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·Original Article·

Knock-down of postsynaptic density protein 95 expression by antisense oligonucleotides protects against apoptosis-like cell death induced by oxygen-glucose deprivation *in vitro*

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Abstract: Objective Postsynaptic density protein 95 (PSD-95) plays important roles in the regulation of glutamate signaling, such as that of N-methyl-*D*-aspartate receptors (NMDARs). In this study, the functional roles of PSD-95 in tyrosine phosphorylation of NMDAR subunit 2A (NR2A) and in apoptosis-like cell death induced by oxygen-glucose deprivation (OGD) in cultured rat cortical neurons were investigated. **Methods** We used immunoprecipitation and immunoblotting to detect PSD-95 protein level, tyrosine phosphorylation level of NR2A, and the interaction between PSD-95 and NR2A or Src. Apoptosis-like cells were observed by 4,6-diamidino-2-phenylindole staining. **Results** Tyrosine phosphorylation of NR2A and apoptosis-like cell death were increased after recovery following 60-min OGD. The increases were attenuated by pretreatment with antisense oligonucleotides against PSD-95 before OGD, but not by missense oligonucleotides or vehicle. PSD-95 antisense oligonucleotides also inhibited the increased interaction between PSD-95 and NR2A or Src, while NR2A expression did not change under this condition. **Conclusion** PSD-95 may be involved in regulating NR2A tyrosine phosphorylation by Src kinase. Inhibition of PSD-95 expression can be neuroprotective against apoptosis-like cell death after recovery from OGD.

Keywords: postsynaptic density protein 95; N-methyl-*D*-aspartate receptor; oxygen-glucose deprivation; tyrosine phosphorylation; Src; cortical neurons

1 Introduction

Postsynaptic density protein 95 (PSD-95) belongs to the membrane-associated guanylate kinase superfamily, which consist of three PSD-95/discs large/zona occludens-1 (PDZ) domains, one Src homology 3 (SH3) domain and one guanylate kinase-like domain. Members of the PSD-95 family are thought to act as molecular scaffolds that regulate the assembly and function of the multiprotein-signaling complex found at the postsynaptic density (PSD) of excitatory synapses[1-3]. In excitatory synapses, glutamate receptors are principal components of the postsynaptic specialization and are highly concentrated in the PSD. The major postsynaptic glutamate receptors include the N-methyl-*D*-aspartate receptor (NMDAR), the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR), and the group I metabotropic glutamate receptor (mGluR). It has been reported that PSD-95 links NMDARs to downstream signaling proteins such

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as Src, a member of the Src family in the central nervous system (CNS), which plays important roles in the synaptic targeting and signaling of NMDARs[4,5]. However, the mechanisms remain elusive.

NMDARs are crucial for CNS development, neuroplasticity, and pathophysiology. They exist as heteromers composed of both NR1 and NR2 (NR2A–NR2D) subunits, and in some cases NR3 subunits (NR3A and NR3B)[6-9]. NR2 subunits form and contribute to the binding site of many NMDAR modulators, and influence receptor desensitization. In PSDs, binding of the cytosolic C-terminals of NR2 subunits to the PDZ domains of PSD-95 is the major component[10,11]. The interaction between NMDAR NR2 subunits and PSD-95 is important for specific localization of NMDARs in the PSD and for the coupling of NMDARs to cytoplasmic signaling pathways. Tyrosine phosphorylation, which is important for the regulation of protein function under physiological and pathological conditions, controls the activity of the NMDAR ion channel. NMDAR NR2 subunits contain many tyrosine residues in their cytoplasmic tails, and activation of NMDARs is mediated by protein tyrosine kinases (PTKs). In fact, 1%–2% of NR2A and NR2B subunits are tyrosine-phosphorylated in the brain. Moreover, tyrosine phosphorylation of NMDAR subunits is increased during long-term potentiation and taste learning[12-14]. Src, a non-receptor PTK, up-regulates NMDAR channel activity[5,15]. The up-regulation of NMDAR activity by Src is necessary for the long-term potentiation of synaptic transmission at Schaffer collateral-CA1 synapses in the hippocampus, the predominant cellular model for learning and memory[16].

Among the five members of the Src family of non-receptor PTKs expressed in the CNS (Src, Fyn, Lyn, Lck and Yes), Src and Fyn play important roles in synaptic transmission and plasticity at excitatory synapses[15]. The Src family of PTKs contain highly homologous regions, including an SH2 domain, an SH3 domain and a catalytic domain at the C-terminal. In the CNS, Src is prominently located in the PSD. Both *in vivo* and *in vitro* studies[17-19] have suggested an association of Src with the prominent PSD scaffolding protein PSD-95. Furthermore, a growing body of evidence indicates that the regulation of glutamatergic neurotransmission and synaptic plasticity is the key function of Src in the CNS. At glutamatergic synapses, Src modulates the activity of NMDARs[15,20,21].

The present study investigated the effects of antisense oligonucleotides against PSD-95 on NR2A tyrosine phosphorylation, on the interaction between NR2A and PSD-95 or Src, and on the apoptosis-like cell death induced by oxygen-glucose deprivation (OGD) in cultured rat cortical neurons.

2 Materials and methods

2.1 Cortical neuron cultures Neurons were derived from cerebral cortex of fetal Sprague-Dawley rats (18 days gestation). Fetal rats were anesthetized by cooling on ice and brains were immediately removed. Briefly, neocortex was carefully isolated in ice-cold high-glucose Dulbecco's modified Eagle's medium (h-DMEM; Gibco-BRL, Grand Island, NY, USA). Cortical cells were dissociated by trypsinisation (0.25% *w*/*v* trypsin and 0.05% EDTA in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution) at 37°C for 15 min, followed by gentle trituration in plating medium (h-DMEM supplemented with 10% fetal bovine serum and 10% horse serum; Gibco-BRL). Cells were seeded onto poly-*L*-lysine-coated wells (Sigma, St. Louis, MO, USA) or coverslips at 1×10^5 cells/cm^2 and incubated at 37°C with 5% CO_2. After 18–24 h, cells were incubated in neurobasal medium supplemented with B-27 (Gibco-BRL) and 0.5 mmol/L glutamine and then half-replaced twice a week. Cells showed over 95% neurofilament immunostaining. All the cultures were randomly divided into sham group (with neither oligonucleotide pretreatment nor OGD treatment), OGD/recovery group (with no oligonucleotide pretreatment), antisense oligonucleotide pretreatment + OGD/recovery group (AS), missense oligonucleotide pretreatment + OGD/recovery group (MS) and vehicle pretreatment + OGD/recovery group (TE).

2.2 OGD and drug treatment Cultures were exposed to OGD at 37°C after 16-day incubation when the cells were vulnerable to OGD insult[22]. Briefly, cultures were washed three times with glucose-free Krebs-Ringer (KR) buffer

(stored at 37°C) containing (in mmol/L) 5.36 KCl, 1.26 $CaCl_2$, 0.44 KH_2PO_4, 0.49 $MgCl_2$, 0.41 $MgSO_4$, 137 NaCl, 4.17 $NaHCO_3$, 0.34 Na_2HPO_4, and 10 HEPES, pH 7.4, and incubated in glucose-free KR buffer gassed with 95% N_2/5% CO_2 at 37°C for 60 min in an anerobic chamber (Forma Scientific, Inc., Marietta, OH, USA) containing the same gas mixture. During OGD, the oxygen tension in the incubator solution was 1%. OGD was terminated by incubating the cultures in neurobasal medium supplemented with B-27 and 0.5 mmol/L glutamine in a normoxic incubator maintained at 37°C with 5% CO_2. Neurons in the sham group were incubated in KR buffer containing 10 mmol/L glucose in a normoxic atmosphere containing 5% CO_2. End-phosphorothioated antisense oligonucleotides against PSD-95 were added to the cultures every 24 h for 5 days before OGD, at a final concentration of 1 μmol/L in medium. The same concentration of missense oligonucleotides was used as a control. Vehicle controls were treated only with Tris-EDTA (TE) buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA). The antisense (AS: 5' TGTGATCTCCTCATACTC 3') and missense (MS: 5' AAGCCCTTGTTCCCATTT 3') oligodeoxynucleotides were synthesized by Invitrogen Co. (Japan).

2.3 Assessment of apoptosis-like cell death After OGD, live cells on each coverslip (6×10^4 cells/cm^2) were restored with l-DMEM. The coverslips were incubated with 10 μg/mL fluorescent DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) at 37°C for 30 min, then washed with PBS and excited at 400 nm. With the emission collected at 455 nm, apoptosis-like cells were characterized by the presence of condensed and fragmented nuclei, as opposed to the diffuse staining observed in non-apoptotic cells. Each sample was pooled from three coverslips. The proportion of apoptosis-like cells was calculated as the percentage of the total cells counted in 10 microscopic fields.

2.4 Cell extract preparation Cells at different time points (1, 6, 12, 18 and 24 h) during recovery after OGD were rinsed three times with Hank's balanced salt solution, scraped off the plate in 80 μL ice-cold homogenization buffer containing (in mmol/L) 50 3-(N-morpholino) propane-sulfonic acid (MOPS, pH 7.4), 20 sodium pyrophosphate, 20 β-phosphoglycerol, 320 sucrose, 0.2 dithiothreitol, 1 EDTA, 1 EGTA, 100 KCl, 50 NaF, 0.5 $MgCl_2$, 1 Na_3VO_4, and 1 p-nitrophenyl phosphate and protease inhibitors [containing 1 mmol/L phenyl-methylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin A, and 16 μg/mL benzamidine]. The collected cells were frozen quickly in liquid nitrogen until use. They were sonicated three times, and the protein concentration was determined by the Lowry method using bovine serum albumin as standard. Samples (total proteins) were stored at -80°C until use and were thawed only once.

2.5 Immunoprecipitation Briefly, 400 μg total proteins were diluted four-fold with 0.05 mol/L HEPES buffer (pH 7.4) (Amresco, Solon, OH, USA) containing 10% glycerol, 0.15 mol/L NaCl, 1% Triton X-100, 0.5% Nonidet P-40 (Amresco), as well as EDTA, EGTA, PMSF and Na_3VO_4, 1 mmol/L each. Samples were preincubated for 45 min with 25 μL protein A/G-sepharose CL-4B (Amersham, Buckinghamshire, UK) and proteins nonspecifically adherent to the protein A/G were removed by centrifugation. The supernatants were incubated with 1–2 μg anti-Src or anti-phosphotyrosine antibody for 4 h or overnight at 4°C. An additional 25 μL protein A/G-sepharose was added and the incubation continued for 2 h. After that, the samples were centrifuged at 10 000 g, and the pellets of immunocomplexes were collected and washed three times with HEPES buffer for immunoblotting.

2.6 Immunoblotting Equal amounts of proteins (100 μg) or proteins purified by immunoprecipitation were separated by 7.5% SDS-PAGE and electrotransferred onto nitrocellulose membranes (0.45 mm; Amersham Pharmacia, Buckinghamshire, UK) for immunoblotting. Membranes were blocked with 3% (*w*/*v*) BSA (fraction V) and then incubated with rabbit anti-NR2A polyclonal antibody (1:500) (Chemicon, Temecula, CA, USA), mouse anti-phosphotyrosine (pY20) monoclonal antibody (Pharmingen, Lexington, KY, USA), mouse anti-Src monoclonal antibody (Calbiochem, Cambridge, MA, USA), or mouse anti-PSD-95 (clone 7E3-1B8) monoclonal antibody (Oncogene, San Diego, CA, USA) at 4°C overnight. After washes, the mem-

branes were incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit (Sigma, St. Louis, MO, USA) or anti-mouse (Sigma) IgG (1:10 000) for 2 h. Proteins were visualized with NBT/BCIP color substrate (Promega, Madison, WI, USA) as described by the manufacturer. Protein bands were scanned and the mean optical densities were analyzed with Lab Works Software (UVP, Upland, CA, USA).

2.7 Statistical analysis Data are presented as mean ± SD from five independent cultures. Statistical analysis was performed using one-way analysis of variance followed by the Duncan's new multiple range method. P <0.05 was considered as statistically significant.

3 Results

3.1 Effects of suppression of PSD-95 expression on apoptosis-like cell death induced by OGD/recovery in cultured rat cortical neurons At the time point of 18 h of recovery after OGD, immunoblotting analysis showed that the protein expression of PSD-95 was reduced to 65% of the sham level, while the missense oligonucleotides or vehicle (TE buffer) treatment induced no significant changes (Fig. 1A, B). Besides, as measured at 24 h of recovery after OGD, DAPI staining showed that the apoptosis-like cell death was increased after OGD/recovery, while suppression of PSD-95 expression protected against the injury induced by OGD, decreasing the proportion of apoptosis-

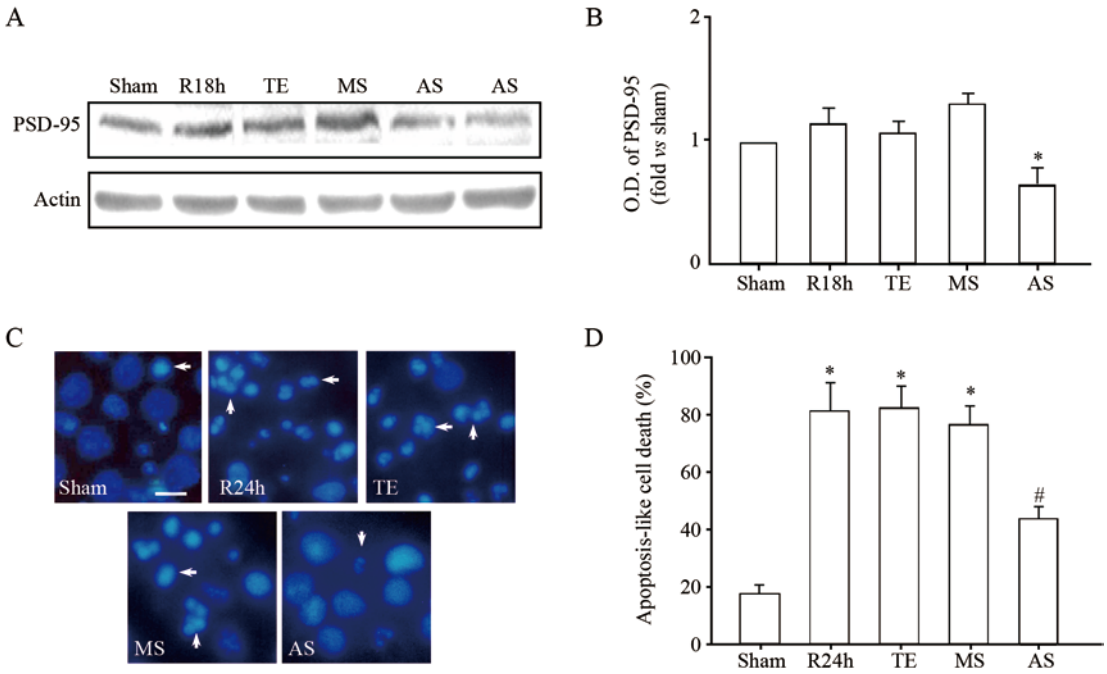

Fig. 1. Effects of PSD-95 antisense oligodeoxynucleotides on apoptosis-like cell death induced by recovery following oxygen-glucose deprivation (OGD). Antisense oligodeoxynucleotides against PSD-95 (1 μmol/L), missense oligodeoxynucleotides (1 μmol/L), or the same dose of vehicle (Tirs-EDTA buffer) were added to the cultures every 24 h for 5 days before OGD. A: Immunoblotting analysis at 18 h of recovery from OGD, of PSD-95 protein levels after treatment with antisense or missense oligonucleotides, or vehicle, using anti-PSD-95 antibody. B: Quantitative analysis of the protein levels of PSD-95. The protein expression of PSD-95 was reduced to 65% of the sham level (*P <0.05) by pretreatment with antisense oligonucleotides. Data are expressed as fold *vs* sham. C: Typical photomicrographs of DAPI staining which was carried out at 24 h of recovery. Apoptosis-like cells were characterized by the presence of condensed and fragmented nuclei, as opposed to the diffuse staining in nonapoptotic cells. Arrows show nuclear condensation and fragmentation typical of apoptosis. Scale bar, 10 μm. D: Percentage of apoptosis-like cells to the total cells counted in 10 microscopic fields. *P <0.05 *vs* sham. #P <0.05 *vs* R24h. Sham: Neurons receiving neither oligonucleotide pretreatment nor OGD. R18h/R24h: Neurons at 18 h/24 h of recovery following OGD, with no oligonucleotide pretreatment. TE: Neurons with vehicle (Tris-EDTA) pretreatment and OGD. MS: Neurons with missense oligonucleotide pretreatment and OGD. AS: Neurons with antisense oligonucleotide pretreatment and OGD. Cells of the control group were restored for 24 h.

like cells from 81% to 44%. However, the missense oligonucleotides and vehicle had no such effects (Fig. 1C, D). These results suggested that suppression of PSD-95 expression protected against the apoptosis-like cell death induced by OGD/recovery in cultured rat cortical neurons.

3.2 Enhanced tyrosine phosphorylation of NR2A induced by OGD/recovery in cultured rat cortical neurons Immunoprecipitation and immunoblotting showed that tyrosine phosphorylation of NR2A reached a peak of up to 4.9-fold *vs* sham at 18 h of recovery, while the level of NR2A protein remained unchanged (Fig. 2A, B). The results showed that tyrosine phosphorylation of NR2A increased during recovery following OGD in cultured cortical neurons. In either immunoprecipitation with anti-NR2A antibody followed by immunoblotting with anti-phosphotyrosine antibody, or immunoprecipitation with anti-phosphotyrosine antibody followed by immunoblotting with anti-NR2A antibody, a band was detected at 175 kDa, and a band of the same molecular weight in the "input" lane was also detected (Fig. 2C). In immunoprecipitation with nonspecific

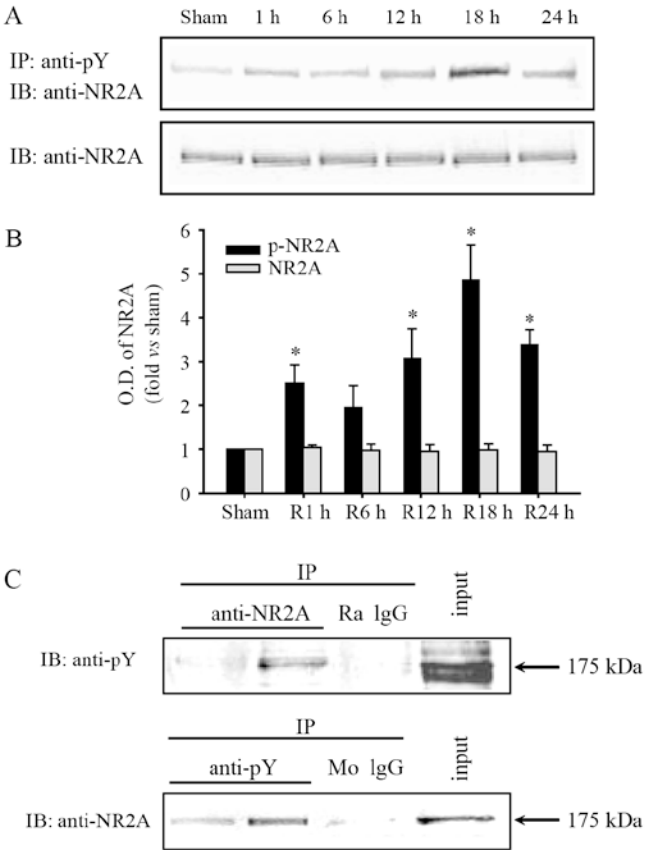

Fig. 2. Time-course of tyrosine phosphorylation of NR2A induced by oxygen-glucose deprivation (OGD)/recovery in cultured cortical neurons. A: Tyrosine phosphorylation of NR2A at different time points during recovery was assessed by immunoprecipitation with anti-phosphotyrosine (pY) followed by immunoblotting with anti-NR2A. Total NR2A was detected by immunoblotting. B: The protein levels of phosphorylated NR2A and NR2A were quantitatively analyzed and expressed as the ratios to the sham level. $^{*}P$ <0.05 *vs* sham. C: NR2A tyrosine phosphorylation at 18 h of recovery was assessed by immunoprecipitation with anti-NR2A antibody or non-specific rabbit IgG followed by immunoblotting with anti-pY antibody, or immunoprecipitation with anti-pY antibody or non-specific mouse IgG followed by immunoblotting with anti-NR2A antibody. NR2A tyrosine phosphorylation was remarkably enhanced in OGD/recovery neurons (the second lane) than in sham control neurons (the first lane). Input lane: proteins were loaded directly without immunoprecipitation. Data for each point were obtained from five independent cultures and one typical experiment is presented (n = 5). p-NR2A: phosphorylated NR2A. IP: immunoprecipitation. IB: immunoblotting. Ra: rabbit. Mo: mouse.

mouse or rabbit IgG, no significant band corresponding to NR2A was detected. The results showed that NR2A tyrosine phosphorylation was induced by OGD/recovery.

3.3 Effects of suppression of PSD-95 expression on tyrosine phosphorylation of NR2A and interaction between NR2A and PSD-95 or Src induced by OGD/recovery in cultured rat cortical neurons To further examine the effects of suppressing PSD-95 expression, NR2A tyrosine phosphorylation was measured at 18 h of recovery from OGD using immunoprecipitation with anti-phosphotyrosine antibody followed by immunoblotting with anti-NR2A antibody. Results showed that suppression of PSD-95 expression significantly inhibited the elevation of NR2A tyrosine phosphorylation from 5.4- to 1.4-fold, while the missense oligonucleotides and vehicle had no effect (Fig. 3A, B). Meanwhile, suppression of PSD-95 expression significantly attenuated the interaction between NR2A and PSD-95 from 3.6- to 1.5-fold, and the interaction between NR2A and Src from 3.8- to 1.2-fold, while the missense oligonucleotides and vehicle had no such effects. Furthermore, the protein level of NR2A remained unchanged after the above treatment (Fig. 3A, B). The results indicated that suppression of PSD-95 expression may protect cortical neuronal cultures against OGD insults by diminishing the interactions between NR2A and PSD-95 or Src.

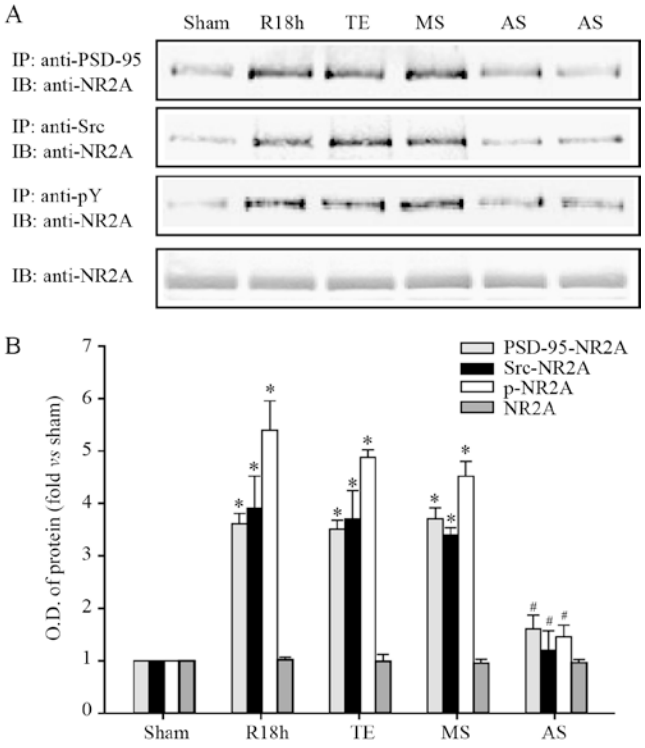

Fig. 3. Effects of PSD-95 antisense oligodeoxynucleotides on NR2A tyrosine phosphorylation and on the interactions of NR2A with PSD-95 or Src in cultured cortical neurons subjected to oxygen-glucose deprivation (OGD). A: Interactions of NR2A with PSD-95 or Src and NR2A tyrosine phosphorylation were assessed through immunoprecipitation with anti-PSD-95, anti-Src, or anti-phosphotyrosine (pY) antibody, followed by immunoblotting with anti-NR2A antibody, at 18 h of recovery from OGD. B: Quantitative analysis of the protein levels of NR2A tyrosine phosphorylation and the interactions of NR2A with PSD-95 or Src. Data are expressed as fold *vs* sham. **P* <0.05 *vs* sham. #*P* <0.05 *vs* R18h. *n* = 6 (independent cultures). R18h: Neurons at 18 h of recovery following OGD, with no oligonucleotide pretreatment. TE: Neurons with vehicle (Tris-EDTA buffer) pretreatment and OGD. MS: Neurons with missense oligonucleotide pretreatment and OGD. AS: Neurons with antisense oligonucleotide pretreatment and OGD. IP: immunoprecipitation. IB: immunoblotting. p-NR2A: phosphorylated NR2A.

4 Discussion

The brain is known to be particularly vulnerable to ischemia. Cerebral ischemia triggers excessive presynaptic release of the excitatory neurotransmitter glutamate. The excess of glutamate over-stimulates postsynaptic receptors such as NMDARs, thereby opening the associated ion channels and leading to calcium overload[23]. The calcium influx activates intracellular proteases and initiates a cascade of further energy-consuming and ultimately lethal processes. Src family kinases are activated rapidly, leading to up-regulation of NMDAR function[15]. Our previous study has shown that NR2A tyrosine phosphorylation induced by transient ischemia and reperfusion exhibits a rapid and sustained rise, a process mediated by PSD-95[19] that also correlates with Src[24]. In this study, we examined the alteration of NR2A tyrosine phosphorylation and the association between NR2A and Src or PSD-95 to reveal the mechanisms underlying the elevation of NR2A tyrosine phosphorylation levels and neuroprotection by PSD-95 antisense oligonucleotides against apoptosis-like cell death induced by OGD/recovery in cultured cortical neurons.

In cerebral ischemia, glutamate excitotoxicity, calcium overload, and free radical damage are important factors affecting the survival of neurons. Neuronal cell death resulting from ischemic events may be associated with abnormal activity of NMDARs. The NMDAR has a specific permeability to calcium, therefore, over-activation of the receptor causes intracellular calcium overload, resulting in neurotoxicity and cell death. So it is possible that suppressing the over-activation of NMDARs can protect neurons against ischemic damage. Here, we showed that NR2A tyrosine phosphorylation was increased after OGD/recovery. Suppression of PSD-95 expression attenuated the increase of NR2A tyrosine phosphorylation induced by OGD/recovery. These results indicate that PSD-95 may regulate tyrosine phosphorylation of NR2A.

PSD-95 binds to postsynaptic NMDARs, causing receptor clustering to the plasma membrane and creating a scaffold for numerous downstream signaling cascades[25,26], and regulating NMDA channel gating[27]. The PDZ domains of PSD-95 interact with the C-terminal tail of NR2A, which specifically localizes NMDARs to the PSD. The SH3 domain of members of the Src family may also interact with the PDZ domains of PSD-95. The Src family is critical for regulating the tyrosine phosphorylation of NMDARs and the opening of NMDAR ion channels[5,19,24]. At the same time, cerebral ischemia increases the interaction of PSD-95 with neuronal nitric oxide synthase (nNOS). Disruption of the interaction between PSD-95 and nNOS may prevent cerebral ischemic damage[28]. Our current study showed that the increases of the interactions between NR2A and PSD-95 or Src were attenuated by suppressing PSD-95 after OGD/recovery in cultured cortical neurons. The interactions between PSD-95 and nNOS were also diminished by suppressing PSD-95. These results suggest that PSD-95 mediates tyrosine phosphorylation of NR2A by recruiting Src kinase.

Taken together, the above results demonstrate that PSD-95 is partially responsible for the opening of NMDAR ion channels and the activity of NMDARs facilitated by Src family kinases. Suppression of PSD-95 expression results in neuroprotection of cortical neuronal cultures against OGD insults.

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