ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8): 715 - 720
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Changes and mechanisms of protein-tyrosine kinase and protein-tyrosine phosphatase activities after brain ischemia/reperfusion[1]

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KEY WORDS ketamine; nifedipine; protein-tyrosine kinase; protein-tyrosine phosphatase; hippocampus; synaptosomes; cerebral ischemia; reperfusion injury

ABSTRACT

AIM: To study the changes and mechanisms of protein-tyrosine kinase (PTK) and protein-tyrosine phosphatase (PTP) activities in the hippocamal synaptosome following cerebral ischemia/reperfusion (I/R) in gerbil. **METHODS**: Transient (15 min) global ischemia was produced by bilateral carotid artery occlusion. Total PTK and PTP activities were measured by [r-^{32}P] incorporation and colorimetric analysis, respectively. Src and proline-rich tyrosine kinase2 (PYK_2) activities were measured by immunoprecipitation and [r-^{32}P] incorporation. **RESULTS**: Total PTK activity increased significantly after I/R, but the PTP activity did not change. The Src activity was much higher than PYK_2 activity in sham-operated controls. I/R mainly caused a pronounced increase in Src activity, but not PYK_2 activity. The increase in Src activity had no relation to the expression of Src protein. Administration of ketamine (KT) or nifedipine (ND) 20 min before ischemia caused a decrease in total PTK and Src activities, and no change in the PYK_2 and PTP activities. **CONCLUSION**: The increase in PTK activity caused by I/R may be mainly due to the increase in Src activity. This increase in Src activity has no relation to the expression of Src protein. But it is related to the activation of NMDA (*N*-methyl-*D*-aspartate) receptor (NR) and *L*-type voltage-gated calcium channel (*L*-type VGCC). In other words, the increase in total PTK and Src activities induced by I/R may be mediated via NR and *L*-type VGCC. The PTP activity did not change during I/R.

[1] Project support by the National Natural Science Foundation of China, № 39770177.
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Received 1999-08-30 Accepted 2000-04-11

INTRODUCTION

Ischemia is a pathological condition in which the brain experiences a local or extensive shortage of glucose and oxygen[1] and is associated with many disease processes including cerebrovascular disease, brain trauma, epilepsy, and cardiac arrest. One of the major pathologies resulting from ischemic insults is the neuronal degeneration in certain brain areas, and the hippocampus is particularly vulnerable to such insults. The specific biochemical mechanisms that underlie ischemia-induced cell death remain unknown; a change in tyrosine-specific protein phosphorylation has recently been implicated[2-4].

Protein tyrosine phophorylation plays a principal and critical role in signal transduction pathways for a wide range of cellular processes, such as cell growth and differentiation[5,6]. Both protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) are highly expressed in the central nervous system[5,7]. However, little is known about the changes in PTK and PTP activities and the mechanisms inducing these changes during ischemia/reperfusion (I/R). However, there is evidence to indicate that the activation of proline-rich tyrosine kinase2 (PYK_2) and Src activities which belong to nonreceptor PTK, may be related to the elevation in the intracellular Ca^{2+} concentration[8,9]. The *N*-methyl-*D*-aspartate receptor (NR) has received special attention in recent studies regarding the pathogenesis of ischemia-induced neurodegeneration due to its characteristic high permeability to the divalent ion, Ca^{2+} [2]. It has recently been reported that both NR and *L*-type voltage-gated calcium channels (*L*-type VGCC) play an important role in the process of ischemia-induced damage in the brain[10-12].

In the present study, we have investigated the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

changes of the PTK (including total PTK, Src, and PYK_2) activities and PTP activity during I/R as well as the effects of ketamin (KT), a noncompetitive antagonist of NR, and nifedipine (ND), an *L*-type VGCC antagonist on the activities of total PTK, Src, PYK_2, and PTP during I/R in the hippocampal synaptosomes in gerbil. The effects of I/R on the expression of Src and PYK_2 were also investigated.

MATERIALS AND METHODS

Chemicals and regents Polypeptide (Glu : Tyr = 4 : 1), p-nitrophenyl-phosphate (PNPP), ATP, KT, ND, leupeptin, pepstatin A, Tween-20, Triton X-100, sodium dodecyl sulfate (SDS), and phenlmethyl-sulphonyl fluoride (PMSF) were purchased from Sigma. Nitrocellulose (NC) membrane were ordered from Amersham. Anti-Src monoclonal antibody (Oncogene, USA), anti-PYK_2 monoclonal antibody (Transduction Laboratories, USA). [r-^{32}P] ATP was from Beijing Ya-Hui Biomedical Technical Co. Ethylene-glycol-bis-tetra-acetate (EGTA), ethylene diamine tetra-acetate (EDTA) and other reagents were all of analytical grade.

Gerbil model of transient forebrain ischemia and reperfusion Mature Mongolian gerbils (60 – 75 g, The Experimental Animal Center, Xuzhou Medical College, Grade Ⅱ, certificate №98001) were used. Transient forebrain ischemia was induced using a method as described by Toshiho[13]. Gerbils were anesthetized with choral hydrate (300 – 350 mg·kg^{-1}, ip). Both carotid arteries were simultaneously occluded with clamps for 15 min. Body temperature was monitored with a rectal thermometer and regulated between 37 ℃ – 37.5 ℃ by a heating lamp. Animals killed by decapitation at 6 h of reperfusion, and the brain was frozen *in situ* under liquid nitrogen and dissected out. The hippocampus was removed and stored at −80 ℃ until use.

Experimental protocol Each gerbil was randomly assigned to one of the following groups: drug-treated groups, treated with KT 12.5, 25, and 50 mg·kg^{-1} ip or ND 10, 15, and 20 mg·kg^{-1} ip, in 80 – 100 μL vehicle, just before ischemia; ischemia groups and sham-operated (control) groups, treated with the same vehicle, just before ischemia.

Preparation of synaptosome Synaptosome was prepared essentially according to the procedures of Hu *et al*[3].

Protein was determined by the method of Lowry[14]. Bovine serum albumin was used as a standard.

Protein tyrosine kinase assays PTK activity was measured as described previously[4] using a synthetic peptide, polypeptide (Glu: Tyr = 4 : 1), as a substrate. Phosphorylation was carried out for 10 min at 30 ℃ in the presence or the absence of 250 μg substrate, using 20 – 40 μg of homogenate protein. PTK activity was defined as the activity measured in the presence of the substrate peptide minus the activity measured in the absence of the peptide.

Src and PYK_2 tyrosine kinase assays The synaptosomal tissues were immunoprecipitated with anti-Src or anti-PYK_2 for 2 h and protein A-Sepharose 4B for 4 h (Pharmacia LKB). The immunocomplexes were washed three times with lysis buffer A (containing HEPES 20, DTT 10, NaF 50, edetic acid 1, egtazic acid 2, PMSF 1 mmol·L^{-1} and Triton X-100 1 %, leupeptin 10 and pepstatin A 10 g·L^{-1}, pH 7.4) and twice with buffer B (containing HEPES 20, DTT 1 mmol·L^{-1}, pH 7.4), and suspended in 50 μL of kinase assay buffer. The rest of the procedure was the same as for total protein tyrosine kinase measurements.

Phosphotyrosine phosphatase assays Measurement of PTP activity was carried out according to the method of Braunton *et al*[4]. Phosphatase activity was assessed by measuring the absorbance at 420 nm ($OD_{420\ nm}$). The vanadate-sensitive tyrosine posphatase activity was defined as the absorbance in the absence of Na_3VO_4 minus the absorbance in the presence of Na_3VO_4.

Electrophoresis and immunoblotting Electrophoresis was carried out on 7.5 % SDS-PAGE according to the method of Laemmli[15]. Following electrophoresis, proteins on the gel were electrotransferred onto NC membrane according to the method of Hu *et al*[3]. The bound antibody was detected by NBT/BCIP (Sigma) as described by the manufacturer. Intensities of the bands were measured using image analysis software (Gene Company) and the results expressed as percentage of sham control.

Immunoprecipitation Synaptosomal proteins were solubilized under nondenaturing or denaturing conditions. Nondenaturing conditions: ice-cold immunobuffer containing Mops 20, NaCl 150, edetic acid 1, egtazic acid 1, sodium orthovanadate 0.2, PMSF 0.2 mmol·L^{-1}, Triton X-100 1 % and Nonidef P-40 0.5 %. Denaturing conditions: boiling in DTT 1 mmol·L^{-1} and SDS 0.5 % for 5 min followed by fourfold dilution with ice-cold immunobuffer. Following incubation with anti-Src (1μg) or

anti-PYK_2 (1 μg) monoclonal antibody overnight at 4 ℃. Immunocomplexes were isolated by the addition of 50 μL of protein A-Sepharose 4B beads followed by incubation for 4 h at 4 ℃. Immunoprecipitates were then washed three times with immunobuffer. The pellets were suspended in 2.5 fold SDS sample buffer [containing Tris-HCl 0.15, EDTA 12.5 mmol·L^{-1}, SDS 6 %, β-mercaptoethanol 12.5 %, glycerol 10 % and bromophenol (blue) 0.05 %, pH 6.8] boiled for 10 min. The samples were subjected to 7.5 % SDS-PAGE and transferred to NC membrane. Analysis was performed by probing with anti-Src antibody or anti-PYK_2 antibody.

Statistical analysis All values are shown as the $\bar{x} \pm s$. Statistical analysis was by student's t-test, for comparison of more than two groups ANOVA was used with significance defined as $P < 0.05$.

RESULTS

Effects of KT on the total PTK and PTP activity The PTK activity increased to 157 % of control after I/R 6 h in the synaptosomes of gerbil hippocampus. KT 12.5, 25, and 50 mg·kg^{-1} ip, 20 min before ischemia caused gradual decrease in total PTK activity to 145 %, 128 %, and 118 % of control, respectively. (*vs* group of I/R: 12.5 mg·kg^{-1} group, $P > 0.05$; 25 and 50 mg·kg^{-1} groups, $P < 0.01$). In contrast, the PTP activity did not change significantly (Tab 1).

Effects of ND on the total PTK and PTP activities PTK activity increased to 151 % of control after I/R 6 h in the synaptosomes of gerbil hippocampus. ND 10, 15, and 20 mg·kg^{-1} ip 20 min before ischemia caused gradual decrease in total PTK activity to 133 %, 119 %, and 114 % of control, respectively (*vs* group of I/R: 10mg·kg^{-1} group, $P > 0.05$; 15 and 20 mg·kg^{-1} groups, $P < 0.01$). In contrast, under the above conditions, the PTP activity did not change significantly (Tab 2).

Effects of ischemia-reperfusion on Src and PYK_2 activities The Src activity was 5.3 folds of PYK_2 activity in control group. The Src activity increased significantly after I/R [from (333 ± 23) cpm·min^{-1} to (507 ± 28) cpm·min^{-1}], but the PYK_2 activity did not change significantly (Fig 1).

Tab 1. Effects of ketamine (KT) on protein-tyrosine kinase (PTK) and protein-tyrosine phosphatase (PTP) activities after I/R 6 h in synaptosome of gerbil hippocampus. n = number of gerbils. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^fP < 0.01$ *vs* group of I/R 6 h.

	Control	I/R 6 h	I/R 6 h + IT /12.5 mg·kg^{-1}	I/R 6 h + KT /25 mg·kg^{-1}	I/R 6 h + KT /50 mg·kg^{-1}
PTK activites (nmol·min^{-1}·g^{-1})	68 ± 4	108 ± 6^c	99 ± 3^{bd}	88 ± 7^{bf}	81 ± 6^f
% of Control	100	157 ± 9	145 ± 4	128 ± 10	118 ± 9
n	6	6	6	6	5
PTK activites ($OD_{420\ nm}$)	0.90 ± 0.06	0.91 ± 0.05^a	0.94 ± 0.07^{ad}	0.96 ± 0.10^{ad}	0.95 ± 0.11^{ad}
% of Control	100	101 ± 3	104 ± 8	107 ± 11	106 ± 12
n	5	5	5	5	5

Tab 2. Effects of nifedipine (ND) on PTK and PTP activites after I/R 6 h in synaptosomes of gerbil hippocampus. n = number of gerbils. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^fP < 0.01$ *vs* group of I/R 6 h.

	Control	I/R 6 h	I/R 6 h + ND /10 mg·kg^{-1}	I/R 6 h + ND /15 mg·kg^{-1}	I/R 6 h + ND /20 mg·kg^{-1}
PTK activites (nmol·min^{-1}·g^{-1})	68 ± 6	103 ± 4^c	90 ± 3^b	81 ± 9^{bf}	78 ± 6^f
% of Control	100	151 ± 6	133 ± 4	119 ± 13	114 ± 9
n	6	6	6	6	5
PTK activites ($OD_{420\ nm}$)	0.90 ± 0.13	0.93 ± 0.05^a	0.89 ± 0.13^{ad}	0.92 ± 0.05^{ad}	0.93 ± 0.09^{ad}
% of Control	100	99 ± 5	95 ± 14	98 ± 5	99 ± 9
n	6	6	6	6	6

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
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Effects of KT and ND on Src activities during I/R Administration of KT (25 $mg \cdot kg^{-1}$) or ND (15 $mg \cdot kg^{-1}$) ip 20 min before ischemia caused the Src activity to change from (306 ± 27) $cpm \cdot min^{-1}$ to (360 ± 27) $cpm \cdot min^{-1}$ or (304 ± 27) $cpm \cdot min^{-1}$ respectively ($P < 0.01$ *vs* I/R group) (Fig 2).

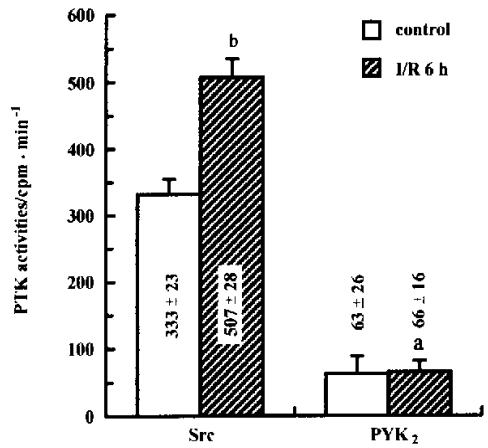

Fig 1. Effects of I/R on Src and PYK_2 activities after I/R 6 h in synaptosome of gerbil hippocampus. $n = 4$ **gerbils.** $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* **control.**

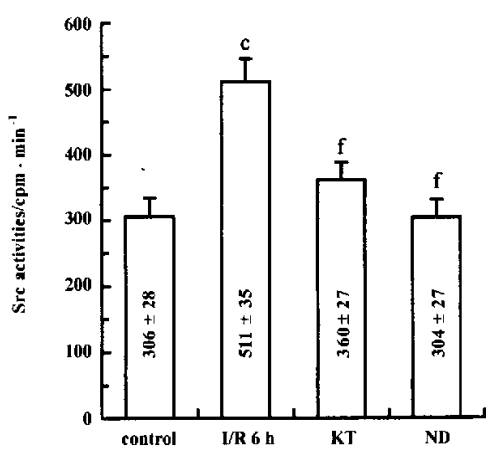

Fig 2. Effects of KT and ND on Src activities after I/R 6 h in synaptosome of gerbil hippocampus. $n = 5$ **gerbils.** $\bar{x} \pm s$. $^cP < 0.01$ *vs* **control.** $^fP < 0.01$ *vs* **Group of I/R 6 h.**

Effects of KT and ND on the expression of Src and PYK_2 during I/R Intensities of the Src bands and PYK_2 bands were measured using image analysis software and the results expressed as percentages of sham control: I/R 6 h (99 ± 3) and (98 ± 2), I/R 6 h + 25KT (98 ± 3) and (98 ± 3), and I/R 6 h + 15ND (99 ± 3) and (97 ± 2), respectively. The results suggested that the increase in PTK activity caused by I/R may be mainly due to Src activity. This increase in Src activity had no relation to the expression of Src, but was related to the activation of NR channel and VGCC (Fig 3).

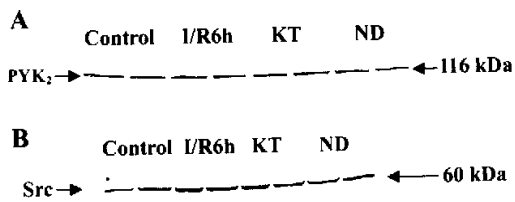

Fig 3. A: Effects of KT (25 $mg \cdot kg^{-1}$) and ND (15 $mg \cdot kg^{-1}$) on the expression of PYK_2. B: Effects of KT (25 $mg \cdot kg^{-1}$) and ND (15 $mg \cdot kg^{-1}$) on the expression of Src.

DISCUSSION

The present results demonstrated that brief periods of global ischemia followed by reprefusion resulted in the increase of total PTK activity in the hippocampal synaptosomes, but PTP activity did not change in the same fashion. As described above, protein tyrosine phosphorylation plays an important role in the regulation of protein function and signal tranduction. Protein tyrosine phosphorylation levels are dynamically controlled by balancing of PTK and PTP activities. Thus, the above results suggest that the destruction of the PTK-PTP activity equilibrium during I/R in the hippocampal synaptosames in gerbil, results in the increase of protein tyrosine phosphorylation. These results are in consistence with the hypothesis that protein kinase activities are activated by extracellular signals[16]. The increase of protein tyrosine phophorylation, therefore, will undoubtedly lead to an alteration of intracellular signal transduction.

PTKs fall into two main groups: transmembrane and cytoplasmic PTKs. Transmembrane PTKs are activated by extracellular signalling molecules, such as growth factors. In contrast, cytoplasmic PTKs are not directly activated by extracellular ligands and are typified by the Src families such as Src, Fyn, and Yes[17]. Both transmembrane and cytoplasmic PTKs have been shown to be expressed within the CNS[18], particularly at synapses[19], and have been implicated in modification of neuronal

functions in physiological processes such as synaptogenesis and long term potentiation and pathological conditions such as ischemia[2]. As activity of both families of PTKs can be altered during ischemic stimulation, our total PTK activity assay may be consistent with changes in activity of one or more Src-related PTKs, PYK_2, which is Ca^{2+}-dependent tyrosine kinase, and other kinases. To thoroughly investigate which PTK activity alters during ischemia, we obtained the Src protein and PYK_2 protein using immunoprecipitation with anti-Src and anti-PYK_2 monoclonal antibody and measured the Src and PYK_2 activities. The results indicate that the Src activity in synaptosome are higher than PYK_2. The Src activity increased significantly 6 h after 15 min of ischemia, but the PYK_2 activity did not. However, whether Fyn and Yes participate in the process of cerebral I/R has to be further studied.

Since our assays indicate that there is no evidence of an increase in PYK_2 activity, it appears that this increase of Src kinase activity is responsible for the total PTK activity increase seen under I/R conditions. Another possible explanation for the increase of Src activity may be an increase in the amount of protein present. However, this possibility can be ruled out by our sequential immunoblotting of the homogenates of each group with anti-Src. The anti-Src probing showed comparable levels of immunoreactivity in each group. The results indicated the amount of Src protein expression did not change.

The increase in total PTK and Src activities may be mediated through a mechanism by which cerebral I/R leads to the elevation in the concentration of intracellular Ca^{2+}. To investigate this mechanism, we examined the effects of KT, a noncompetitive antagonist of NR and ND, an antagonist of *L*-type VGCC on total PTK and Src activities. The results showed that administration of KT or ND 20 min before cerebral ischemia attenuated the stimulation of total PTK and Src activities caused by I/R. The results suggested that the increase in total PTK and Src activities induced by I/R may be related to the activation of NR and *L*-type VGCCs during I/R. Protein tyrosine phosphorylation results in the potentiation of the NR[20], leading to the elevation of intracellular Ca^{2+}. Thus, the drugs, which inhibit tyrosine phosphorylation including the inhibitors of PTK or Src and the antagonists of NR and *L*-type VGCCs, may be the candidates for the cure of ischemic brain injury.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
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脑缺血再灌注后酪氨酸蛋白激酶和蛋白磷酸酶的活性变化及其机制[1]

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关键词 氯胺酮; 硝苯地平; 蛋白质酪氨酸激酶; 蛋白质酪氨酸磷酸酶; 海马; 突触体; 脑缺血; 再灌注损伤

目的: 研究脑缺血再灌注后沙土鼠海马突触体蛋白质酪氨酸激酶(PTK)和蛋白质酪氨酸磷酸酶(PTP)活性的变化及其引起变化的机制. **方法**: 双侧颈总动脉结扎(15 min)形成全脑缺血模型; 放射性同位素 γ-^{32}P掺入法和比色法分别测定了总 PTK 和 PTP 的活性, 免疫沉淀和 γ-^{32}P 放射性同位素测定 Src 和 PYK_2 活性, 免疫印渍测定 Src 和 PYK_2 蛋白表达量. **结果**: ① 脑缺血再灌注引起 PTK 活性升高, 而 PTP 活性不变. ②在假手术对照组中, Src 比 PYK_2 活性高, 脑缺血再灌引起 Src 活性明显升高, 而 PYK_2 活性无显著变化. ③脑缺血前腹腔分别给予氯胺酮和硝苯地平, 都能拮抗脑缺血再灌注引起的 PTK 和 Src 活性的升高, 但对 PTP 活性无影响. **结论**: 脑缺血再灌注能诱导沙土鼠海马突触体总的 PTK 活性升高, 而对 PTP 活性无影响, PTK 活性的升高主要是 Src 活性的增加而与 PYK_2 无关; 脑缺血再灌注诱导 PTK 和 Src 活性升高是通过 NR 和 L-型电压门控钙通道介导的, 即与这两种钙通道的激活有关, 而与其蛋白表达量无关.

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