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Suppression of postsynaptic density protein 95 by antisense oligonucleotides diminishes postischemic pyramidal cell death in rat hippocampal CA1 subfield

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Abstract

Our previous investigation has shown that postsynaptic density protein 95 (PSD-95) is critical for the Src family kinases-mediated tyrosine phosphorylation of *N*-methyl-D-aspartate receptor subunit 2A (NR2A) in the postischemic hippocampus. To clarify the roles of PSD-95 in the ischemic brain damage, histological method was performed to examine the effects of PSD-95 antisense oligonucleotides (AS) on the postischemic delayed cell death in rat hippocampus. Transient (15 min) brain ischemia was induced by the four-vessel occlusion method in Sprague–Dawley rats. Five days of reperfusion following brain ischemia (I/R5d) led to hippocampal CA1 pyramidal cell death upward of 90%. Intracerebroventricular infusion of AS (every 24 h for 3 days before ischemia) not only decreased the PSD-95 expression but also increased the number of surviving pyramidal neurons, while missense oligonucleotides (MS) had no effects. To further investigate the mechanisms underlying the neuroprotection of PSD-95 deficiency, the interaction of proline-rich tyrosine kinase 2 (Pyk2) with NR2A as well as autophosphorylation (Tyr402) of Pyk2 were detected. Immunoprecipitation and immunoblot analysis showed that preischemic treatment with AS, but not MS or vehicle, attenuated the I/R6h-induced increases in Pyk2–NR2A association and Pyk2 autophosphorylation. The protein levels of NR2A and Pyk2 had no differences under the above conditions. Our data suggest that the recruitments of ion channels and signaling molecules may be involved in the PSD-95 neurotoxicity in the postischemic hippocampus.

Keywords: Brain ischemia; Neuroprotection; Postsynaptic density protein 95; *N*-methyl-D-aspartate receptor; Proline-rich tyrosine kinase 2

Postsynaptic density protein 95 (PSD-95), also known as synapse-associated protein 90 (SAP90), is a predominant kind of scaffold proteins concentrated in glutamatergic postsynaptic density. Although accumulating evidence has indicated that PSD-95 is involved in the ischemic events [1,5–7,15], the exact roles of PSD-95 in the postischemic neuronal cell death have not been elucidated.

PSD-95 proteins associate NR2 subunits of *N*-methyl-D-aspartate (NMDA) receptors with an array of protein tyrosine kinases (PTKs) including Src family of kinases and proline-rich tyrosine kinase 2 (Pyk2) [18,19]. Src family kinases belong to the family of membrane-associated non-receptor PTKs. Within nine of Src family members, Src and Fyn kinases are highly expressed in the central nervous system. Electrophysiological studies have shown that NR2A tyrosine phosphorylation by Src family kinases enhances NMDA channel responses [9]. The associations of Src and Fyn with NR2A and NR2A tyrosine phosphorylation were found increased significantly in the postischemic rat hippocampus [5,10]. Knockdown of PSD-95 by antisense oligonucleotides reduced the above increases [7]. These data suggest that PSD-95 may be responsible for the excessive activation of NMDA receptor channel activity and thus contributes to the delayed neuronal cell death in the postischemic hippocampus. To prove the hypothesis, the neuroprotective action of PSD-95 suppression by antisense oligonucleotides on the delayed hippocampal cell death after brain ischemia was evaluated by histological method.

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Pyk2, also designated CAKβ, RAFTK, CADTK or FAK2, may also be involved in the postischemic facilitation of NR2A tyrosine phosphorylation [11]. It has been demonstrated that Pyk2 interacts with NMDA receptors via PSD-95 [18]. In recent experiments, the enhanced associations involving PSD-95, Pyk2 and NR2A as well as autophosphorylation of Pyk2 on Tyr402 in the postischemic hippocampus have been established [5,10,13]. The autophosphorylation of Pyk2 is essential to the enhancement of Pyk2 kinase activity and activation of Src family kinases that in turn induce ion channel function up-regulation and activation of downstream signaling cascades [14]. To reveal the mechanisms underlying PSD-95 neurotoxicity, the effects of PSD-95 antisense oligonucleotides on the binding of Pyk2 to NR2A and Pyk2 autophosphorylation were examined by immunoprecipitation and immunoblot.

Adult male Sprague–Dawley rats weighing 250–300 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science. End-phosphorothioated antisense oligonucleotides (AS) specifically against PSD-95 (Invitrogen, Japan K.K., Tokyo, Japan) were dissolved in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and administrated to the rats every 24 h for 3 days by means of unilateral intracerebroventricular (i.c.v.) infusion as described previously [7]. The same doses of end-phosphorothioated missense oligonucleotides (MS) or vehicle (TE) were used as control. The sequences of oligonucleotides (AS: 5′-GAATGGGTCACCTCC-3′; MS: 5′-CCGCTCTATCGAGGA-3′) were from Sattler et al. [16].

After pretreated with AS or MS or the same dose of TE, the rats were subjected to 15 min of transient brain ischemia followed by 6 h or 5 days of reperfusion. Transient brain ischemia was induced by the method of four-vessel occlusion with a little modification as indicated before [5]. Sham-operated rats received the same surgical treatment, except for the occlusion of carotid arteries.

For histology, the rats subjected to 5 days of reperfusion were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under anesthesia. The paraffin-embedded brain sections (5 μm) were prepared and stained with hematoxylin and eosine.

For immunoprecipitation and immunoblot, the rats subjected to 6 h of reperfusion were decapitated, and the hippocampi were removed and rapidly frozen in liquid nitrogen. The frozen tissue samples were homogenized in 1:10 (w/v) ice-cold homogenization buffer containing 50 mM MOPS (3-(*N*-morpholino)propanesulfonic acid; pH 7.4), 100 mM KCl, 320 mM sucrose, 0.5 mM $MgCl_2$, 0.2 mM dithiotheitol, and some inhibitors of phosphatase and protease. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C and the supernatants were collected. Supernatant proteins (400 μg) were used to immunoprecipitate or immunoblot with the indicated antibodies as described formerly [5].

To prove the suppression efficiency of AS on PSD-95 level, immunoblot with mouse monoclonal anti-PSD-95 antibody (clone 7E3-1B8; Oncogene, San Diego, CA, USA) was carried out to detect the alteration of PSD-95 expression. As shown in Fig. 1, repeated infusion (i.c.v.) of rats with AS (50 or 100 μg) every 24 h for 3 days evidently reduced the protein level of PSD-95, while MS (100 μg) or TE did not affect PSD-95 expression.

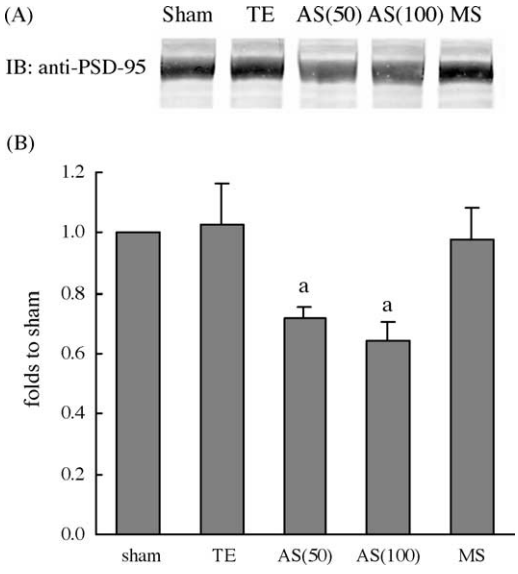

Fig. 1. The effect of pretreatment with PSD-95 antisense oligonucleotides (AS) on PSD-95 expression in rat hippocampus. (A) Immunoblot analysis of the protein levels of PSD-95 with anti-PSD-95 antibody. (B) Quantitative representation of the protein levels of PSD-95. Data are mean ± S.D. ($n = 3$ rats) and expressed as folds vs. sham. [a] $P < 0.05$ vs. sham (ANOVA followed by Newman–Keuls test).

Hematoxylin and eosine staining was performed to show the survival of hippocampal CA1 pyramidal neurons after ischemia. The normal cells showed round cell body and plain stained nuclei (Fig. 2A and E). The neuronal density of the CA1 pyramidal cells was expressed as the number of normal cells per 1 mm length counted under a light microscope (×400) and presented in the Fig. 3. After 5 days of reperfusion following brain ischemia, most of hippocampal CA1 pyramidal neurons were shrunken with pyknotic nucleus and counted as dead cells (Figs. 2B, F and 3). Repeated pretreatment with 50 μg of AS before ischemia markedly increased the number of surviving neurons (Fig. 3) and 100 μg of AS showed more significant neuroprotection (Figs. 2C, G and 3). Infusion of 100 μg MS had no effect on the postischemic cell death (Figs. 2D, H and 3). Above results revealed that PSD-95 is responsible for the delayed hippocampal cell death after ischemia.

Previously, both Pyk2–NR2A binding and Pyk2 autophosphorylation have been shown to increase rapidly and sustainedly following brain ischemia and reach a peak level at 6 h of reperfusion [10,13]. To further detect the role of PSD-95 in the facilitation of NR2A tyrosine phosphorylation, we inspected the effect of PSD-95 AS pretreatment on these 6 h of reperfusion-induced increases.

The Pyk2–NR2A association was inspected by co-immunoprecipitation under non-denatured conditions.