Fyn Kinases Play a Critical Role in Neuronal Apoptosis Induced by Oxygen and Glucose Deprivation or Amyloid- β Peptide Treatment

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SUMMARY

Aims: Src family protein tyrosine kinases (SrcPTKs) have been implicated in the pathogenesis of brain ischemia and Alzheimer's disease (AD). In this study, we investigated whether Src and Fyn kinases, two major members of SrcPTKs in the brain, have distinct roles in the oxygen and glucose deprivation (OGD) and amyloid- β peptide ($A\beta$)-induced neuronal apoptosis. **Methods and results:** The DAPI staining and caspase-3 activation analysis showed that small interfering RNAs (siRNAs) knockdown of Src or Fyn attenuated SH-SY5Y cells apoptosis after OGD and $A\beta$ treatment. Fyn knockdown had a more potent neuroprotective effect than Src knockdown, suggesting a principal pathological significance of Fyn in brain ischemia and AD. Previously, we reported that brain ischemia promotes the phosphorylation of postsynaptic density protein 95 (PSD-95) at tyrosine 523 (Y523), which is associated with postsynaptic mechanisms of excitotoxicity. Here, immunoblot analysis indicated that not only OGD but also $A\beta$ incubation increased the PSD-95Y523 phosphorylation. Src knockdown, especially Fyn knockdown, significantly inhibited such phosphorylation. **Conclusion:** Fyn mediates PSD-95Y523 phosphorylation, which may be responsible for the excitotoxic signal cascades and neuronal apoptosis in brain ischemia and $A\beta$ neurotoxicity. Fyn is a potential therapeutic target for the treatment of ischemic stroke and AD.

Aims

Glutamate excitotoxicity is involved in the pathogenesis of neurodegenerative disorders resulting from brain ischemia and Alzheimer's disease (AD) [1–3]. During the pathological processes, over-activation of glutamate receptors triggers excessive postsynaptic signaling, eventually leading to neuronal cell death, a process collectively known as excitotoxicity [1]. Considering the clinical side effects of glutamate receptor antagonists, exploring the postsynaptic signaling molecules responsible for excitotoxicity may offer effective neuroprotective therapies for the treatment of ischemic stroke and AD.

Src family protein tyrosine kinases (SrcPTKs), the membrane-associated nonreceptor tyrosine kinases family, have been indicated in the neuronal injury resulting from brain ischemia or AD [1,4–7]. We have reported that PP2, a potent SrcPTKs inhibitor, not only protects against hippocampal CA1 pyramidal cell death after brain ischemia [4], but also presents neuroprotective effect on the amyloid- β peptide ($A\beta$) neurotoxicity that has been widely believed to be associated with AD pathogenesis [7]. There are nine members of SrcPTKs, and five members (Src, Fyn, Lyn, Lck, and

Yes) are ubiquitously expressed in the mammalian central nervous system. Both Src and Fyn are highly expressed in the brain, but it is unclear whether Src and Fyn have distinct roles in the pathogenesis of brain ischemia and AD. In this study, we applied small interfering RNA (siRNA) to knock down the expression of Src or Fyn in undifferentiated human neuroblastoma SH-SY5Y cells and then evaluated the apoptosis induced by oxygen and glucose deprivation (OGD) or $A\beta$ treatment.

Postsynaptic density protein 95 (PSD-95) has been proved to be an important organizer of postsynaptic signal transduction. PSD-95 binds to the membrane-associated excitatory glutamate receptors, including *N*-methyl-D-aspartate (NMDA) receptors and kainate receptors [8,9], as well as to various downstream signaling proteins, including SrcPTKs [10,11]. We previously reported that brain ischemia promotes the phosphorylation of PSD-95 at tyrosine 523 (Y523) which is concerned with the over-activation of NMDA receptors. PSD-95Y523 phosphorylation was reduced by specific SrcPTKs inhibitor PP2, indicating SrcPTKs participate in such phosphorylation [12]. To elucidate the mechanism of Src- or Fyn-mediated neuronal apoptosis in brain ischemia and AD, we investigated the effects of Src and Fyn knockdown on the

PSD-95Y523 phosphorylation induced by OGD or A β incubation in SH-SY5Y cells.

Methods

Antibodies

Mouse monoclonal anti-PSD-95, rabbit polyclonal anti-actin and alkaline phosphatase (AP)-conjugated secondary antibodies were purchased from Sigma (MO, USA). A mouse monoclonal anti-Src antibody was obtained from Upstate Biotechnology/Millipore (NY, USA). A mouse monoclonal anti-Fyn antibody was obtained from Santa Cruz Biotechnology (CA, USA). A rabbit polyclonal anti-cleaved caspase-3 antibody was purchased from Cell Signaling Technology (MA, USA). A rabbit polyclonal antibody against phospho-PSD-95Y523 (p-Y523) was raised in New Zealand white rabbits with pY-containing synthetic peptide (keyhole limpet hemocyanin-CREDSVLSpyETVT) as immunogen and then purified by two-step immunoaffinity chromatography (AbMART, Shanghai, China) [12].

Cell Culture and Reverse Transcription-PCR

The undifferentiated human neuroblastoma SH-SY5Y cell line (CRL-2266) is characterized by a typical neuron-like morphology (i.e., neurite formation) and possesses similar physiological functions to normal neurons. SH-SY5Y cells were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 1:1 Mixture; Gibco/Invitrogen, CA, USA) with 10% (v/v) fetal bovine serum (Biocrom AG, Berlin, Germany) at 37°C/5% CO₂.

The total RNAs were extracted, and then, RT-PCR (reverse transcription-PCR) was performed to check the mRNA levels of Src and Fyn. RT-PCR primers for partial fragments of Src, Fyn, and GAPDH were shown in Table 1.

siRNA Transfection

Src and Fyn siRNAs (Table 2) were designed and synthesized by GenePharma (Shanghai, China). The siRNAs were transfected SH-SY5Y cells at 80–90% confluence with LipofectamineTM 2000 (Invitrogen, CA, USA) following the manufacturer's protocol. Briefly, Lipofectamine and siRNAs were diluted in Opti-MEM I + GlutaMAXTM-1 Reduced Serum Medium (Gibco/Invitrogen) respectively. After 5-min incubation, the diluted siRNAs and Lipofectamine were combined, mixed gently, and then incubated for

20 min at room temperature. The siRNA–Lipofectamine complexes were then added to the cells that were also incubated with Opti-MEM I+GlutaMAXTM-1 Reduced Serum Medium. After 4–6 h, the cells were maintained in DMEM/F-12 medium with serum-free N-2 supplements (Gibco/Invitrogen) at 37°C/5% CO₂. The efficiencies of siRNA transfection were determined by both RT-PCR and immunoblot 48 h after transfection. OGD or A β _{25–35} treatment was performed 48 h after siRNAs transfection.

OGD and A β Treatment

For OGD experiments, SH-SY5Y cells in DMEM with low glucose (Gibco/Invitrogen) were kept at a reduced oxygen condition (1% O₂) by continuously infusing the incubator with 5% CO₂ and 95% N₂ gas mixture. For reoxygenation, the cells were maintained in DMEM/F-12 medium with N-2 supplements at 37°C/5% CO₂.

Amyloid- β peptide neurotoxicity is involved in AD pathogenesis, and soluble oligomeric A β _{25–35} (Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met) shows similar toxic effect with A β _{1–40} and A β _{1–42} [13]. Synthetic A β _{25–35} peptides (Sigma) were dissolved in sterile water at 1 mM and incubated at 37°C for 7 day to prepare oligomeric forms [14–16]. Soluble oligomeric A β _{25–35} peptides were added to the cell cultures at 10 μ M and incubated for the indicated times.

Cell Extracts Preparation

The cells were lysed in an ice-cold lysate buffer [in mM: Mops (pH 7.4) 50, KCl 100, sucrose 320, MgCl₂ 0.5, and phosphatase and pro-

Table 1 RT-PCR primers for Src and Fyn

Gene	Primers	Sequence	Product (bp)
Src	Forward	5'-GGGAGTCAGAGCGTTAC-3'	605 bp
	Reverse	5'-GTACTTGCCTGTCTCCCC-3'	
Fyn	Forward	5'-GACTCTTAACACAGGCAC-3'	715 bp
	Reverse	5'-CAGGTTTTCACAGGTTG-3'	
GAPDH	Forward	5'-CGGATTGGTCGTATTG-3'	212 bp
	Reverse	5'-GCTCCTGGAAGATGGTG-3'	

Table 2 siRNAs for Src and Fyn

Name	siRNAs	Sequence
Src siRNA	S1 Sense	5'-CCAAGGGCCUCAACGUGAATT-3'
	Antisense	5'-UUCACGUUGAGGCCUUGGCG-3'
	S2 Sense	5'-CGUCCAAGCCGACAGUCATT-3'
	Antisense	5'-UGAGUCUGCGGCUUGGACGTG-3'
	S3 Sense	5'-GGCUCAUUGAAGACAAUGATT-3'
	Antisense	5'-UCAUUGUCUCAAUGAGCCGA-3'
	S4 Sense	5'-UCAUUGUCUCAAUGAGCCGA-3'
	Antisense	5'-UUCUCCGAACGUGUCACGUTT-3'
Fyn siRNA	F1 Sense	5'-GGUGCCUUAUACUUUUCUATT-3'
	Antisense	5'-UAGAAAGUGAAUAGGCACCTT-3'
	F2 Sense	5'-CGAGUAUAUGAACAAAGGATT-3'
	Antisense	5'-UCCUUUGUUAUUAUCUCGGT-3'
	F3 Sense	5'-GAAAGGAGACCAUGUCAATT-3'
	Antisense	5'-UUUGACAUGGUCUCCUUUCAT-3'
	F4 Sense	5'-GAUUGAUAAGAAGACAUGATT-3'
	Antisense	5'-UCAUUGUCUUAUCAAUCCGG-3'
Negative control	Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'
Scramble	Sense	5'-GAUUCAGGGAAGCUAACUTT-3'
Src siRNA (S3)	Antisense	5'-AGUUAAGUUUCCUGAAUUCTT-3'
Scramble	Sense	5'-GGUCAAUAAGGAAGUAUATT-3'
Fyn siRNA (F4)	Antisense	5'-UAUACUCCUUUUUAGACCTT-3'

tease inhibitors (β -glycerophosphate 20, sodium pyrophosphate 20, NaF 50, 1 mM each of EGTA, EDTA, sodium orthovanadate, *p*-nitrophenyl phosphate, phenylmethylsulfonyl fluoride and benzamide, and 5 μ g/mL each of aprotinin, leupeptin, and pepstatin A)] [15]. The lysates were ultrasonic treated in ice-cold bath, and protein concentrations were determined by Lowry assay.

Immunoblot

Samples proteins were denaturated by boiling for 5 min in Laemmli sample buffer [62.5 mM Tris/HCl (PH 6.8), 2% (w/v) SDS, 1% 2-mercaptoethanol, 10% (v/v) glycerol, and 0.003% Bromophenol Blue]. Then, the equal amounts of protein samples were separated by SDS-PAGE and electrotransferred onto the nitrocellulose membranes (Amersham Pharmacia, Buckinghamshire, UK). After blocking in bovine serum albumin, the membranes were incubated with specific primary antibodies, then incubated with AP-conjugated secondary antibodies, and developed with the BCIP/NBT Color Development Substrate (Promega, WI, USA) following the manufacture's protocol. The bands on the membrane were scanned and analyzed with Quantity One 1-D Analysis Software (Bio-Rad, CA, USA).

DAPI Staining

The cells grown on 24-well plates were fixed with 4% paraformaldehyde for 30 min at room temperature, incubated with 10 mg/L fluorescent DNA binding dye DAPI (4',6-diamidino-2-phenylindole; Sigma) at 37°C for 10 min, and then washed with phosphate-buffered saline. The morphological evaluation of cells was performed using an Olympus fluorescent microscope with excitation fluorescence at 400 nm and emission wavelength at 455 nm. Apoptotic cells were identified by bright condensed, fragmented nuclei, as opposed to the flat diffused staining in normal cells. The total number of cells with apoptotic morphology was counted in ten randomly chosen fields (400 $\times$) per well (150–200 cells). The apoptosis rate was calculated as the ratio of apoptotic cells compared to the total number of cells for each treatment.

Statistical Analysis

For each type of experiment, data were obtained from at least three independent culture preparations. The results are expressed as mean $\pm$ standard deviation (SD). Comparison of each group to control was carried out by LSD (least significant difference) test, and others were by the *q* (Newman-Keul's) test, with *P* < 0.05 considered significant.

Results

Expression and Knockdown of Src or Fyn in SH-SY5Y Cells

To study the role of Src and Fyn kinases in brain ischemia and AD, we intended to use SH-SY5Y cells treated with OGD or oligomeric A β to construct pathological cell models. The expression of Src and Fyn was first checked in SH-SY5Y cells. RT-PCR was per-

formed using specific primers designed according to the conserved motif of Src and Fyn respectively, which could not cross-recognize each other (Table 1). As shown in Figure 1A1, both Src and Fyn mRNAs were expressed in SH-SY5Y cells. The protein expression of Src and Fyn was also confirmed by immunoblot (Figure 1A2).

To clarify the role of Src and Fyn, we set out to screen the specific siRNAs to knock down Src or Fyn in SH-SY5Y cells. The siRNAs, designed from the conserved motif of human *c-src* and *c-fyn* genes, recognized mRNAs of Src and Fyn specifically (Table 2). Nonsilencing siRNA did not disturb the expression of *c-src*, *c-fyn*, or housekeeping gene GAPDH and was used as a negative control (NC). As shown in Figure 1B1,B2, after transient transfection of SH-SY5Y cells with Src siRNA (S1, S2, S3, and S4) or Fyn siRNA (F1, F2, F3, and F4), the significantly reduced mRNA and protein levels of Src and Fyn were observed after 48 h of transfection in Src siRNA S3 and Fyn siRNA F4 groups compared with their respective NC groups, while the Src expression in F4 transfection and Fyn expression in S3 transfection showed no change. Next, we determined the effective dosage of Src siRNA (S3) and Fyn siRNA (F4) by incubating SH-SY5Y cells with different concentrations (50, 100, and 120 nM) of S3 and F4. The S3 and F4 transfection in 100 nM or 120 nM significantly decreased the expression of Src and Fyn (Figure 1C1,C2). The expression reduced about 50% at 100 nM. As shown in Figure 1D1,D2, the siRNA (100 nM) transfection significantly reduced the expression of Src and Fyn after 48 h of transfection and maintained up to 72 h. In addition, scramble-siRNAs against Src siRNA and Fyn siRNA did not affect the protein expression of Src and Fyn. Therefore, siRNAs transfection (100 nM; 48 h) for S3 and F4 was applied for the following experiments. These results indicate that Src siRNA (S3) or Fyn siRNA (F4) transfection specifically down-regulates the expression of Src and Fyn at both mRNA and protein levels in SH-SY5Y cells.

Neuroprotection of Src and Fyn Knockdown Against OGD- or A β -Induced Neurotoxicity

Previously, we have reported that specific SrcPTKs inhibitor PP2 rescues the hippocampal CA1 pyramidal cell death after brain ischemia or A β treatment in the rat [4,7]. In this study, we investigated whether Src and Fyn kinases have distinct roles in OGD and A β -induced neuronal apoptosis.

After various times of reoxygenation following 0.5 h of OGD treatment, the SH-SY5Y apoptosis was first assessed by DAPI staining (Figure 2A1). The ratio for the apoptosis-like SH-SY5Y cells increased in a time-dependent manner after OGD. Next, the activation of caspase-3, one of the key executioners of apoptosis, was examined by immunoblot analysis with an anti-cleaved caspase-3 antibody which specifically recognized the large fragment of activated caspase-3. As shown in Figure 2A2, reoxygenation following OGD increased the level of cleaved caspase-3 in a time-dependent manner, which was consistent with the DAPI staining results.

Both DAPI staining and caspase-3 activation analysis showed that Src or Fyn knockdown led to obvious decreases in the OGD/reoxygenation (24 h)-induced cells apoptosis (Figure 2B1,B2). Compared with the OGD/reoxygenation groups, the apoptotic rate decreased 22% by Src knockdown and 33% by Fyn

Figure 1 Expression and knockdown of Src and Fyn by siRNAs transfection in SH-SY5Y cells. **(A)** The expression of Src and Fyn in SH-SY5Y cells. Total RNAs were extracted from SH-SY5Y cells, and RT-PCR was used to assess the mRNA expression of Src and Fyn (A1). Immunoblot analysis of cell lysate with an anti-Src or anti-Fyn antibody was used to determine the protein expression of Src or Fyn (A2). **(B)** The selection of siRNAs for knockdown of Src and Fyn in SH-SY5Y cells. The cells were transfected with 100 nM Src siRNAs (S1, S2, S3 and S4), Fyn siRNAs (F1, F2, F3 and F4), and corresponding negative control (NC) for 48 h. Con, control group was transfected with the same volume of transfection reagent without siRNA. The mRNA levels of Src (B1) and Fyn (B2) were detected by RT-PCR, and the protein levels of Src (B1) and Fyn (B2) were detected by immunoblot with an anti-Src or an anti-Fyn antibody. GAPDH and actin were used as internal control. **(C)** Determination of siRNA effective concentration of S3 and F4. SH-SY5Y cells were transfected with different concentrations (50, 100 and 120 nM) of S3 or F4, and the expression of Src (C1) or Fyn (C2) was examined by immunoblot after 48 h of transfection. **(D)** Determination of siRNA effective period of S3 and F4. SH-SY5Y cells were transfected with S3 or F4 (100 nM) for 24, 48 and 72 h. The protein levels of Src (D1) and Fyn (D2) were checked by immunoblot analysis.

knockdown, respectively (Figure 2B1). After $A\beta$ treatment for 24 h, the percentage of SH-SY5Y cells apoptosis increased up to 60%. In comparison with the cells only treated with $A\beta$, the apoptotic rate reduced 24% by Src knockdown and 31% by Fyn knockdown (Figure 2C1). In addition, immunoblotting analysis with an anti-cleaved caspase-3 antibody also displayed the similar conse-

quences with the DAPI staining results (Figure 2C2). These results suggest that both Src and Fyn participate in SH-SY5Y cells apoptosis conditioned with OGD or $A\beta$ treatment. Fyn knockdown presents a higher neuroprotective effect compared with Src knockdown, indicating Fyn may be a more potent therapeutic target for the treatment of ischemic stroke and AD.

Figure 2 Protection of Src and Fyn knockdown against OGD- or A β -induced apoptosis in SH-SY5Y cells. **(A)** The time course of SH-SY5Y cell apoptosis induced by 0.5 h of OGD followed by reoxygenation for 0, 6, 12 and 24 h. DAPI staining was performed to detect the apoptotic SH-SY5Y cells (A1) and immunoblot analysis was performed to check the activation level of caspase-3 using an anti-cleaved caspase-3 antibody (A2). Typical representation of SH-SY5Y apoptosis by DAPI staining was shown in the left panel of A1. Arrows direct apoptotic cells. Scale bar = 10 μ m. Quantitative analysis of apoptotic rate was shown in the right panel of A1. Data are mean $\pm$ SD (n = 5). ^aP < 0.05 in comparison to OGD without reoxygenation. **(B and C)** Protection of Src and Fyn knockdown against SH-SY5Y cell apoptosis induced by OGD/reoxygenation (B1 and B2) or A β treatment (C1 and C2). Before treatments, SH-SY5Y cells were transfected with Src siRNA (100 nM), Fyn siRNA (100 nM), and corresponding negative control (NC) for 48 h. The control group (Con) and OGD/A β treatment only (-) group were transfected with the same volume of transfection reagent without siRNA. The apoptotic cells were evaluated by DAPI staining (B1 and C1), and the activity of caspase-3 was checked by immunoblot (B2 and C2). Data are mean $\pm$ SD (n = 5). ^aP < 0.05 in comparison to respective control group, and ^{ab}P < 0.05 in comparison to respective (-) group.

PSD-95Y523 Phosphorylation after OGD or A β Treatment in SH-SY5Y Cells

Src or Fyn knockdown restrains the SH-SY5Y cells apoptosis after OGD or A β treatment, and then the molecular mechanism of Src- or Fyn-induced neuronal apoptosis was identified. Previously, we reported that brain ischemia and reperfusion promote the Src/Fyn-mediated phosphorylation of PSD-95 at Y523 in the rat hippocampus, one of the most vulnerable brain regions [12]. In this study, we detected the PSD-95Y523 phosphorylation in human cell models of brain ischemia and AD.

Immunoblot analysis showed that the phosphorylation level of PSD-95Y523 markedly increased following 0.5 h of OGD. Such increase dropped at 1 and 2 h of OGD compared with 0.5 h but still remained at a higher level than normal control group (Figure 3A). Following A β treatment (1–24 h) in SH-SY5Y cells, the PSD-95Y523 phosphorylation level increased to a maximum at 6 h and remained evident at 24 h. No pronounced difference was observed in the expression of PSD-95 proteins among the control groups and A β treatment groups (Figure 3B). These results suggest that PSD-95Y523 phosphorylation may play a pathologic significance in both brain ischemia and AD.

Src and Fyn Mediate PSD-95Y523 Phosphorylation after OGD or A β Treatment in SH-SY5Y Cells

A hetero-expression system and *in vitro* kinase assay showed that both Src and Fyn mediate the PSD-95Y523 phosphorylation [12]. Here, we clarified whether the molecular mechanism of Src- or Fyn-mediated neurotoxicity is correlated with PSD-95Y523 phosphorylation in cell models of brain ischemia and AD.

The phosphorylation of PSD-95Y523 was checked in SH-SY5Y cells pre-incubated with selective siRNAs of Src or Fyn for 48 h

following management of OGD (0.5 h) or A β (6 h). Src knockdown decreased the PSD-95Y523 phosphorylation in both OGD- (Figure 4A) and A β -treated SH-SY5Y cells (Figure 4B). In addition, a more weakened phosphorylation was observed in Fyn-deficient groups in comparison with Src-deficient groups. The expression of Src was attenuated about 60% and Fyn about 50% by the respective siRNA transfection, but Src or Fyn expression was not influenced by OGD or A β treatment. These results suggest that both Src and Fyn participate in the OGD- or A β -induced PSD-95Y523 phosphorylation, and Fyn kinases appear to be the main reason contributing to such phosphorylation compared with Src kinases.

Conclusion

Excitotoxicity mediated by excessive activation of postsynaptic glutamate receptors is associated with neurodegenerative disorders resulting from brain ischemia and AD [1–3]. Postsynaptic signaling cascades by over-activation of glutamate receptors may present a potential target for ischemic stroke and AD therapy. Src family kinases regulate the function and signaling of NMDA receptors [17–19]. We have demonstrated that SrcPTKs are responsible for the neuronal cell death following brain ischemia and A β treatment [4,7] but which family member plays a major role remains unclear. The present results indicate that both Src and Fyn are involved in neuronal SH-SY5Y cell apoptosis after OGD or A β treatment and Fyn plays a considerable role in the pathogenesis of brain ischemia and AD.

Inhibition of SrcPTKs activity abolishes the brain ischemia- and A β -mediated tyrosine phosphorylation of NMDA receptor subunit GluN2A [4,7], which facilitates the openness of NMDA receptors [20]. Therefore, the neuroprotection of Src knockdown or Fyn knockdown against the damage of OGD and A β treatment may partially be due to the down-regulation of

Figure 3 The increased PSD-95Y523 phosphorylation in SH-SY5Y cells after OGD or A β treatment. The phosphorylation of PSD-95Y523 after 0.5, 1 or 2 h of OGD (A) or 1, 3, 6, 12 or 24 h of A β (10 μ M) treatment (B) were determined by immunoblot with an anti-p-Y523 antibody that specifically recognizes phosphorylated Y523 of PSD-95. Immunoblot with an anti-PSD-95 antibody was performed to check the protein expression. Con, control group for untreated SH-SY5Y cells. Relative levels were normalized to respective controls. Data are mean $\pm$ SD ($n = 3$), ^a $P < 0.05$ in comparison to respective control group.

Figure 4 Effects of Src and Fyn knockdown on the OGD- or A β -induced PSD-95Y523 phosphorylation in SH-SY5Y cells. The cells were performed with 0.5 h of OGD (**A**) or 6 h of A β (10 μ M) (**B**) after transfected with Src siRNA (100 nM), Fyn siRNA (100 nM), and corresponding negative control (NC) for 48 h. The control group (Con) and OGD/A β treatment only (-) group were transfected with the same volume of transfection reagent without siRNA. The phosphorylation of PSD-95Y523 was examined by immunoblot with an anti-p-Y523 antibody. The expression of target proteins was confirmed by immunoblot analysis using the indicated antibodies. Actin serves as an internal control. Relative levels were normalized to respective controls. Data are mean $\pm$ SD ($n = 3$). ^a $P < 0.05$ in comparison to respective control group, and ^b $P < 0.05$ in comparison to respective (-) group.

NMDA receptor function. However, whole-cell patch-clamp could not record the NMDA-triggered currents from SH-SY5Y cells [21], although the expression of NMDA receptors has been proved [22], and NMDA receptor antagonists prevent the apoptosis of such cell line connected with the over-activation of NMDA receptors [21]. It seems that SH-SY5Y cell apoptosis does not result from the excessive openness of glutamate receptors but rather from the intracellular signal transduction. Taken together, besides its effect on the up-regulation of NMDA receptors, Fyn may be involved in the downstream signal events of excitotoxicity. However, the mechanism connected with the initiation of intracellular signaling has remained largely unknown.

Postsynaptic density protein 95 integrates and modulates the postsynaptic signaling of glutamate receptors. On the one hand, PSD-95 binds to NMDA receptors and up-regulates the receptor function via Fyn-mediated phosphorylation [10,23]. On the other hand, PSD-95 interacts with downstream signal molecules such as mixed lineage kinase 3 (MLK3) and mediates the kainate receptors-evoked excitotoxic signal cascades [24,25]. The phosphorylation of PSD-95Y523 has been found to be increased in a rat model of global brain ischemia [12]. In this study, increased PSD-95Y523 phosphorylation is observed not only in cell model of ischemia, but also in AD cell model, implicating a pathological role of such phosphory-

lation. Protein phosphorylation usually results in a conformational change in the modified protein. PSD-95Y523 is located between the SH3 and GK domains. There is a direct intramolecular interaction between the SH3 domain and the GK domain in the PSD-95 subfamily [26]. It is possible that phosphorylation of PSD-95Y523 opens up this stable intramolecular structure, facilitates the association of MLK3 with the SH3 domain of PSD-95, and increases the activation of MLK3 as well as the downstream apoptotic signal pathway.

Using siRNAs transfection to knock down the expression of Src or Fyn kinases, the results suggest that Src, especially Fyn, mediates the PSD-95Y523 phosphorylation in SH-SY5Y cells. Electrophysiological experiments revealed that such phosphorylation contributes to the up-regulation of NMDA receptors in cultured hippocampal neurons [12]. The Fyn-mediated PSD-95Y523 phosphorylation in the SH-SY5Y cells that do not present functional NMDA receptors implicates that Fyn-facilitated PSD-95 signaling is responsible for the cell apoptosis. Development of the drugs targeting the Fyn-PSD-95 complex may offer an opportunity for treatment of ischemic brain damage and AD neurotoxicity.

Excitotoxic signal cascade is considered to be a predominant impairment mechanism in many central nervous system diseases, including brain ischemia [2], trauma [27], and also play a role in neurodegenerative diseases, such as AD [3], amy-

trophic lateral sclerosis [28]. Besides brain ischemia and AD, the pathogenesis mechanisms of these diseases may also be related with the Fyn-mediated PSD-95Y523 phosphorylation. So Fyn may be a versatile therapy target, but this deduction still needs to be investigated.

In conclusion, this study suggests that Fyn kinases principally contribute to the cell apoptosis resulting from brain ischemia and A β neurotoxicity. PSD-95Y523 phosphorylation by Fyn kinases is involved in the postsynaptic mechanisms of excitotoxicity. Fyn is a promising therapeutic target for future neuroprotective therapy in ischemic stroke and AD.

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Conflict of Interest

The authors declare no conflict of interest.