Mechanisms of regulation of tyrosine phosphorylation of NMDA receptor subunit 2B after cerebral ischemia/reperfusion

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KEY WORDS cerebral ischemia; ketamine; nifedipine; vanadates; genistein; tyrosine; phosphorylation; N-methyl-D-aspartate receptors; calcium channels

ABSTRACT

AIM: To study the mechanisms of the regulation of the tyrosine phosphorylation of N-methyl-D-aspartate (NMDA) receptor subunit 2B (NR2B) in the gerbil hippocampal synaptosomes following ischemia/reperfusion (I/R).

METHODS: Transient (15 min) cerebral ischemia was produced by bilateral carotid artery occlusion procedure. The tyrosine phosphorylation of NR2B was analyzed by immunoprecipitation and immunoblot assay.

RESULTS: Transient forebrain ischemia for 15 min caused a marked decrease in the levels of tyrosine phosphorylation of many protein bands including 180 kDa protein. Transient ischemia followed by reperfusion induced rapid (within 15 min of reperfusion), and sustained (for at least 48 h) increase in the tyrosine phosphorylation of many protein bands including 180 kDa protein. Immunoprecipitation and immunoblot confirmed that NR2B is among the phosphorylated 180 kDa protein. Maximal phosphorylation of 180 kDa band corresponding to NR2B (1.8 fold relative to sham-operated controls) was reached at 6 h of reperfusion following 15 min of cerebral ischemia. But the level of protein expression of NR2B did not change. Administration of ketamine (KT), a non-competitive NMDA receptor antagonist, or nifedipine (ND), an L-type voltage gated calcium channel (L-type VGCC) blocker, 20 min before ischemia attenuated stimulation of the tyrosine phosphorylation of NR2B without affecting the level of protein expression of NR2B. Under these conditions, non-NMDA receptor antagonist 6,7-dinitroquinlazoline-2,3-dione (DNQX) had no effect on the level of tyrosine phosphorylation. Protein tyrosine phosphatase (PTP) inhibitor vanadate and protein tyrosine kinase (PTK) inhibitor genestein resulted in the increase and the decrease of the tyrosine phosphorylation of NR2B, respectively. Src coprecipitated with NR2B protein. CONCLUSION: The increase of the tyrosine phosphorylation of NR2B induced by I/R has relation to NR and L-type VGCC; PTK and PTP participate in the regulation of the tyrosine phosphorylation of NR2B during I/R. Src that associates with NR2B may play an important role in the regulation of the tyrosine phosphorylation of NR2B during I/R.

INTRODUCTION

Transient cerebral ischemia initiates a series of pathophysiological changes that lead to the degeneration of neurons in vulnerable brain regions several days after the ischemic episode. The specific biochemical mechanisms involved in the process of neuronal degeneration following ischemia are not fully understood, but there is considerable evidence to indicate that they involve overactivation of both the N-methyl-D-aspartate (NMDA) receptor (NR) and the L-type voltage gated calcium channel (L-type VGCC)\textsuperscript{1,2}. Although a role for NR in excitotoxic cell death and focal ischemia has been well documented\textsuperscript{3,4}, the contribution of NR activation to delayed neuronal death resulting from transient ischemia remains controversial\textsuperscript{5}. There are two pharmacologically distinct ionotropic excitatory amino acid (EAA) receptors, NMDA and non-NMDA subfamilies. The NMDA subfamily has received special attention in recent studies of the pathogenesis of ischemia-induced neurodegeneration due to its characteristic high permeability to the divalent ion, $\text{Ca}^{2+}$\textsuperscript{6}. The NR is composed of two major types of subunits, NR1 and NR2 subunits, there are four

\textsuperscript{1} Project supported by the National Natural Science Foundation of China, No 39770177.
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Received 1999-09-06 Accepted 2000-03-28
subtypes of NR2 (NR2A-D). Whereas NR1 subunits form fully functional homooligomeric channels, NR2 subunits do not form functional channels but rather serve to modulate the properties of heteromeric receptors consisting of NR1 plus one or more NR2 subunits\(^5\). It has recently been suggested that NR is modulated by the tyrosine phosphorylation pathway. NMDA currents are potentiated by protein tyrosine kinase (PTK)\(^6,7\) and depressed by activity of protein tyrosine phosphatase (PTP)\(^1,7\). This modulation may result from direct tyrosine phosphorylation of the channel itself since regulation of the channel function can be observed in excised membrane patches\(^3\) and residues\(^6\). More recently, there is evidence to indicate that Src PTK, which belongs to a nonreceptor PTK, may participate in the regulation of NR channel function\(^6\), and its activity may be related to the elevation of the intracellular Ca\(^{2+}\) concentration\(^10\). In the present study, we investigated the effects of five drugs including ketamine (KT), a noncompetitive antagonist of NR, nifedipine (ND), an L-type VGCC blocker, 6,7-dinitroquinoxaline-2,3-dione (DNQX), a non-NMDA receptor antagonist, vanadate, an inhibitor of PTP, and genistein, an inhibitor of PTK, on the tyrosine phosphorylation of NR2B in synaptosomes of gerbil hippocampus following brain ischemia/reperfusion (I/R). Our objective was to understand the mechanisms of the regulation of the tyrosine phosphorylation of NR2B in the hippocampus during I/R.

MATERIALS AND METHODS

Chemicals and reagents KT, DNQX, ND, genistein, vanadate, leupeptin, pepstatin A, Tween-20, Triton X-100, and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma. NC membrane was ordered from Amersham. Anti-Phosphotyrosine (PY20) monoclonal antibody (Pharmingen, USA), anti-Src monoclonal antibody (Oncogene, USA), anti-NR2B monoclonal antibody (Transduction Laboratories, USA). Other reagents were all AR.

Gerbil model of transient forebrain ischemia and reperfusion Mature Mongolian gerbils (60 - 75 g, The Experimental Animal Center, Xuzhou Medical College, Grade II, Certificate No 98001) were used. Transient forebrain ischemia was induced using a method as described by Toshino\(^11\). Gerbils were anesthetized with choral hydrate (300 - 350 mg \( \cdot \) kg\(^{-1}\), ip). Both carotid arteries were simultaneously occluded with clamps for 15 min, and body temperature was monitored with a rectal thermometer and regulated between 37 \( \circ\)C - 37.5 \( \circ\)C by a heating lamp. Animals were 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 \(-80\) \( \circ\)C until used.

Experimental protocol Each gerbil was randomly assigned to one of the following groups: Drug-treated groups, treated with the different drugs 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 procedure of Hu et al\(^12\).

Protein was determined according to the method of Lowry\(^13\). Bovine serum albumin was used as a standard.

Electrophoresis and immunoblot Electrophoresis was carried out on 7.5 % SDS-PAGE according to the method of Laemmli\(^14\). Following electrophoresis, proteins on the gel were electrotransferred onto nitrocellulose membrane according to the method of Hu et al\(^12\). 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.

Statistical analysis All values are shown as the \(x \pm s\). 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 ischemia/reperfusion on the tyrosine phosphorylation of synaptosomal proteins in the gerbil hippocampus Transient forebrain ischemia for 15 min caused a marked decrease in the levels of tyrosine phosphorylation of many protein bands including proteins of 180 kDa, 120 kDa, and 95 kDa. Transient ischemia followed by reperfusion induced rapid (within 15 min of reperfusion), and sustained (for at least 48 h) increase in the tyrosine phosphorylation of many protein bands including 180 kDa protein, compared with sham-operated control. Maximal phosphorylation of 180 kDa bands (1.8 fold relative to sham-operated controls) was reached at 6 h of reperfusion following 15 min of cerebral ischemia (Fig 1).
Relationship between the tyrosine phosphorylated 180 kDa protein and NR2B. To examine the relationship between the tyrosine phosphorylated 180 kDa protein and NR2B, we combined immunoprecipitation (IP) with immunoblot (Blot) to detect the 180 kDa protein. Whether using IP with anti-tyrosine phosphate antibody (anti-P-tyr) followed by Blot with anti-NR2B antibody (anti-NR2B) (Fig 2A) or using IP with anti-NR2B followed Blot with anti-P-tyr (Fig 2B), we detected one band of 180 kDa. These results were in consistence with the results obtained using the anti-P-tyr antibody alone (Fig 1). Thus, the results confirmed that the tyrosine-phosphorylated 180 kDa was NR2B, in other words, NR2B was phosphorylated at tyrosine residues. The results showed that the tyrosine phosphorylation of NR2B from the L/R samples was higher than the corresponding subunit from sham-operated controls. In this experiment, the average increase in the tyrosine-phosphorylated NR2B, in relation to sham-operated controls, was 187% (n = 3 gerbils, P < 0.01 vs sham-operated controls, Fig 2).

Effects of ischemia/reperfusion on the expression of NR2B. In contrast, under above conditions, when the proteins were analyzed by immunoblotting with anti-NR2B, we could only see a protein band of 180 kDa (NR2B) which did not change significantly (Fig 3).

Effects of KT, ND, and DNQX on the tyrosine phosphorylation of NR2B. To characterize the mechanisms of the tyrosine phosphorylation of NR2B, the samples were examined by IP with anti-P-tyr followed by Blot with anti-NR2B. A marked elevation (182%, n = 3 gerbils, P < 0.01 vs sham-operated controls) of the tyrosine phosphorylation was seen in NR2B after 6 h reperfusion following a 15-min episode of ischemia (Fig 4). KT ip 12.5, 25, and 50 mg kg⁻¹, respectively 20 min before ischemia resulted in a marked decrease in the tyrosine phosphorylation of NR2B to 120%, 118%, and 111% of sham-operated control (n = 3 gerbils, P < 0.01 vs group of L/R). ND ip 10, 15, and 20 mg kg⁻¹ 20 min before ischemia caused a marked decrease in the tyrosine phosphorylation of NR2B to 126%, 121%, and 115% of sham-operated control (n = 3 gerbils, P < 0.01 vs group of L/R). DNQX ip 10, 20, and 30 mg kg⁻¹ 20 min before ischemia resulted in insignificant changes in the tyrosine phosphorylation of NR2B to 182%, 181%, and 180% of sham-operated control (n = 3 gerbil, P > 0.05 vs L/R). Fig 4 represented only the effects of KT 25 mg kg⁻¹ and ND 15 mg.
• kg⁻¹ on the tyrosine phosphorylation of NR2B, respectively.

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<th>IP: Anti-P-tyr</th>
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Fig 4. Effects of ketamine (KT) and nifedipine (ND) on the tyrosine phosphorylation of NR2B in the gerbil hippocampal synaptosomes. Sh: sham operated controls. I/R: ischemia/reperfusion. Time of ischemia: 15 min. Time of reperfusion: 6 h. KT: 25 mg·kg⁻¹. ND: 15 mg·kg⁻¹. n = 3 gerbils.

In contrast, under above conditions, when the proteins were analyzed by Blot with anti-NR2B, we could only see a protein band of 180 kDa (NR2B) which did not change significantly (data not shown).

**Effects of vanadate and genistein on the tyrosine phosphorylation of NR2B** To characterize the mechanisms of phosphorylation, we examined the effects of vanadate, an inhibitor of PTP, and genistein, an inhibitor of PTK, on the tyrosine phosphorylation of NR2B. A marked elevation of the tyrosine phosphorylation was seen in all major bands after 6 h reperfusion following a 15-min episode of ischemia, especially the 180 kDa band (NR2B) was observed to significantly increase to 184% of sham-operated controls. Vanadate or genistein ip 15 mg·kg⁻¹ 20 min before ischemia resulted in a marked increase in the tyrosine phosphorylation of 180 kDa (NR2B) to 195% (n = 3 gerbils, P < 0.05 vs group of I/R), and decreased to 110% of sham-operated control (n = 3 gerbil, P < 0.01 vs group I/R) respectively (fig 5). In contrast, under above conditions, when the proteins were analyzed by Blot with anti-NR2B, we could only see a protein band of 180 kDa (NR2B) which did not change significantly (data not shown).

**Association of Src and NR2B** To determine whether Src and NR2B were associated physically, we immunoprecipitated synaptosomal proteins with antibodies specific for the NR2B or for Src. When we used non-denaturing conditions to solubilize proteins, immunoprecipitation with anti-NR2B resulted in coprecipitation of Src (fig 6A), conversely, immunoprecipitation with anti-Src resulted in coprecipitation of NR2B (fig 6B). The coprecipitation of Src by anti-NR2B and of NR2B by anti-


**A**

- Anti-Src → --- --- → IgG
- Anti-NR2B → --- --- → 180 kDa

**B**

- Anti-NR2B → --- --- → 180 kDa
- Anti-Src → --- --- → 60 kDa

Fig 6. Association of Src and NMDA channel proteins. SM: solubilized membrane. NDN: non-denaturing. DN: denaturing. n = 3 gerbils.

**DISCUSSION**

The present results identified that NR2B was phosphorylated at tyrosine residues, and demonstrated that transient forebrain ischemia for 15 min caused a marked decrease of the tyrosine phosphorylation of many proteins including NR2B. But, transient ischemia followed by reperfusion induced rapid and sustained increase in the ty-
rosine phosphorylation of many proteins including NR2B. Although the decrease in the tyrosine phosphorylation of NR2B during ischemia remains unknown, the increase in the tyrosine phosphorylation of NR2B following I/R may be due to the increase in PTK activity, but not the decrease in PTP activity according to our previous study. In addition, we found that vanadate, an inhibitor of PTK activity, and genistein, an inhibitor of PTK activity, increased and decreased the tyrosine phosphorylation of NR2B, respectively, further suggesting that PTK and PTP participate commonly in the regulation of the tyrosine phosphorylation of NR2B during I/R, although PTP activity had no change during I/R.

We found that the increase in total PTK activity caused by I/R may be mainly due to Src activity and it was related to the activation of NR and L-type VGCC (data not shown). In this study we further found that Src may have been physically associated with NR2B, suggesting that the increased tyrosine phosphorylation of NR2B may result in altered signal transduction in the postischemic brain. Src appears to play an important role in the regulation of the tyrosine phosphorylation of NR2B. More recently, evidence is accumulating that tyrosine phosphorylation results in the potentiation of the NR<sup>[6,7]</sup>. In other words, the tyrosine phosphorylation of NR2B is an up-regulating NR channel activity, causing the influx of Ca<sup>2+</sup>. Thus, we speculate that the increase of the tyrosine phosphorylation of NR2B induced by I/R may aggravate ischemic brain injury.

To characterize the relationship between the tyrosine phosphorylation of NR2B and NR or L-type VGCC during I/R, we examined the effects of KT, an antagonist of NR, and ND, an blocker of L-type VGCC, as well as DNQX, an antagonist of the non-NMDA receptor (antagonist of AMPA receptor, not antagonist of mGluR), on the tyrosine phosphorylation of NR2B during I/R. The results showed that both KT and ND can prevent the tyrosine phosphorylation of NR2B, but DNQX failed to prevent it. These results indicated that the tyrosine phosphorylation of NR2B is related to the activation of NR and L-type VGCC, but has no relation to AMPA receptor.

Taken together, the increase in the tyrosine phosphorylation of NR2B induced by I/R may mediate via the NR and L-type VGCC; PTK, in particular Src that associates with NR2B physically, and PTP participate in the regulation of the tyrosine phosphorylation of NR2B during I/R.

REFERENCES

缺血性再灌注后 NMDA 受体亚基 2B 酪氨酸磷酸化的调节机制

黄林,...

关键词 脑缺血；氨酰；硝苯地平；乙酰基；金雀仁素；氨酰胺；N-甲基-D-天冬氨酸受体；钙通道

目的：研究鼠缺血再灌注后海马突触对 N-甲基-D-天冬氨酸(NMDA)受体亚基 2B(NR2B)酪氨酸磷酸化调节的机制。方法：鼠脑缺血再灌注后,动物脑组织的 NR2B 酪氨酸磷酸化水平明显下降;再灌注后包括 180 kDa 蛋白在内,多种蛋白酪氨酸磷酸化水平快速下降(再灌注 15 分钟),持续至少 48 小时升高。免疫沉淀和免疫印迹证实,180 kDa 条带为 NR2B。缺血 15 分钟,再灌注 6 小时, NR2B 酪氨酸磷酸化明显高于对照组,为对照组的 3.8 倍,而 NR2B 蛋白表达量则无变化。

7th National Congress on Anti-inflammation and Immunopharmacology

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