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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 activited 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

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cassette[8].

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[9,10], 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 separatively 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-isoquinolinesulphonyl)-*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_2SO) 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_3VO_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 ºC. Protein concentration was determined by the Bradford method[14]. Followingly, the extracted proteins were denatured in sodium dodecyl sulfate (SDS) sample buffer at 100 ºC 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,

China), and enhanced with nitro-blue tetrazolium / 5-bromo-4-chloro-3'-indolyl phosphate (NBT/BCIP) color substrate (Promega). The bands on the membrane were scanned and analyzed with an image analyzer (UVP image analyzer software, CA, USA).

2.4 Data disposal All data were expressed as means ± SD from three independent animals. Statistical analysis and comparison of the results were carried out by one-way ANOVA followed by Newman-Keul's test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Time courses of expression and phosphorylation of ERKs, Src and CaMKII caused by ischemia alone As shown in the upper panels of Fig. 1A, B and C, ischemia alone resulted in rapid activation of Src and sustained activation of CaMKII, with their peaks at 5 min or so, through the phosphorylation at Tyr-416 and Thr-286 (3.6 and 2.6 folds separatively *vs* sham control, $P < 0.05$), respectively. However, transient ischemia (5 min, I-5) caused no alteration of ERK phosphorylation *in vivo* ($P > 0.05$). Src and ERK activities were at the lowest when ischemia time prolonged to 15 min (I-15, $P < 0.05$). Moreover, severe ischemia (30 min, I-30) also triggered obvious expression rebound of the Src and ERK, which is obviously different from their expression at I-15 ($P < 0.05$). Protein levels of Src, CaMKII and ERKs kept stable during 30 min ischemia ($P > 0.05$, down panel of the Fig. 1A, B and C), suggesting that their activities were closely associated with the phosphorylation level but not the protein level in cerebral ischemia.

3.2 Time courses of phosphorylation and expression of ERKs, Src and CaMKII caused by ischemia-recovery Recovery after 15 min ischemia contributed to sustained activation of Src kinase with another peak at about 6 h (R6, 2.7 folds *vs* the Sham, upper panels of Fig. 2A) and rapid phosphorylation of CaMKII with its highest level at 15 min (R15', 3.2 folds *vs* sham, upper panels of Fig. 2B). However, the activation of ERKs at 15 min of ischemia and 24 h of recov-

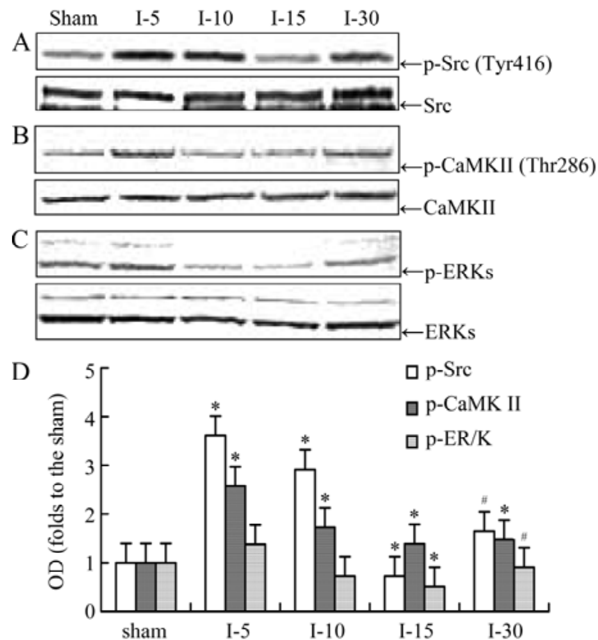

Fig.1 Temporal courses of the protein and phosphorylation level of ERKs, Src and CaMKII induced by ischemia in rat hippocampi. The samples were obtained from rat hippocampi subjected to sham, different times (5, 10, 15 and 30 min) of ischemia (I-5, I-10, I-15, and I-30, respectively). Immunoblotting analyses were performed with phospho and non-phospho antibodies against: (A) ERKs, (B) Src kinase and (C) CaMKII. D, Bands on western blotting were scanned and the intensities were determined by optical density (OD). Data were expressed as means ± SD from three independent experiments ($n = 3$). * $P < 0.05$ *vs* Sham, # $P < 0.05$ *vs* I-15.

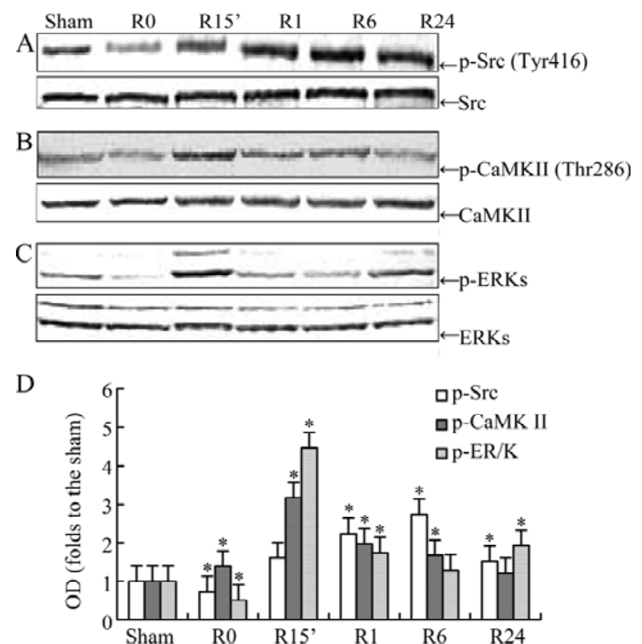

Fig. 2 Time courses of the protein and phosphorylation levels of ERKs, Src and CaMKII induced by post-ischemia recovery in rat hippocampus. The rat brain samples obtained from the sham group and ischemia-recovery groups (15 min, 1 h, 6 h, and 24 h recoveries after 15 min of ischemia, represented by R15', R1, R6, and R24 respectively) were used for the present study. The phosphorylation and protein levels of ERK (A), Src kinases (B) and CaMKII (C) were determined by immunoblot. D: Semiquantitative assay was exerted according to the method mentioned above, * $P < 0.05$ *vs* Sham.

ery (R15' and R24, 4.5 and 2.0 folds respectively *vs* the Sham, upper panels of Fig. 2C) was significantly different from that of Src and CaMKII with a biphasic but not sustained or rapid activation. The total protein of Src, CaMKII and ERK (down panel of Fig. 2A, B and C, respectively) did not show any change in all the operation groups ($P > 0.05$).

3.3 Involvement of Src family tyrosine kinases and CaMKII in NMDA receptor-dependent ERK activation resulting from post-ischemia recovery To explore the molecular mechanism underlying ischemia-induced ERK activation, a few inhibitors were administered in the following studies. Ketamine, a selective NMDA receptor inhibitor, might perform its exogenous neuroprotection through blocking NMDA receptor-dependent calcium influx and the subsequent calcium-dependent activation of multiple protein kinases like Src, pyk2 and CaMKII[3,13]. As shown in Fig. 3A, an effective dose of ketamine attenuated ERK phosphorylation elicited by early recovery (15 min) after ischemia ($P < 0.05$), indicating that the upregulated NMDA receptor complexes might be implicated in control of ischemia-induced activation of ERKs *in vivo*. To further clarify whether NMDA receptor-dependent upregulation of Src or CaMKII activity is involved in ERK cascades following brain ischemia, PP2 (a selective inhibitor of Src family tyrosine kinases) and KN62 (a specific inhibitor of CAMKII) were injected (i.c.v.) separatively to the rats. PP2 significantly reduced the phosphorylation of ERKs at 15-min recovery ($P < 0.05$, Fig. 3B), in accordance to the *in vitro* studies on hypoxia and calcium signaling[5,6,8]. Pretreatment with KN62 also obviously repressed ischemia-induced activation of ERKs during 15-min recovery, significantly different from the solvent control (Me_2SO) ($P < 0.05$, Fig. 3C). Moreover, no change in protein level of ERKs was found in all administration groups ($P > 0.05$, Fig. 3D).

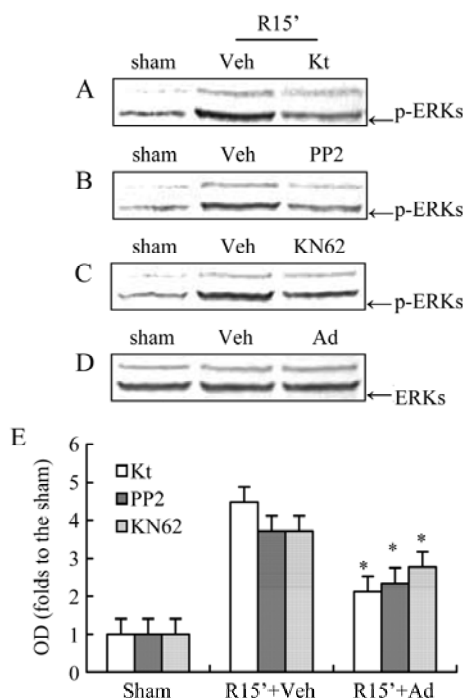

Fig.3 Effects of the NMDA receptor inhibitors, Src family tyrosine kinases and CaMKII on the diphosphorylation of ERKs resulting from ischemia-recovery in rat hippocampus. Ketamine (Kt) and the same volume of its vehicle (Veh, saline) were administered (i.p.) to the rats, respectively; PP2, KN62 and the same volume of their vehicle (Me_2SO) were administered (i.c.v.) separatively 30 min before ischemia, and then these rats were exposed to 15 min of ischemia and recovered for 15 min (R15'). A–C: A role that ketamine, PP2 or KN62 played in EKR activation after ischemia-recovery. D: Representative protein content of ERKs subjected to various stimuli mentioned above. Ad: administration group. E, Semiquantitative alterations of biphosphorylation of ERKs after ischemia-recovery were determined by optical density (OD). Data were expressed as means ± SD (*n* = 3). **P* < 0.05, administration group *vs* its vehicle group.

4 Discussion

It has been suggested that the *in vivo* mechanism of cell response to ischemic stress might be different from the *in vitro* one[1]. Actually, the former might be more complex, just as the complex association of ERKs with Src kinase and CaMKII in the post-ischemia hippocampus that we have demonstrated in the present study. *In vitro* studies have shown that Src kinase is closely related to ERK activation in response to hypoxia/anoxia[6,8]. However, active Src kinase is not always associated with the upregulation of ERKs during ischemia and recovery in the present *in vivo* study. The present studies suggested that the Src activation with a peak was approximately linked to blocking or loss of ERK phosphorylation at 5 min ischemia or 6 h recovery after ischemia, indicating that the intensive activation of Src did not implicate in the upregulation of ERK activity resulting from brain ischemia *in vivo*.

Recent studies showed that a few new signaling molecules mediated by Src kinase were involved in the inactivating pathway of ERKs. Members of Sprouty family like Sprouty 2 have been identified to suppress Ras-ERK cascades through Src-induced phosphorylation at its Tyr-55 residue[15,16]. Spred, the Sprouty-related protein, was also found as a negative regulator of ERK pathway and to be upregulated directly by tyrosine kinases[17]. Our data also showed that inhibiting Src family tyrosine kinase activity

with PP2 could effectively attenuate the ERK phosphorylation elicited by post-ischemia recovery, and ERK activation with a peak or distinct rebound usually occurred when Src kinase was activated mildly. These results indicate that Src activity might be necessary to ERK phosphorylation in cerebral ischemia as well. So Src family tyrosine kinases seem to perform stimulatory and inhibitory functions in ERK pathway through diverse signaling molecules *in vivo*. Both Src family tyrosine kinases and ERKs are closely associated with NMDA receptor in ischemia lesion[2,3,6]. The result that Src kinases had dual effects on the ERK pathway after ischemia may be in agreement with the studies that Chandler *et al*. have done, in which NMDA receptor is thought to involve bidirectional modulation of ERK cascade in response to various level of NMDA exposure[18].

Furthermore, CaMKII was also found participate in ERK pathway after ischemia in our study. Possible mechanism of CaMKII involved in ERK activation is attributed to SynGAP, an abundant synaptic Ras GTPase-activating protein. SynGAP is most highly expressed in the brain than other tissues and is chiefly localized at glutamatergic synapses, similar to the distribution of CaMKII. Recent studies showed that in neuron active SynGAP might repress ERK activation by inhibiting Ras protein, while active CaMKII could reversibly repress SynGAP activity by inducing its phosphorylation[2,9-11]. Therefore, CaMKII may positively regulate ERK pathway through double inhibition. The mechanism of CaMKII involved in ERK activation might be supported by another fact that in striatal neurons CaMKII could regulate the ERK phosphorylation induced by amphetamine[19], which had been shown to increase glutamate release and induce glutamate receptors upregulation in the striatum, and inhibiting CaMKII with KN62 obviously attenuated amphetamine-induced ERK activation. Considering that the upregulation of CaMKII activity induced by ischemia was not always associated with ERK activation like 5 min ischemia, we conjecture that the roles of activated CaMKII played in ERKs cascades might be indirect or weak *in vivo*.

In brief, the present results showed primarily that Src family tyrosine kinases and CaMKII might be implicated together in NMDA receptor-dependent ERK activation in the early recovery after ischemia. However, intense activation of Src kinases by ischemia might perform diverse modulation to ERKs *in vivo*, involving non-decisive attenuation pathway in response to brain ischemia stimuli. The precise mechanism is being studied further in our laboratory.

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References:

[1] Nishizawa Y. Glutamate release and neuronal damage in ischemia. Life Sci 2001, 69: 369-381.

[2] Xia Z, Dudek H, Miranti CK, Greenberg ME. Calcium influx via the NMDA receptor induces immediate-early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 1996, 16: 5425-5436.

[3] Nicole O, Ali C, Docagne F, Plawinski L, MacKenzie ET, Vivien D, *et al*. Neuroprotection mediated by glial cell line-derived neurotrophic factor: involvement of a reduction of NMDA-induced calcium influx by the mitogen-activated protein kinase pathway. J Neurosci 2001, 21: 3024-3033.

[4] Guo DJ, Zhao YB, Chen YH. Effect of acupuncture on expression of GDNF and activation of MAPKs in the rat brain after focal cerebral ischemic injury. Neurosci bull 2005, 21: 385-390.

[5] Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, *et al*. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J Clin Invest 1997, 100: 1813-1821.

[6] Crossthwaite AJ, Valli H, Williams RJ. Inhibiting Src family tyrosine kinase activity blocks glutamate signalling to ERK1/2 and Akt/PKB but not JNK in cultured striatal neurons. J Neurochem 2004, 88: 1127-1139.

[7] Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci 2004, 5: 317-328.

[8] Rusanescu G, Qi H, Thomas SM, Brugge JS, Halegoua S. Calcium influx induces neurite growth through a Src-Ras signaling cassette. Neuron 1995, 15: 1415-1425.

[9] Chen HJ, Rojas-Soto M, Oguni A, Kennedy MB. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. Neuron 1998, 20: 895-904.

[10] Miyabe T, Miletic V. Multiple kinase pathways mediate the early sciatic ligation-associated activation of CREB in the rat spinal dorsal horn. Neurosci Lett 2005, 381: 80-85.

[11] Knuesel I, Elliott A, Chen HJ, Mansuy IM, Kennedy MB. A role for synGAP in regulating neuronal apoptosis. Eur J Neurosci 2005, 21: 611-621.

[12] Zalewska T, Domanska-Janik K. Brain ischaemia transiently activates Ca^{2+}/calmodulin-independent protein kinase II. Neuroreport 1996, 7: 637-641.

[13] Guo J, Meng FJ, Zhang GY, Song B. Cerebral ischemia induced phosphorylation of c-Src and its combination with Pyk2 and the inhibitory effect of ketamine in rat hippocampus. Chin J

Neurosci 2003, 19: 303-307.
[14] Bradford MM. A rapid and sensitive for the quantitation of microgram quantitities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72: 248-254.
[15] Hanafusa H, Torii S, Yasunaga T, Nishida E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. Nat Cell Biol 2002, 4: 850-858.
[16] Li X, Brunton VG, Burgar HR, Wheldon LM, Heath JK. FRS2-dependent SRC activation is required for fibroblast growth factor receptor-induced phosphorylation of Sprouty and suppression of ERK activity. J Cell Sci 2004, 117 (Pt 25): 6007-6017.
[17] Kato R, Nonami A, Taketomi T, Wakioka T, Kuroiwa A, Matsuda Y, *et al*. Molecular cloning of mammalian Spred-3 which suppresses tyrosine kinase-mediated Erk activation. Biochem Biophys Res Commun 2003, 302: 767-772.
[18] Chandler LJ, Sutton G, Dorairaj NR, Norwood D. *N*-methyl-*D*-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. J Biol Chem 2001, 276: 2627-2636.
[19] Choe ES, Wang JQ. CaMKII regulates amphetamine-induced ERK1/2 phosphorylation in striatal neurons. Neuroreport 2002, 13: 1013-1016.

脑缺血时 NMDA 受体通过 Src 激酶和 Ca^{2+}/ 钙调蛋白依赖性蛋白激酶 II 调控 ERKs 激活

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摘要：目的 ERKs 是钙依赖性激活蛋白，本研究旨在探讨钙依赖性蛋白激酶是否参与了脑缺血后 ERK 级联的调控。**方法** 采用四动脉结扎诱导大鼠前脑缺血，用免疫印迹的方法观察几个钙依赖性蛋白激酶含量及活性的变化。**结果** 致死性脑缺血以 NMDA 受体依赖的方式激活 ERKs，并差异性上调 Src 和 Ca^{2+}/ 钙调蛋白依赖性蛋白激酶 II（CaMKII）的活性。Src 激酶和 CaMKII 的抑制剂 PP2 和 KN62 能显著的阻止缺血诱导的 ERKs 激活。然而，缺血诱导的 Src 过度激活也伴随着 ERKs 的活性抑制。**结论** 致死性脑缺血刺激 NMDA 受体通过 Src 激酶和 CaMKII 介导 ERKs 活性上调，但是脑缺血诱导的 Src 过度激活可能也参与了 ERKs 信号通路的负性调控。
关键词：脑缺血；细胞外信号调节激酶；NMDA 受体；Src 家族蛋白激酶；CaMKII