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Apoptosis Induced by Src-Family Tyrosine Kinase Inhibitors in Cultured Rat Cortical Cells

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Abstract In the central nervous system, members of the Src family of tyrosine kinases (SFKs) are widely expressed and are abundant in neurons. The purpose of this study is to examine whether glycogen synthase-3 (GSK-3) is involved in SFK inhibitor-induced apoptosis. PP2 and SU6656, SFK inhibitors, increased apoptotic cell death with morphological changes that were characterized by cell shrinkage, chromatin condensation, or nuclear fragmentation. Moreover, both activation of caspase-9 and caspase-3 were accompanied by the cell death. GSK-3 inhibitors, such as alsterpaullone and SB216763, prevented the PP2-induced apoptosis. In addition, insulin-like growth factor-I prevented the PP2-induced cell death and PP2 inhibited phosphorylation of focal adhesion kinase (FAK). Phosphorylation of FAK on Tyr 576 by Src activates FAK. These results suggest that inhibition of SFK induces apoptosis possibly via blocking of FAK/phosphatidylinositol-3 kinase/Akt signaling pathway and activation of GSK-3 is involved in the cell death in rat cortical neurons.

Keywords Glycogen synthase kinase-3 · Src-family kinase · Caspase · Apoptosis

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Introduction

In the central nervous system (CNS), members of the Src family of tyrosine kinases (SFKs) are widely expressed, as well as in neurons (Thomas and Brugge 1997). The SFKs are non-receptor protein tyrosine kinases and are involved in neurogenesis, differentiation, migration, neurite extension, and synaptic transmission (Thomas and Brugge 1997; Morgan et al. 2000; Zhao et al. 2003; Kalia et al. 2004).

SFKs have been shown to rescue several cell types from apoptotic death induced by removal of cytokines, irradiation, or chemotherapeutic drugs (Thomas and Brugge 1997; Johnson et al. 2000; Lee et al. 2004). However, it is not clear that SFKs play essential roles in neural cell death.

We have reported that inhibitors of glycogen synthase kinase-3 (GSK-3) reduced caspase-dependent apoptosis in rat cortical neurons, when death was initiated by *N*-methyl-D-aspartate (NMDA) receptor antagonist (Takadera et al. 2004, 2006). Recent reports suggest that GSK-3 affects many fundamental cellular functions, including the cell cycle, gene transcription, cytoskeletal integrity, and apoptosis (Cross et al. 1995; Grimes and Jope 2001; Hetman et al. 2000). The phosphatidylinositol-3 kinase/Akt signaling pathway is one of the signaling systems implicated in the survival of neurons that leads to inhibition of GSK-3 by increasing Ser^9 phosphorylation (Cross et al. 1995; Pap and Cooper 1998). Ca^{2+}-influx through NMDA receptors activates Akt/protein kinase B (PKB) through a PI3-k-dependent pathway and the PI3-k-dependent route to Akt/PKB activation involves possible recruitment of SFK-dependent phosphorylation of a focal adhesion kinase (FAK)–p85 (the regulatory subunit of PI3-k) complex in glutamate stimulation (Crossthwaite et al. 2004).

We, therefore, examined whether SFK inhibitors induce caspase-dependent apoptotic cell death in cultured cells

and whether GSK-3 is involved in the SFK inhibitor-induced cell death. We report here that the SFK inhibitors induce apoptosis and that GSK-3 inhibitors block the apoptosis in rat cultured cortical neurons.

Methods

Materials

4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2), SU6656 and 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) were purchased from Merck (Darmstadt, Germany). Alsterpaullone, protease inhibitor cocktail, and mouse anti-β-actin monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, USA). Rabbit anti-phospho-GSK-3β (Ser9) antibody, anti-GSK-3β antibody, and anti-FAK antibody were purchased from Cell Signaling Technology, Inc. (Boston, USA). Rabbit (monoclonal) anti-phospho-FAK (Tyr 576) antibody was acquired from GeneTex, Inc. (Irvine, USA). Rabbit anti-phospho-Akt (Ser473) antibody and anti-AKT antibody were purchased from BioSource International, Inc. (Camarillo, USA). Rabbit anti-phospho-Src (Tyr418) was purchased from Invitrogen Co. (Carlsbad, USA). Insulin-like growth factor-I (IGF-I) was acquired from Roche Diagnostics. 3-(2,4-Dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763) was purchased from Tocris Cookson (Bristol, UK). Hoechst 33258 (bis-benzimide) was purchased from Invitrogen (Carlsbad, USA). Ac-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), Ac-Leu-Glu-His-Asp-4-methyl-coumaryl-7-amide (Ac-LEHD-MCA), and 7-amino-4-methyl-coumarin (AMC) were acquired from the Peptide Institute (Osaka, Japan). Other chemicals were purchased from Wako Pure Industries (Osaka, Japan).

Cell Culture

Cerebral cortical cells were obtained and cultured essentially as described by Dichter (1978) and Choi et al. (1988) from fetal rats (Wistar) after 18–19 days of gestation. Whole cerebral neocortices were removed from fetal mice, taking care to discard the hippocampal formation, basal ganglia, and most of the meninges. The tissue was then minced, incubated in 0.25% trypsin for 30 min at 37°C, and then DMEM plus fetal calf serum was added. The tissue was triturated with Pasteur pipette. Clumps of cells were removed by filtering through a double layer of lens paper.

The dissociated cortical cells were cultured on poly-D-lysine-coated 35 mm dishes (Falcon 3001) (2×10^6 cells/dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. 10 μM of cytosine-β-D arabinofuranoside was added to the culture medium on day 3 after plating. The cells were cultured for 9–10 days and then used in the experiments. We examined the relative proportion of neurons to other cell types by staining the cells with anti-neuronal nuclei (NeuN) monoclonal antibody and anti-glial fibrillary protein (GFAP) visualized with an immunohistochemical staining kit, Vectastain ABC kit (Vector). The relative proportion of neurons (NeuN+) to glia cells (GFAP+) was about 95%.

Cell Treatment and Cell Viability

The cells were washed twice with Tris-buffered salt solution containing (in mM): NaCl 120, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, Tris–HCl 25, and glucose 15, at pH 6.5, and then replaced with 2 ml of DMEM. We used washing buffer at pH 6.5 to block the glutamate neurotoxicity during the treatment. Reagents such as PP2 were then added to the cells. The treatment was carried out for 1–2 days at 37°C. Morphological cell changes were observed by phase-contrast microscopy during the experiment.

Apoptotic cell death was determined by staining the cells with Hoechst dye H33258. Cells were fixed with a 10% formalin neutral phosphate buffer solution (pH 7.4) for 5 min at room temperature. After washing the cells with distilled water, they were stained with 8 μg/ml H33258 for 5 min. The nuclear morphology was observed under a fluorescent microscope (Olympus IX70 model). Apoptosis was quantitated by scoring the percentage of cells with apoptotic nuclear morphology at the single cell level. Condensed or fragmented nuclei were scored as apoptotic. A total of 5–7 randomly selected fields were captured using Polaroid PDMC II software. At least 200 cells were counted per condition, and each experiment was repeated three times.

Caspase Activity

The caspase activity was measured as described previously (Takadera and Ohyashiki 1997). In brief, the cells were washed with phosphate-buffered saline and suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 10 mM EGTA. The cells were treated with 10 μM digitonin for 10 min. The lysates were obtained by centrifugation at 10,000×*g* for 5 min, and the cleared lysates containing 50–100 μg of protein were incubated with 50 μM enzyme substrate Ac-LEHD-MCA (caspase-9) and Ac-DEVD-MCA (caspase-3) for 1 h at 37°C. The specificities of the peptide substrates have been reported (Thornberry et al. 1997). The reaction was terminated by addition of monoiodoacetic acid (5 mM). AMC levels were

measured using a spectrofluorometer (Hitachi F4500, Japan) with excitation at 380 nm and emission at 460 nm, and the activity is expressed as pmol or nmol of AMC released/min/mg of protein.

Western Blotting

Primary cultured cells were scraped off the dish and collected by centrifugation ($400\times g$ for 5 min) followed by homogenization in ice-cold buffer (50 mM Tris–HCl buffer containing 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM benzamide, and 10% protease inhibitor cocktail, pH 7.4). The cell suspension was placed on ice for 30 min and centrifuged at $18{,}000\times g$ for 30 min. The supernatant was diluted with an equal volume of sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue, heated at 95°C for 5 min, and stored at −20°C. Protein concentration was determined by the bicinchoninic acid assay. Each sample (20–30 μg/lane) was loaded and separated using 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred on a nitrocellulose membrane and blocked with Tris-buffered saline containing 5% skim milk for 1 h at room temperature and then incubated with anti-Akt, anti-phospho-Akt, anti-GSK-3β, anti-phospho-GSK-3β, anti-phospho-Src, or anti-phospho-FAK antibody in Tris-buffered saline overnight at 4°C. After washing for 5 min with three changes of Tris-buffered saline, the membrane was incubated with a phosphatase-conjugated goat anti-rabbit or mouse antibody for 1 h at room temperature in Tris-buffered saline. After washing for 5 min with three changes of Tris-buffered saline, immunoreactive bands were visualized with a Western blot detection kit BCIP/NBT system. Equal amounts of protein extracts were also analyzed by Western blot analysis with anti-actin antibody.

RT-PCR

RT-PCR was carried out using the Access RT-PCR System (Promega, Madison, USA). Total RNA was isolated from cortical cells on day 10 by the guanidine isothiocyanate method using an RNeasy mini kit (QIAGEN GmbH, Germany) according to the manufacturer's directions. The reverse transcription reaction was performed at 48°C for 45 min. After incubation at 95°C for 9 min, 30 cycles of amplification (30 s at 94°C (denaturation), 1 min at 60°C (annealing), and 2 min at 68°C (extension)) were performed using the following oligonucleotides: 5′-GGTAT TGAGACAGACAGTGG-3′ (caspase-3 sense primer) and 5′-CATGGGATCTGTTTCTTTGC-3′ (caspase-3 antisense primer).

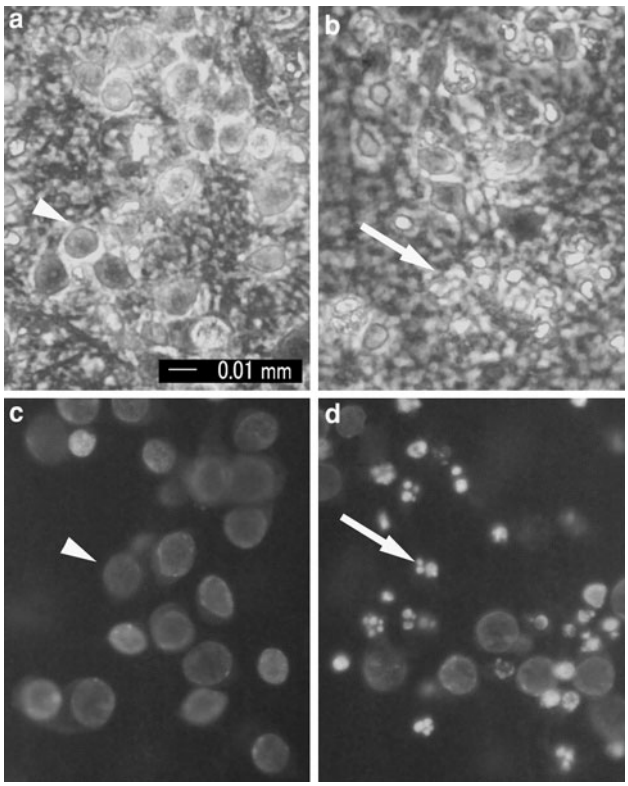


Fig. 1 SFK inhibitor-induced apoptosis. Phase-contrast (**a**, **b**) and H33258 fluorescence (**c**, **d**) microscopy of the cortical cells after exposure to the SFK inhibitor, PP2. Cells were incubated with 0.1% DMSO (**a**, **c**) or 5 μM PP2 (**b**, **d**) for 48 h at 37°C as described in "Methods" section. Scale bars = 10 μm. *Arrowheads* indicate healthy neurons. *Arrows* indicate neurons with apoptotic morphology (condensed or fragmented nuclei)

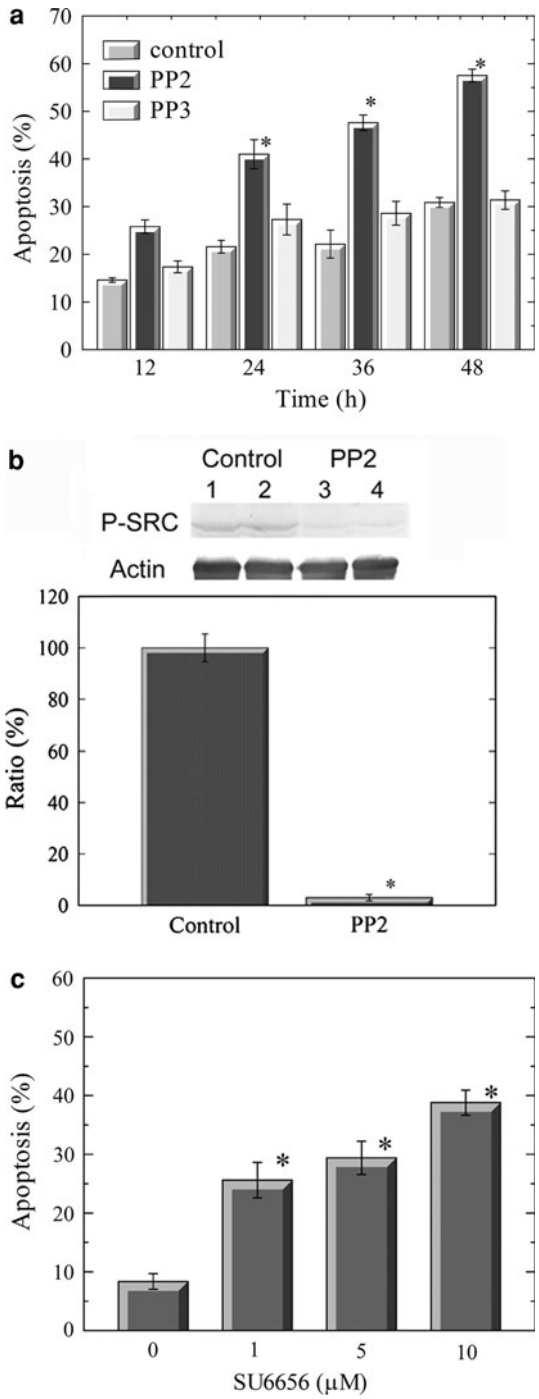
Statistical Analysis

Statistical significance was assessed by one-way ANOVA and post-hoc Scheffe's comparisons.

Results

Apoptosis Induced by SFK Inhibitors

We examined the effect of PP2, a selective SFK inhibitor, on rat cultured cortical cells. Cell treatment with 5 μM PP2 induced apoptotic morphology, such as shrunken cell bodies, fragmented processes, and condensed or fragmented nuclei (Fig. 1). Cell viability was checked by staining with both trypan blue and Hoechst 33258. More than 50% of cells showed apoptotic cell death by treatment with 5 μM PP2 for 48 h (Fig. 2a). PP3, a negative control for the Src family of protein kinase inhibitor PP2, did not induce cell death (Fig. 2a). Dimethyl sulfoxide (DMSO), in which PP2 was dissolved, had no effect on cell viability and nuclear morphology in cortical cells (data not shown).



◀ **Fig. 2** **a** Time-dependent SFK inhibitor-induced cell death. The cells were incubated with or without PP2 (5 μM) or PP3 (5 μM) for 12–48 h at 37°C. Apoptotic cell death was checked by staining with Hoechst 33258 as described in "Methods" section. Apoptosis was quantitated by scoring the percentage of cells with apoptotic nuclear morphology at the single cell level. Condensed or fragmented nuclei were scored as apoptotic. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells). **b** Effect of PP2 on Src phosphorylation levels. The cells were incubated in the presence or absence of PP2 (5 μM) for 1 h at 37°C. Cells were then lyzed and the lysate was immunoblotted with anti-Src (Tyr418) or anti-actin antibody. Data are expressed as a percentage of optical density value for control. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells). **c** SFK inhibitor (SU6656)-induced apoptosis. The cells were incubated with SU6656 (1–10 μM) in the presence or absence of for 48 h at 37°C. Apoptotic cell death was measured as described in "**a**". Each value represents the mean ± SEM. $^*P < 0.05$ (vs. control cells)

Using an antibody that recognizes activated Src, which is phosphorylated on Tyr^{418} in the catalytic domain (Cooper and MacAuley 1988), we assessed that PP2 (5 μM) blocked Src activity in the cortical cells (Fig. 2b). Another SFK inhibitor, SU6656, also induced apoptotic cell death in a dose-dependent manner (Fig. 2c).

Caspases are known to be involved in apoptosis in many injured cells. Knock-out mice have been used to highlight the critical importance of caspase-9, an initiator caspase, and caspase-3, an effector caspase, in neuronal apoptosis during development (Kuida et al. 1996, 1998; Hakem et al. 1998). Therefore, we measured caspase-9 and caspase-3 activity after treatment of the cells with PP2 and PP3 using peptide substrates. Treatment of the cells with 5 μM PP2 for 12 h increased the activities of caspase-9 and caspase-3 (Fig. 3a, b). The negative control PP3 had no effect on the activities of caspase-3 and caspase-9. The expression of caspase-3 was detected in these cells using RT-PCR methods in the rat cortical cells (Fig. 3c).

The apoptotic cell death induced by 5 μM PP2 was attenuated by the presence of 0.1 μg/ml cycloheximide, indicating that the synthesis of new proteins is required for PP2-induced apoptosis, a characteristic of programmed cell death (Fig. 4).

Insulin Growth Factor-I (IGF-I) Protected the Cells from PP2-Induced Cell Death

IGF-I has been reported to play a role in differentiation and survival in CNS via the PI3-K/Akt signaling pathway (Stewart and Rotwein 1996). We reported previously that IGF-I attenuated NMDA receptor antagonist-induced apoptotic cell death in the rat cortical cells (Takadera et al. 1999). As shown in Fig. 5, IGF-I at 50 ng/ml completely inhibited PP2-induced apoptosis.

PP2 Decreased Akt Phosphorylation Levels

Akt is phosphorylated at two sites that are associated with activation of enzyme activity: Thr308 in the catalytic domain and Ser473 in the cytoplasmic domain. Phosphorylation of both sites is critically dependent upon PI3-K activity (Alessi et al. 1997). Akt phosphorylation at Ser473 was determined by immunoblot analysis utilizing a phospho-Akt-(Ser473)-specific antibody. PP2 (5 μM) reduced phospho-Akt levels (Fig. 6).

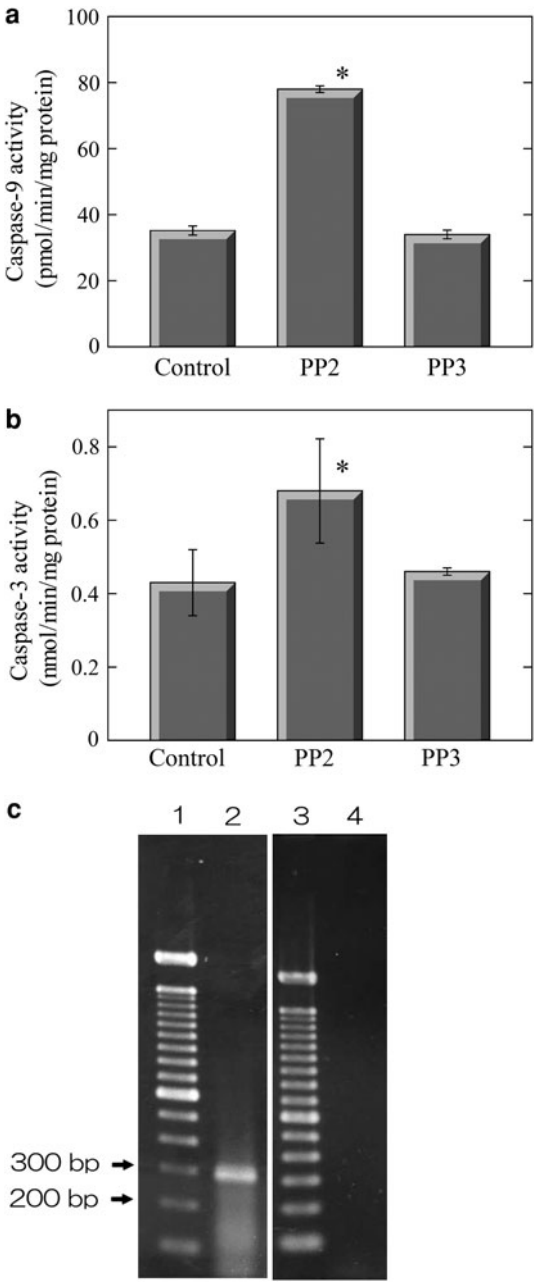


Fig. 3 **a** PP2-induced caspase-9 activation. **b** PP2-induced caspase-3 activation. The cells were incubated with or without 5 μM PP2 or PP3 for 12 h at 37°C. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells). Caspase activity was measured as described in "Methods" section using peptide substrates. Caspase-9 activity and caspase-3 activity were measured using Ac-LEHD-MCA and Ac-DEVD-MCA, respectively. **c** Detection of caspase-3 mRNA by RT-PCR. Amplified products were electrophoresed in 2% agarose gel. The expected product for caspase-3 is 281 bp long (*lane 2*). *Lanes 1 and 3*, size marker of 100-bp DNA ladder. *Lane 4*, no reverse transcriptase reaction

GSK-3 Inhibitors Prevented PP2-Induced Apoptosis

GSK-3 is a principal physiological substrate of Akt and the activity of GSK-3 is inhibited by Akt-mediated

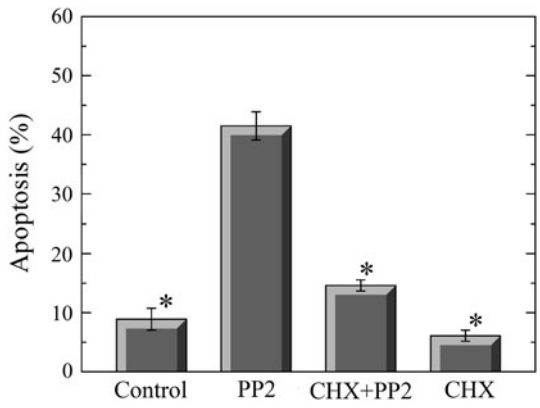


Fig. 4 Effect of cycloheximide on PP2-induced apoptosis. The cells were incubated with PP2 (5 μM) in the presence or absence of cycloheximide (0.1 μg/ml) for 48 h at 37°C. Apoptotic cell death was measured as described in Fig. 2a. Each value represents the mean ± SEM. $^*P < 0.05$ (vs. PP2-only-treated cells)

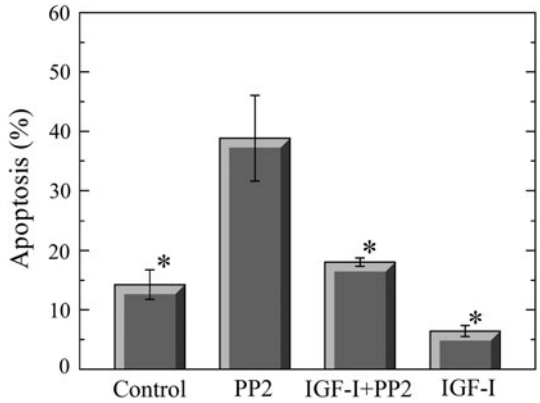


Fig. 5 Effect of IGF-I on PP2-induced apoptosis. The cells were incubated with PP2 (5 μM) in the presence or absence of IGF-I (50 ng/ml) for 48 h at 37°C. Apoptotic cell death was measured as described in Fig. 2a. Each value represents the mean ± SEM. $^*P < 0.05$ (vs. PP2-only-treated cells)

phosphorylation in response to trophic stimulation such as IGF-I. To investigate directly the role of the activity of endogenous GSK-3 in the cell death in response to the SFK inhibitor treatment, we assayed the effect of selective inhibitors, alsterpaullone and SB216763, on PP2-induced apoptosis. The GSK-3 inhibitors showed a protective effect against the PP2-induced apoptosis (Fig. 7a, b).

The kinase activity of GSK-3β is inhibited by phosphorylation on serine-9. We then examined the effect of PP2 on the phosphorylation of GSK-3β on serine-9 using a specific antibody detecting the phosphorylation of GSK-3β on serine-9. As shown in Fig. 8, GSK-3β dephosphorylation was detected in PP2-treated cells.

PP2 Decreased FAK Phosphorylation Levels

Phosphorylation of FAK on Tyr 576 by Src activates FAK (Calalb et al. 1995). The PI3-K p85 subunit interacts with

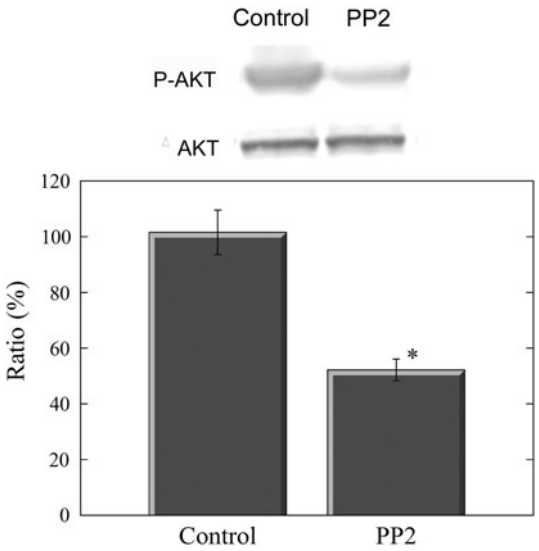


Fig. 6 Effect of PP2 on Akt phosphorylation levels. The cells were incubated in the presence or absence of PP2 (5 μM) for 1 h at 37°C. Cells were then lyzed and the lysate was immunoblotted with anti-Akt or anti-phospho-Akt (Ser473) antibody. Data are expressed as a percentage of optical density value for control. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells)

FAK via its SH2 domain and is activated by FAK (Girault et al. 1999; Xia et al. 2004). We, therefore, examined the effect of PP2 on the phosphorylation of FAK on Tyr576 in the rat cortical cells. As shown in Fig. 9, PP2 markedly decreased the FAK phosphorylation levels.

Discussion

We showed in this report that the GSK-3 inhibitors protected cortical neurons from SFK inhibitor-induced apoptosis, suggesting that GSK-3 activity is critical for neuronal cell death induced by inhibiting Src-family tyrosine kinase activity.

We previously reported that cycloheximide protects apoptosis induced by blocking the trophic effect of NMDA receptor in rat cortical cells (Takadera et al. 1999). Similarly, cycloheximide protected PP2-induced apoptosis, suggesting that PP2-induced apoptosis requires synthesis of proapoptotic protein(s) downstream of the apoptotic pathway (Fig. 4).

The mechanism by which Src inhibitors induce apoptosis in the rat cortical cells is not clear. NMDA receptor antagonist triggers rat or mouse cortical neuron apoptosis in immature rodent, suggesting that NMDA receptor has pro-survival activity during CNS development (Takadera et al. 1999, 2004; Ikonomidou et al. 1999; Hwang et al. 1999). Non-receptor tyrosine kinases of the Src family are closely associated with NMDA receptor (Wang and Salter 1994). Ca^{2+}-influx through NMDA receptors activates Akt/protein

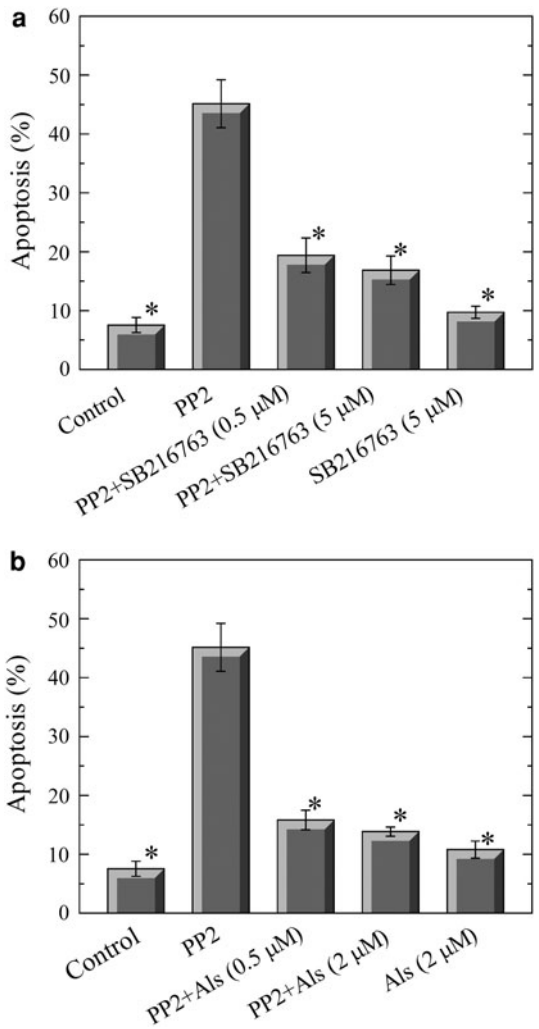


Fig. 7 **a** Effect of SB216763 on PP2-induced apoptosis. The cells were incubated with PP2 (5 μM) in the presence or absence of SB216763 (0.5–5 μM) for 48 h at 37°C. Each value represents the mean ± SEM. $^*P < 0.05$ (vs. PP2-only-treated cells). Apoptotic cell death was measured as described in Fig. 2a. **b** Effect of alsterpaullone on PP2-induced apoptosis. The cells were incubated with PP2 (5 μM) in the presence or absence of alsterpaullone (0.5–2 μM) for 48 h at 37°C. Apoptotic cell death was measured as described in Fig. 2a. Each value represents the mean ± SEM. $^*P < 0.05$ (vs. PP2-only-treated cells). *Als* alsterpaullone

kinase B (PKB) through a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent pathway and the PI 3-kinase-dependent route to Akt/PKB activation involves possible recruitment of an SFK-dependent phosphorylation of a FAK–p85 (the regulatory subunit of PI 3-kinase) complex in glutamate stimulation (Crossthwaite et al. 2004).

FAK has an important role in the prevention of apoptosis by cell attachment. FAK could have a similar function in the nervous system (Girault et al. 1999). Phosphorylation of FAK on Tyr 576 by Src activates FAK (Calalb et al. 1995). The PI3-K p85 subunit interacts with FAK via its SH2 domain and might be activated by FAK (Girault et al.

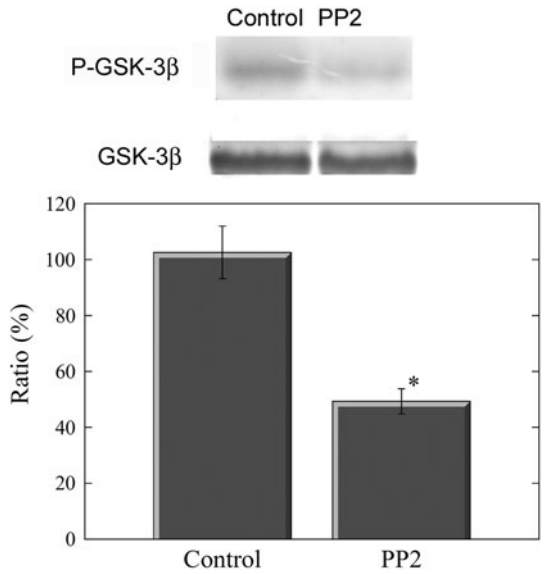


Fig. 8 Effect of PP2 on GSK-3 phosphorylation levels. The cells were incubated in the presence or absence of PP2 (5 μM) for 1 h at 37°C. Cells were then lyzed and the lysate was immunoblotted with anti-GSK-3β or anti-phospho-GSK-3β (Ser9) antibody. Data are expressed as a percentage of optical density value for control. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells)

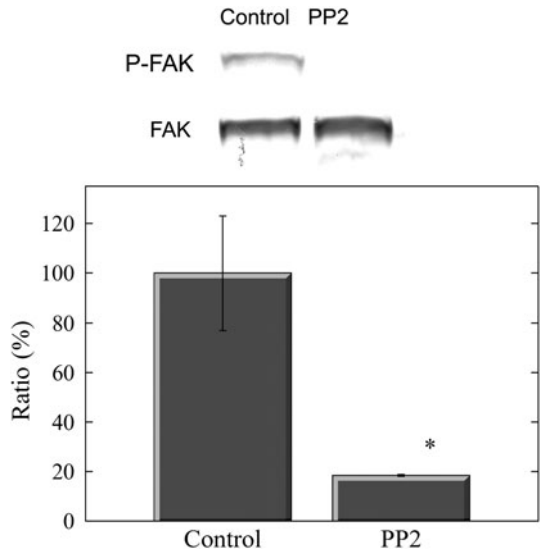


Fig. 9 Effect of PP2 on FAK phosphorylation levels. The cells were incubated in the presence or absence of PP2 (5 μM) for 1 h at 37°C. Cells were then lyzed and the lysate was immunoblotted with anti-FAK or anti-phospho-FAK (Tyr576) antibody. Data are expressed as a percentage of optical density value for control. Data are shown as the mean ± SEM. $n = 3$ per group. $^*P < 0.05$ (vs. control cells)

1999). As shown in Fig. 9, PP2 inhibited the phosphorylation of FAK on Tyr576. The SFK inhibitor may interfere with the survival signal of NMDA receptor via inhibition of FAK/PI3-K/Akt signaling pathway and induce GSK-3-dependent apoptosis.

Glycogen synthase kinase-3 activity is known to be suppressed when it becomes phosphorylated on serine 9 by activation of Akt (Cross et al. 1995; Pap and Cooper 1998). IGF-I has been reported to play a role in differentiation and survival in CNS (Stewart and Rotwein 1996) and to activate the phosphatidylinositol-3 kinase (PI3-K)/Akt signaling pathway (Alessi et al. 1997). As expected, IGF-I completely inhibited PP2-induced apoptosis (Fig. 5).

The critical importance of caspase-9, an initiator caspase, and caspase-3, an effector caspase, in neuronal apoptosis during development has been shown using knock-out mice (Kuida et al. 1996, 1998; Hakem et al. 1998). The apoptosis induced by the SFK inhibitor was accompanied by activation of caspase-9 and caspase-3. We have reported that NMDA antagonist-induced caspase-3 activation is blocked by GSK-3 inhibitors, suggesting that GSK-3 probably acts at a site upstream of caspase-3 (Takadera et al. 2006).

However, the downstream substrates of GSK-3 that ultimately induce neuronal death are not clear. Linseman et al. (2004) have reported that GSK-3 phosphorylates Bax, a pro-apoptotic Bcl-2 family member, and promotes its mitochondrial localization. Bax stimulates the intrinsic (mitochondrial) death pathway, including caspase cascade, by eliciting cytochrome c release from mitochondria,

We showed in this report for the first time that GSK-3 inhibitors protected cortical neurons from SFK inhibitor-induced apoptosis.

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