms of PP2 or PP3 [17] dissolved in 10 μl dimethyl sulfoxide (DMSO) was given 30 min before ischemia by means of intracerebroventricular infusion. 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. Transient brain ischemia was induced by four-vessel occlusion (4-VO) as described before [18]. Briefly, under anesthesia with chloral hydrate (300 mg/kg, i.p.), both vertebral arteries were occluded permanently by electrocautery. On the following day, both carotid arteries were occluded with aneurysm clips to induce cerebral ischemia. After 15 min of the occlusion, the aneurysm clips were removed for reperfusion. Sham controls were performed using the same surgical procedures except that the carotid arteries were not occluded.

2.2. Sample preparation

For brain tissue preparation, rats were killed under anesthesia at 6 h, 3 days and 5 days 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. 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) (Sigma, St. Louis, MO, USA), 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and inhibitors of protease and enzymes. The homogenates were centrifuged at $15000 \times g$ for 20 min at 4 °C, supernatants were collected, and stored at −80 °C for use. The protein concentrations were determined by the method of Lowry with bovine serum albumin (BSA) as standard.

2.3. Antibodies and reagents

The following primary antibodies were used: goat polyclonal anti-HPK1 (sc-6231), rabbit polyclonal anti-MLK3 (sc-13072), anti-p-JNKs (sc-6254), anti-c-Jun (sc-1694), and mouse monoclonal anti-p-c-Jun (sc-822) were purchased from Santa Cruz Biotechnology. Anti-phospho-Src (pY416) (SA-314) was purchased from Biomol Biotechnology Inc. Anti-Src(05-184), rabbit polyclonal anti-JNK3 antibody (06-749), monoclonal antiphosphotyrosine Ab(4G10) were obtained from Upstate Biotechnology Inc. Rabbit polyclonal anti-p-MLK3 was obtained from Cell Signal Biotechnology. The secondary antibodies used in our experiment were goat anti-mouse IgG, goat anti-rabbit IgG and donkey anti-goat IgG. They were from Sigma. PP2(529573) and PP3(529574) were purchased from Calbiochem Biotechnology. MK-801 was purchased from Sigma. All the other chemicals were from Sigma unless indicated otherwise.

2.4. Immunoprecipitation

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 that adhered non-specifically to protein A-Sepharose. The supernatants were incubated with 1–2 μg primary antibodies for 4 h at 4 °C. Protein A was added to the tube for another 2 h incubation. Samples were centrifuged at $10000 \times g$ for 2 min at 4 °C and the pellets were washed with immunoprecipitation (IP) buffer for three times. Bead-bound proteins were eluted by boiling at 100 °C for 5 min in sodium dodecylsulfate–polyacrylamide gel electrophoresis loading buffer and then isolated by centrifuging. The supernatants were used for immunoblot analysis.

2.5. Immunoblot

For immunoblot (IB), proteins were separated on 10% polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After being 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 densities of the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP Upland, CA, USA).

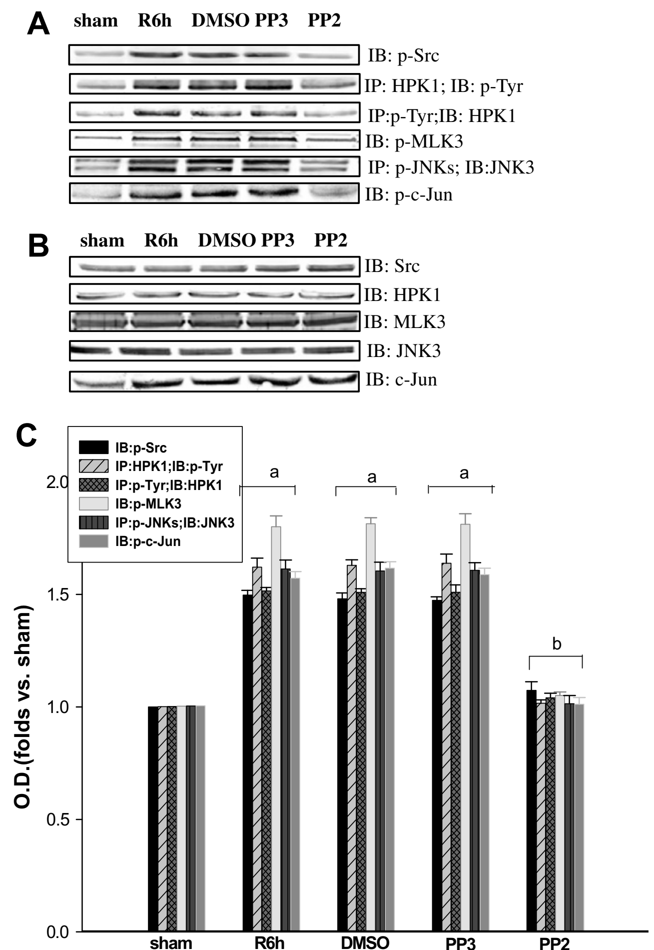

Fig. 1. PP2 inhibits the phosphorylation ofSrc, HPK1, MLK3, JNK3 and c-Jun in the hippocampal CA1 region after cerebral ischemia/reperfusion. (A) Rats were pretreated with 15 μg of PP2 or PP3 in 10 μl DMSO or the same dose of vehicle 30 min before ischemia followed by 6 h of reperfusion (R6h). Phosphorylation of Src, MLK3 and c-Jun were examined by immunoblotting (IB) analysis with the anti-p-Src, anti-p-MLK3 and anti-p-c-Jun antibody, phosphorylation of HPK1 and JNK3 were examined by immunoprecipitation (IP) with anti-p-Tyr and anti-p-JNKs followed by IB with antibody against HPK1 and JNK3. (B) The protein levels of total Src, HPK1, MLK3, JNK3 and c-Jun were examined by IB analysis with anti-Src, anti-HPK1, anti-MLK3, anti-JNK3 or anti-c-Jun antibody. (C) Corresponding bands were scanned and the optical density (OD) was represented as folds versus sham control. Data were the means ± S.D. and were expressed as folds versus sham. $^{a}P < 0.05$ versus sham; $^{b}P < 0.05$ versus respective reperfusion groups ($n = 3$).

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