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Brain Research 1035 (2005) 51–59
BRAIN RESEARCH
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Research report

The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways

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Accepted 19 November 2004
Available online 25 January 2005

Abstract

Increasing evidence suggests that c-Jun N-terminal kinase (JNK) is an important kinase mediating neuronal apoptosis in brain ischemia. To further study the roles of JNK activation in hippocampal CA1 neurons in a rat model of transient global ischemia, we assessed the effect of JNK inhibition by SP600125 on the degree of brain injury. Our results demonstrated that SP600125 significantly increased the number of surviving cells in hippocampal CA1 subfield and decreased the activation of p-JNK1/2 and p-JNK3 at 30 min and 3 days after brain ischemia. Moreover, SP600125 significantly diminished the increased levels of phosphorylated-c-Jun (Ser63/73) and phosphorylated-Bcl-2 (Ser87) at 3 h after brain ischemia. These results indicate that SP600125, a new inhibitor of JNK, protected transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 region at least via suppressing the activation of nuclear substrate (c-Jun) and inactivating non-nuclear substrate (Bcl-2) induced by ischemic insult. Thus, inhibiting JNK activity by SP600125 may represent a new and effective strategy to treat ischemic stoke.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: SP600125; Brain ischemia; JNK; c-Jun; Bcl-2

1. Introduction

Transient global cerebral ischemia leads to cell death of hippocamal CA1 pyramidal neurons starting 2–3 days after reperfusion[16]. Increasing evidence suggests that c-Jun N-terminal kinases (JNKs/SAPKs), members of the mitogen-activated protein kinase (MAPK) superfamily [18,20,31], play important roles in mediating neuronal apoptosis in brain ischemia/reperfusion. JNKs are activated through a kinase cascade in which the MAP kinase kinase kinase MEKK1 phosphorylates the dual specificity Thr-Tyr protein kinase JNK kinase (also termed SEK or MKK), which then phosphorylates JNKs [17]. Activated JNKs in turn phosphorylate the substrates including nuclear substrates such as c-Jun and cytosol substrates including Bcl-2 then leading to neuronal death. Importantly, blocking the JNK signaling pathway inhibits neuronal cell death after growth factor withdrawal in vitro, and disruption of the gene encoding the JNK3 isoform in mice prevents kainic acid-mediated cell death of neurons, which strongly suggests a functional role for JNKs in programmed neuronal cell death [22,37].

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doi:10.1016/j.brainres.2004.11.050

Apoptosis is an active form of cell death, which is closely associated with alteration of gene expression. Gene expression is regulated by immediately early genes (IEGs) such as c-Jun, and therefore, they were considered to be involved in apoptotic neuronal cell death. In fact, some studies revealed that c-Jun plays important roles in neuronal cell death under in vitro and in vivo conditions [26,37]. The c-Jun-activated gene transcription is dependent on its phosphorylation state. Activated JNKs specifically phosphorylate the N-terminal activation domain of transcription factor c-Jun at serine 63 and 73 residues thereby increasing transcriptional activity of c-Jun [26]. And expression of a c-Jun mutant that cannot be phosphorylated on serine 63 inhibits programmed neuronal cell death in vitro [37].

Bcl-2 is the first identified survival gene involved in the control of apoptosis. Post-translational modifications of Bcl-2 proteins may have a great impact on cell fate. Bcl-2 phosphorylation occurs in a large unstructured loop of the protein located between the BH4 and BH3 regions (amino acids 32-80), a proline-rich region called the "loop region," which contains several serine and threonine residues and is therefore amenable to phosphorylation [3]. Several protein kinases including Raf-1 [4], protein kinase C (PKC) [25], cAMP-dependent protein kinase [33], the extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and JNK1 [5,29,35], as well as the cell cycle-regulated kinase cyclin-dependent protein kinase 1 (CDK1) [1], have been suggested to be implicated in these phosphorylations. The functional significance of the Bcl-2 phosphorylation remains highly controversial. Some Bcl-2 phosphorylations have been reported to render the cells more susceptible to apoptosis. The apoptosis regulatory protein Bcl-2/Bcl-xL is phosphorylated and inactivated by microtubule disarraying agents in a cell cycle dependent manner [33]. ASK1/Jun N-Terminal protein kinase pathway phosphorylates Bcl-2 during cell cycle progression as normal physiologic progress to inactivate Bcl-2 [6].

SP600125 (Anthra [1,9-*cd*] pyrazol-6 (2*H*)-one1, 9-pyrazoloanthrone, SAPK Inhibitor II), is a potent, cell-permeable, selective, and reversible inhibitor of c-Jun N-terminal kinase (JNK) (IC_{50} = 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3). The inhibition is competitive with respect to ATP, and is over 300-fold greater selectivity for JNK as compared to ERK1 and p38 MAP kinases. SP600125 inhibits the phosphorylation of c-Jun and blocks the expression of IL-2, IFN-g, TNF-a, and COX-2 cells [2,32], blocks IL-1-induced accumulation of p-Jun and induction of c-Jun transcription [11].

In this study, by using immunoprecipitation, immunoblotting, and histochemistry, we showed the protective effects of SP600125 on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via suppressing both the phosphorylation of nuclear substrate c-Jun and non-nuclear substrate Bcl-2 of JNKs.

2. Materials and methods

2.1. Animal surgical procedures

Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 250 ± 10 g were given free access to food and water before surgery. Transient brain ischemia (15 min) was induced by the four-vessel occlusion method as described previously [28]. Briefly, under anesthesia with chloral hydrate (300–350 mg/kg, i.p.), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rat were allowed to recover for 24 h and fasted overnight. Ischemia was induced by occluding the common arteries with aneurysm clips. Animals meeting the criteria of a completely flat bitemporal electroencephalograph, maintenance of dilated pupils, and the absence of a corneal reflex during ischemia were selected for the present experiments. Carotid artery blood flow was restored by releasing the clips. During ischemia and reperfusion, rectal temperature was maintained at about 37 °C. The sham operation was performed using the same surgical exposure procedures except for occlusion of the carotid artery.

2.2. Drug treatment

SP600125 (molecular weight = 220) was dissolved in 1% DMSO. When necessary, SP600125 (30 μg/10 μl) or vehicle (DMSO) was administered to rats by intracerebral ventricular infusion 20 min before ischemia. Drug infusion (10 μl) was performed using a microinjector through a preimplanted cannula in the left cerebral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm).

2.3. Tissue preparation

Rats were decapitated at specified time points of reperfusion after 15 min of ischemia, and the hippocampal were separated into CA1 and CA3/DG from hippocampal fissure and CA1 were rapidly frozen in liquid nitrogen. Frozen tissue samples were homogenized in 1:10 (w/v) ice-cold homogenization buffer containing 50 mM MOPS [3-(*N*-morpholino) ropanesulfonic acid, pH 7.4], 100 mM KCl, 320 mM sucrose, 0.5 mM MgCl, 0.2 mM dithiothreitol, phosphatase, and protease inhibitors (20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 50 mM NaF, and 1 mM each of EGTA, EDTA, sodium orthovanadate, *p*-nitrodomains phenyl phosphate, phenylmethylsulfonyl fluoride and benzamidine, and 5 mg/ml each of aprotinin, leupeptin and pepstatin A). The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C. Supernatants, as cytosol part, were collected and protein concentrations were determined by the method of Lowry et al. [21]. The nuclear pellets were extracted with 20 mM HEPES, pH 7.9, 20% glycerol, 420 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and enzyme inhibitors for 30 min

at 4 °C with constant agitation. After centrifuged at 12,000 × *g* for 15 min at 4 °C. Supernatants as nuclear parts were collected and protein concentrations were determined by the method of Lowry et al. Samples were stored at −70 °C and were thawed only once.

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitations were performed with the indicated antibodies as described by Liu et al. [19]. In brief, tissue homogenates (400 μg) of hippocampal CA1 were diluted four-fold with immunoprecipitation buffer containing 50 mM HEPES (pH 7.1), 150 mM NaCl, 1 mM ZnCl, 1.5 mM MgCl, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, and inhibitors of phosphatase and protease as indicated above. After preincubation with 25 μl protein A sepharose CL-4B (Amersham, Buckinghamshire, UK) or protein G agarose (Santa Cruz, CA, USA) and centrifugation to remove protein adhering nonspecifically to the protein A/G, the supernatants were incubated with 1–2 μg proper primary antibodies for 4 h or overnight at 4 °C, followed by incubation with protein A/G beads (25 μl) for 2 h. Samples were centrifuged at 10,000 × *g* and the pellets were washed three times with immunoprecipitation

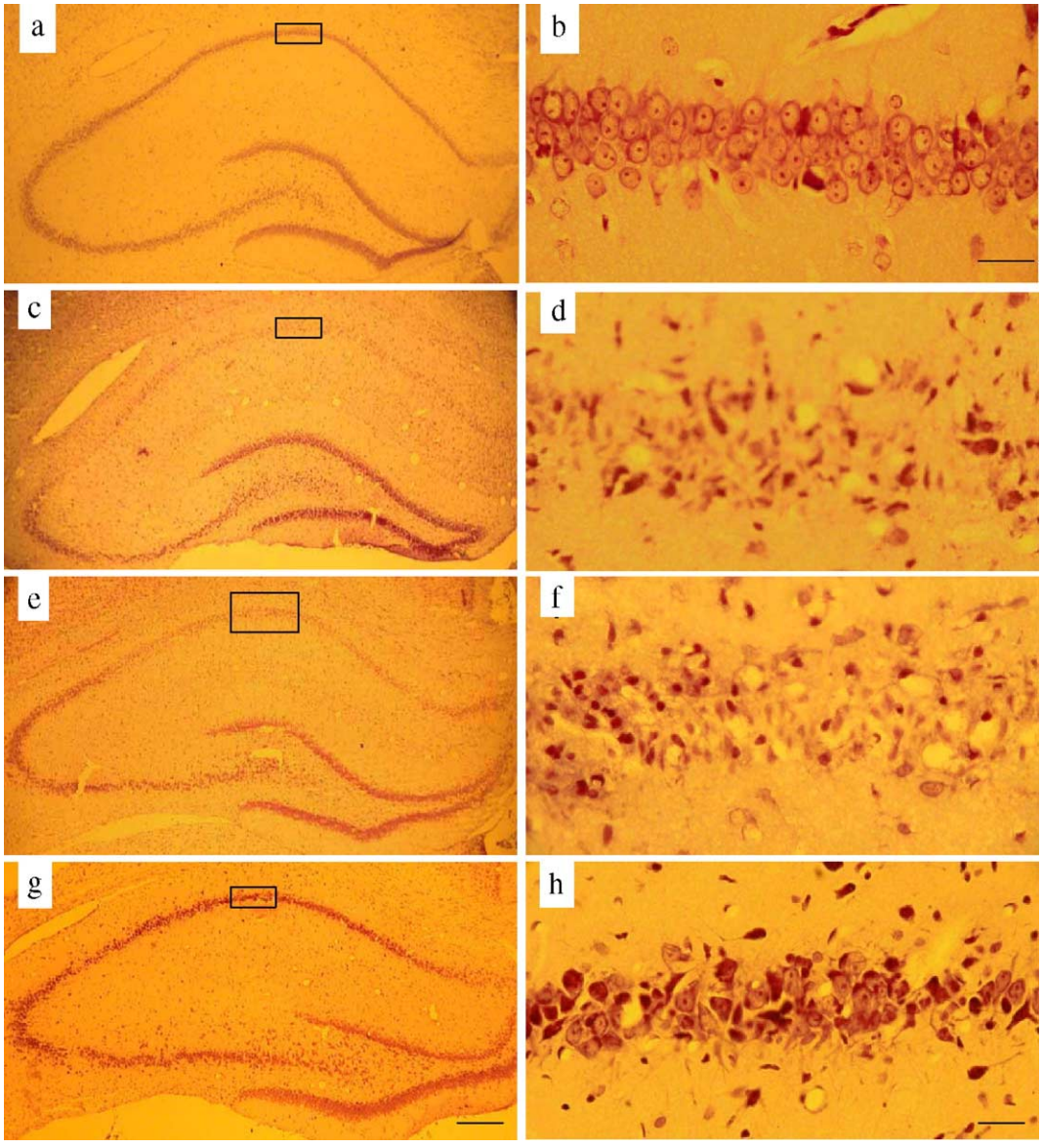

Fig. 1. Example of Cresyl violet-strained sections of the hippocampi of sham operated rats (a, c) and rats subjected to 15 min of ischemia followed by 5 days of reperfusion (c, d), and rats subjected to 15 min of ischemia followed by 5 days of reperfusion, which administration of the vehicle (e, f), 30 μg/10 μl of SP600125 20 min before ischemia (g, h). Data were obtained from seven independent animals in each experimental group and the results of a typical experiment are presented. Scale bars: (a, c, e, g) 200 μm; (b, d, f, h) 20 μm. The areas shown with high magnification on the right panel have been framed in a, c, e, g.

buffer. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE sample buffer. For immunoblotting, proteins were separated on 10% or 12.5% SDS-PAGE and were electrotransferred onto a nitrocellulose membrane (Amersham). The membrane was probed with the indicated antibodies overnight at 4 °C. To detect bound antibodies, alkaline phosphatase conjugate goat anti-rabbit IgG or goat anti-mouse IgG (Sigma) and NBT/BCIP color substrate (Promega, Madison, WI, USA) were used. The bands on the membrane were then scanned and analyzed with an image analyzer (LabWorks software, UVP, Upland, CA, USA).

2.5. Histology

Rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) under anesthesia after 5 days of ischemia/reperfusion. Brains were removed quickly and further fixed with the same fixation solution at 4 °C overnight. Post-fixed brains were embedded by paraffin, followed by preparation of coronal sections 5 μm thick using a microtome. The paraffin embedded brain sections were deparaffinized with xylene and rehydrated by ethanol at graded concentrations of 50–100% (v/v), followed by washing with water. The sections were stained with 0.1% (w/v) cresyl violet and were examined with light microscopy and the number of surviving hippocampal CA1 pyramidal cells per 1 mm length was counted as the neuronal density.

2.6. Antibodies

Mouse monoclonal anti-p-c-Jun (KM-1) and p-JNKs (G-7), rabbit polyclonal anti-Bcl-2 anti-p-Bcl-2(Ser87)and anti-c-Jun antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-JNK3 (06-749) antibody was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-JNK1/2 antibody was from Sigma.

2.7. Statistics

Values are expressed as mean ± SD from three independent rats. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range method or Newman–Keuls tests. P values < 0.05 was considered significant.

3. Results

3.1. The neuroprotective action of SP600125 against ischemic injury

Cresyl violet staining was used to examine the survival of CA1 pyramidal neurons. Normal cells showed round and pale stained nuclei. The shrunken cells with pyknotic nuclei after ischemia were regarded as dead cells. Transient brain ischemia followed by 5 days of reperfusion induced severe cell death. Administration of SP600125 (30 μg/10 μl) 20 min before ischemia significantly decreased neuronal degeneration (Fig. 1, Table 1).

3.2. Effects of SP600125 on the activation of JNKs induced by reperfusion after transient brain ischemia in hippocampal CA1

To investigate the effects of SP600125 on activation of JNKs, p-JNK1/2 and p-JNK3 were examined by immunoprecipitation and immunoblotting. As shown in Fig. 2, administration of SP600125 (30 μg/10 μl) 20 min before ischemia diminished the increases of p-JNK1/2 and p-JNK3 at 30 min and 3 days after ischemia. JNK1/2 and JNK3 were observed had two activated peaks at R 30 min and R 3 days in our previous studies [9,12]. The same dose of vehicle did not affect the increase in the activation.

3.3. Effects of SP600125 on activation and expression of c-Jun induced by reperfusion after transient brain ischemia in hippocampal CA1

To elucidate the downstream mechanisms of JNK function, the effects of SP600125 on activation and expression of c-Jun after brain ischemia was studied. As indicated by immunoblotting, phosphorylation and expression of c-Jun were rapidly increased after ischemia, which reached their peak levels at 3 h and 6 h of reperfusion, respectively. The increase of phosphorylation sustained for at least 5 days (Figs. 3A, B). As shown in Figs. 3C and D, administration of SP600125 (30 μg/10 μl) 20 min before ischemia significantly diminished the increased of p-c-Jun at 3 h after ischemia. The same dose of vehicle did not affect the increase in the activation. The protein levels of c-Jun were not affected by SP600125 or vehicle.

Table 1
Quantitative analysis of the protective effects of SP600125 against transient ischemia followed by reperfusion

Group	The numbers of neuronal profiles (mean ± SD)
Sham	203.0 ± 25.1
I/R5d	20.3 ± 7.2
Pre-DMSO I/R5d	30.2 ± 5.1
Pre-SP600125 I/R5d	103.4 ± 15.4*

* The numbers of neuronal profiles is expressed as the number of surviving neurons per 1 mm length of CA1 pyramidal cells counted under light microscopy. Data are mean ± SD ($n = 5$), *$P < 0.05$ vs. vehicle-treated group. Sham: performed using the same surgical exposure procedures except for occlusion of the carotid artery, I/R5d: the 5 day after ischemia/reperfusion, Pre-DMSO: administration DMSO 20 min before ischemia, Pre-SP600125: administration SP600125 20 min before ischemia.

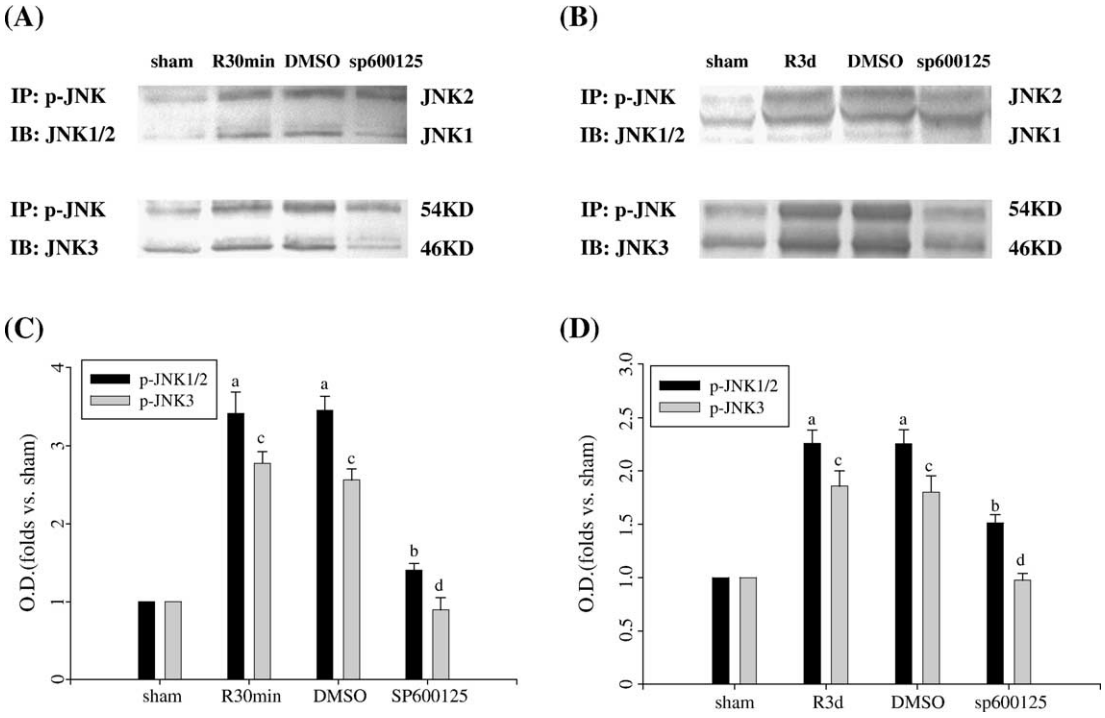

Fig. 2. Effects of SP600125 on I/R induced phosphorylation of JNK1/2, JNK3 in hippocampal CA1. (A, B) Homogenates were immunoprecipitated with p-JNKs (1, 2, 3) antibody and blotted with JNK1/2, JNK3 antibody, respectively. (C, D) Bands corresponding to JNK1/2 and JNK3 were scanned and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD (n = 3). $^{a,c}P < 0.05$ vs. respective sham, $^{b,d}P < 0.05$ vs. respective vehicle-treatment groups. I: ischemia, R: reperfusion.

3.4. Effects of SP600125 on phosphorylation and expression of Bcl-2 induced by reperfusion after transient brain ischemia in hippocampal CA1

To further elucidate the downstream mechanisms of JNK function, the effects of SP600125 on phosphorylation and expression of Bcl-2 after brain ischemia was studied. As indicated by Western blot analysis, phosphorylation of Bcl-2 was significantly increased at 3 h of reperfusion. The expression of Bcl-2 was not significantly changed after ischemia (Figs. 4A, B). As shown in Figs. 4C, D, administration of SP600125 (30 μg/10 μl) 20 min before ischemia significantly diminished the increased of p-Bcl-2 (Ser87) at 3 h after ischemia. The same dose of vehicle did not affect the increase in the activation. The protein levels of Bcl-2 were not affected by SP600125 or vehicle.

4. Discussion

The JNK MAP kinase signaling pathway is activated by stress and cytokines and has been involved in cell death and differentiation. Three JNK genes have been identified: Jnk1, Jnk2, and Jnk3. JNK1 and JNK2 are constitutively expressed in a large variety of tissues, whereas JNK3 seems primarily localized to neuronal tissues and the cardiac myocyte [23]. Mice lacking JNK1 or JNK2 exhibit deficits in T-helper (CD4) cell function [8,30,40]. Double knockout animals are embryonic lethal, although fibroblasts from these animals are viable in vitro and exhibit a remarkable resistance to radiation-induced apoptosis [36]. The JNK3 knockout mouse exhibits resistance to kainic acid-induced apoptosis in the hippocampus and to subsequent seizures [39]. Therefore, JNK activity seems critical for programmed cell death. Therapeutic inhibition of JNK may provide clinical benefit in diseases as diverse as inflammatory, stroke, Parkinson's disease, ischemic injury, and myocardial infarction. Transient global cerebral ischemia/reperfusion lead to cell death of hippocamal CA1 pyramidal neurons and activation of JNK has been reported in our previous studies [12,20]. In this study, we observed the neuroprotective action of SP600125, a potent, cell-permeable, selective, and reversible inhibitor of *c*-Jun N-terminal kinase (JNK), on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1. Our results demonstrate that SP600125 significantly increased the number of surviving hippocampal CA1 pyramidal cells and diminished the activation of JNK1/2 and JNK3 at 30 min and 3 days after ischemia.

c-Jun N-terminal kinase (JNK) is a serine threonine protein kinase that phosphorylates c-Jun (a nuclear substrate of JNK) [7,14], a component of the transcription factor activator protein-1 (AP-1) [13,15]. In complex with other DNA binding proteins, AP-1 regulates the transcription of numerous genes leading to neuronal death and excitotoxicity. Expression of a c-Jun mutant that cannot be

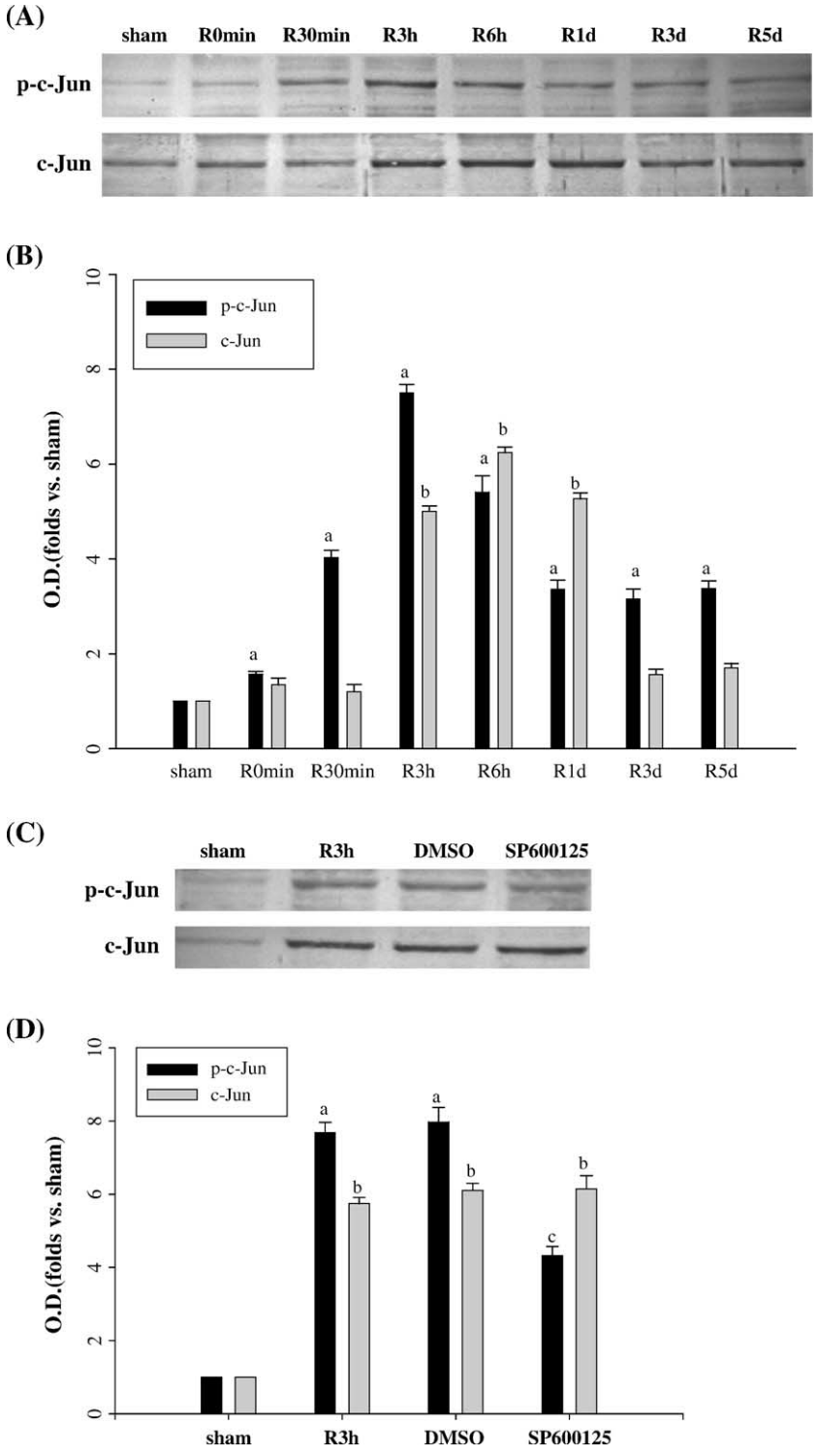

Fig. 3. Effects of SP600125 on I/R induced activation and expression of c-Jun in hippocampal CA1. (A, B) Time courses of c-Jun activation and expression in nucleus in hippocampal CA1 derived from sham-treated rats or rats at various time points after 15 min of ischemia. (C, D) Effects of SP600125 on I/R-induced expression and activation of c-Jun in hippocampal CA1. Western blot probed with antibodies to phosphorylated c-Jun (p-c-Jun, Ser63) and c-Jun. Bands corresponding to p-c-Jun and c-Jun were scanned and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD ($n = 3$). $^{a,b}P < 0.05$ vs. respective sham, $^{c}P < 0.05$ vs. vehicle-treatment group.

phosphorylated on serine 63 inhibits programmed neuronal cell death in vitro [37]. In this study, our results demonstrate that ischemia/reperfusion induced activation of c-Jun and SP600125 significantly diminished the increased of p-c-Jun at 3 h after ischemia. However, the increased expression of c-Jun was not affected by SP600125.

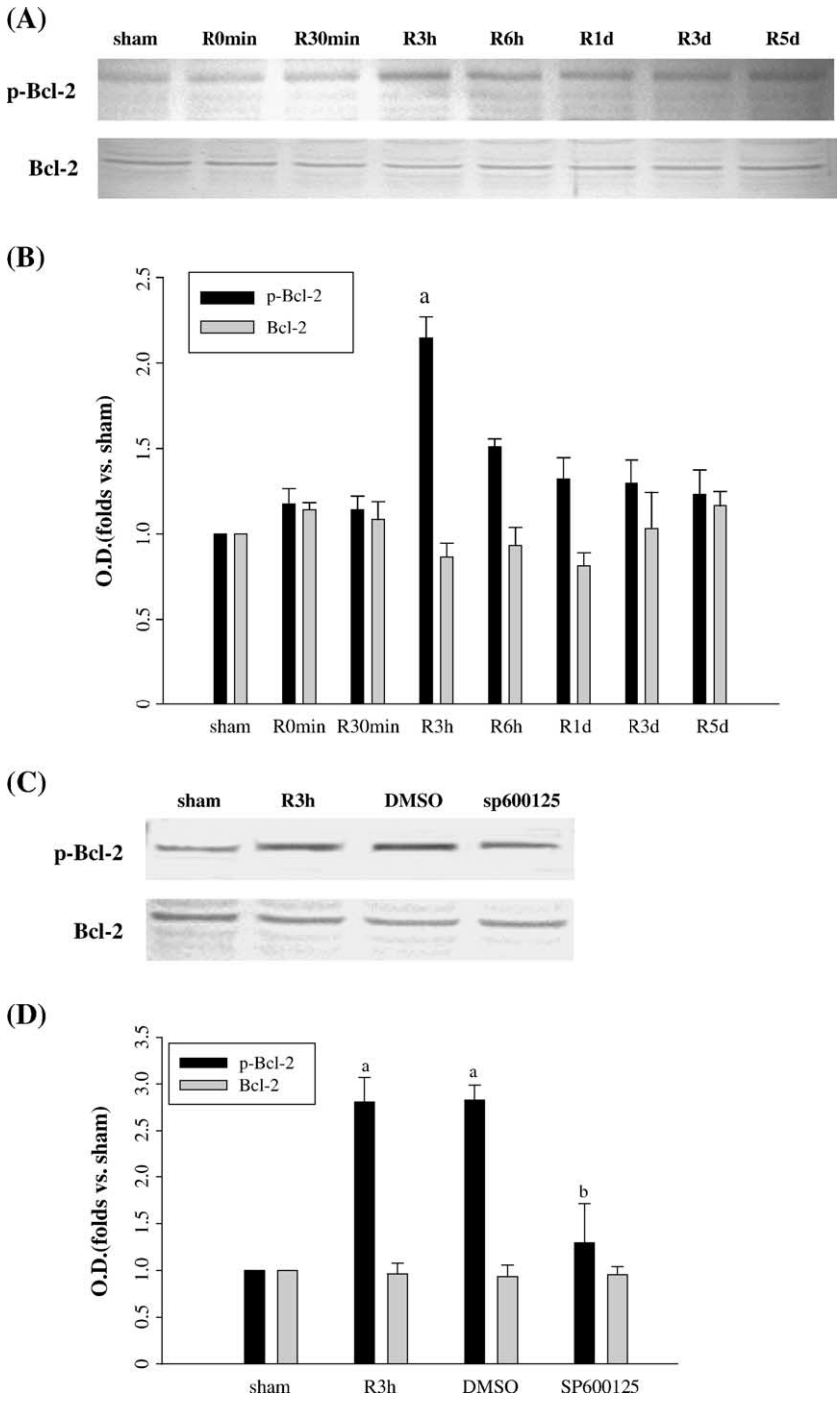

Fig. 4. Effects of SP600125 on I/R induced of Bcl-2 phosphorylation and expression in hippocampal CA1. (A, B) Time courses of Bcl-2 phosphorylation and expression in the cytosol in hippocampal CA1 derived from sham-treated rats or rats at various time points after 15 min of ischemia. (C, D) Effects of SP600125 on I/R induced of Bcl-2 phosphorylation and expression in hippocampal CA1. Western blot probed with antibodies to phosphorylated Bcl-2 (p-Bcl-2, Ser87) and Bcl-2. Bands corresponding to p-Bcl-2 and Bcl-2 were scanned and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD (n = 3). $^{a}P < 0.05$ vs. respective sham, $^{b}P < 0.05$ vs. vehicle-treatment group.

In an earlier study, Maundrell et al. [24] showed that JNK phosphorylated Bcl-2 both in vitro and in Bcl-2-overexpressing COS-7 cells that coexpress JNK and active Rac1. Based on mutagenesis and phosphopeptide sequence analysis, four sites (Thr56, Ser70, Thr74, and Ser87) were identified that are present in a putative unstructured flexible loop. Each of these sites occurs NH2-terminal to a proline residue, a well established recognition determinant for MAPKs [27]. Srivastava et al. [34] demonstrated that paclitaxel mediated phosphorylation of Bcl-2 at Ser70 in breast cancer cells was inhibited by dominant-negative JNK. A very recent study also concluded that JNK is

responsible for paclitaxel-induced Bcl-2 phosphorylation in Jurkat cells overexpressing Bcl-2 [38]. These results collectively suggest that Bcl-2 could act as a non-nuclear substrate of JNK. Bcl-2 is the first identified survival gene involved in the control of apoptosis. Uncertainty still exists whether phosphorylation increases or decreases the anti-apoptotic function of Bcl-2. Most data support the original hypothesis [10] and suggest that phosphorylation inactivates Bcl-2, thus promoting apoptosis, possibly by freeing Bax from Bcl-2/Bax dimmers [3]. However, the functional significance of the Bcl-2 phosphorylation remains highly controversial [6]. Some Bcl-2 phosphorylations have been reported to enhance the cytoprotective effects of Bcl-2, whereas others rendered the cells more susceptible to apoptosis. As recently suggested, it is likely that the role of Bcl-2 phosphorylation in promoting or inhibiting apoptosis may depend on the phosphorylation sites involved and the cellular context where this event takes place, as well as on its duration. In this study, our results demonstrate that ischemia/reperfusion-induced phosphorylation of Bcl-2 and SP600125 significantly diminished the increased of p-Bcl-2 at Ser87 at 3 h after ischemia. The results suggest that JNK could phosphorylate Bcl-2 during ischemia/reperfusion and the phosphorylation inactivates Bcl-2, thus promoting apoptosis.

In conclusion, the present study demonstrates for the first time the neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal cell death in rat hippocampal CA1 via inhibiting the activation of nuclear substrate c-Jun and the inactivation of non-nuclear substrate Bcl-2 of JNKs. These results also indicate that inhibiting JNK activity may represent a new and effective strategy to treat brain stoke.

Acknowledgment

Project supported by the Key Item National Nature Science Foundation (No. 30330190).

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