Original Articles

Involvement of cyclin dependent kinase 5 and its activator p35 in staurosporine-induced apoptosis of cortical neurons[1]

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ABSTRACT

AIM: To investigate whether cyclin-dependent kinase 5 and its regulatory protein p35 was involved in staurosporine-induced apoptosis of cortical neuronal cultures. **METHODS:** Primary cerebral cortical neurons were exposed to 300 nmol/L staurosporine. After incubation for different time, morphological alterations were observed with phase-contrast microscopy, fluorescence microscopy, and transmission electron microscopy. DNA fragmentation was detected by agarose gel electrophoresis. The protein levels of Cdk4, p53, Cdk5, and its regulatory protein p35 following staurosporine treatment were measured by Western blotting. The Cdk5 activity was assayed for histone H1 kinase activity by autoradiography. **RESULTS:** The typical morphological changes of apoptosis were observed and the nuclear DNA fragmentation showed the characteristic "ladder" pattern after the cells were treated by staurosporine. The Cdk5 protein level increased markedly at 3 h and continued to 24 h. The p35 level increased at 3 h after being exposed to staurosporine, and decreased at 12 h. The cleavage of p35 to p25 was also detected at 12 h and increased at 24 h. There was no increase in Cdk5 kinase activity despite the increased cleavage of p35. The protein level of Cdk4 protein increased at 3 h and then decreased gradually from 6 h, but it was still higher than that in the vehicle cultures at 12 h. The p53 level decreased obviously at 3 h after staurosporine treatment and then seemed to increase at 12 h, but remained lower than that of vehicle cultures. **CONCLUSION:** Staurosporine-induced increase in Cdk5 protein levels and the cleavage of p35 to p25 may contribute to neuronal apoptosis.

INTRODUCTION

Neuronal apoptosis occurs during development of the central nervous system (CNS) as well as in pathological situations such as acute injury and progressive degenerative diseases. In order to develop rational, efficacious, and safe therapy against central nervous system disorders, it becomes vital to elucidate the cellular and molecular mechanisms of neuronal apoptosis. Recent evidence suggests that apoptosis in post-mitotic neurons involves an aborted attempt of cells to re-enter the cell cycle and it is characterized by increased expression of cell cycle proteins, such as cyclins or cyclin-dependent kinases (CDKs), prior to death[1]. In dividing cells, CDKs regulate proliferation, differentiation, senescence, and apoptosis. In contrast, all CDKs in post-mitotic neurons, with the notable exception of Cdk5, are silenced. Cdk5 and cycling CDKs may have little in common in the healthy CNS. Cdk5 is activated

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by the specific regulatory proteins p35, p39 rather than cyclins, and not involved in the progression of the cell cycle[2]. Cdk5 plays a critical role in neuronal migration, synaptogenesis and regulation of synaptic transmission[3]. Deregulation of Cdk5 has been linked to the pathology of neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis[3,4]. Recently, there is evidence to suggest that Cdk5 may facilitate the progression of apoptosis[5,6]. However, the mechanisms involved have not been elucidated.

We have investigated the expression of Cdk5 and its activator p35 in staurosporine-induced apoptosis of NG108-15 cells. Staurosporine (STS) is a potent and non-specific inhibitor of protein kinases. There is also evidence of STS being a potent inducer of apoptosis. Our data indicates that Cdk5 protein level do not have obvious changes after the NG108-15 cells were exposed to STS. We also did not see any significant conversion of p35 to p25[7]. To investigate whether Cdk5 was involved in STS-induced apoptosis, we looked at the protein level of Cdk5 and Cdk4 in cortical neuronal cultures treated with STS to produce apoptotic death.

MATERIALS AND METHODS

Materials Poly-*L*-lysine was purchased from Sigma Chemical Co. Fetal bovine serum (FBS), heat-inactivated horse serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco/BRL. STS (Sigma) was dissolved in dimethyl sulfoxide to make a stock solution. Primary antibodies used for immunohistochemistry were monoclonal mouse anti-neurofilaments (NF68 & 200 kDa, MS-359-R7) and polyclonal rabbit anti-GFAP (glial fibrillate acid protein, ZA-0117). Primary antibodies used for Western blots included mouse monoclonal antibodies to human β-actin (C-2, sc-8432), p53 (Pab 240, sc-99), and rabbit polyclonal antibodies to human Cdk4 (C-22, sc-260) and p35(C-19, sc-820) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody to human Cdk5 (Ab-2) was purchased from Calbiochem, Cdk5 polyclonal antibody (C-8) was purchased from Santa Cruz. An enhanced chemiluminescent detection system (ECL kit) from Amersham was used for immunodetection. All other reagents were of analytical reagent quality.

Cell culture Mixed cortical cell cultures, containing both neuronal and glial elements were prepared from 16-day-old embryos of Kunming mice. In brief, following dissociation in 0.25 % trypsin, cerebrocortical cells were plated at a density of 5×10^5 cell / ml in DMEM supplemented with 10 % FBS, 2 mmol/L *L*-glutamine in Falcon 35-mm dishes, or 24-well culture plates, which were previously coated with 12.5 mg/L poly-*L*-lysine. Half-hour after plating, the medium that contained non-attached cells and debris was aspirated and replaced with fresh medium containing 10 % FBS. After 3 d *in vitro*, non-neuronal cell division was halted by 2 d to 40 mmol/L cytosine arabinoside (Ara-C), and the cells were then shifted into a maintenance medium identical to the plating media, but 5 % heat-inactivated horse serum substitute was used for fetal serum. Cells were treated for experiment at seven days in culture.

Immunocytochemistry Neurons were stained with an antibody against NF (68 & 200 kDa) and astrocytes were stained with an antibody against GFAP. Cells were rinsed with PBS for three times and fixed in pre-chilled acetone for 12 min, then exposed to 0.3 % H_2O_2 for 10 min to block endogenous peroxidase activity. After washing with PBS, the samples were incubated with different primary antibodies for 1 h at 37 ºC, and then incubated with secondary antibodies followed by SABC reagent each for 20 min at room temperature. After visualization with diaminobenzidine (DAB), the samples were washed with tap water and dehydrated. Positive reaction was seen as brown staining. In three-day-old cultures, GFAP positive astrocytes were about 30 percent and NF positive neurons were 70 percent. If the cells were exposed to Ara-C to inhibit astrocytic growth, GFAP positive astrocytes were less than 5 percent and NF positive neurons were more than 95 percent in seven-day-old cultures.

Hoechst 33258 staining After treatment with STS for 12 h, the cells were harvested and fixed for 30 min in pre-chilled phosphate buffered saline (PBS) containing 4 % paraformaldehyde with gentle agitation. After fixation at room temperature, the cells were washed with pre-chilled PBS for three times and then exposed to 2.5 mg/L Hoechst 33258 in PBS for 5 min at room temperature. After washing, all samples were analysed under a fluorescence microscope.

Transmission electron microscopy Analysis of transmission electron microscopy was made as described previously[7].

Assay of DNA fragmentation After treatment with vehicle or STS, the cells were scraped from the dishes using a rubber policeman and DNA were iso-

lated according to the literature[8]. DNA fragments thus obtained were electrophoretically separated on a 1 % agarose gel for 30 min at 100 V. The gel was stained with ethidium bromide and photographed under UV transillumination.

Western blot analysis Western blots were prepared following a protocol described previously, with little modification[7].

Immunoprecipitation and kinase assay An *in vitro* Cdk5-associated histone H1 kinase activity (Cdk5 kinase activity) assay was carried out as described by Kerokoski *et al*[9]. Immunoprecipitation of active Cdk5 from primary cortical cells was performed using polyclonal anti-Cdk5 (C-8, 5 ml per sample). The antibody was preincubated with protein G-sepharose 4B (GIBCO BRL). The immuno-complexes were used to determine the kinase activity at 30 ºC for 30 min in a final volume of 50 μL containing a kinase buffer (30 mmol/L MOPS, 10 mmol/L $MgCl_2$, pH 7.4), 2 mg of histone H1 peptide (P^9KTPKKAKKL^{18}) and 5 μCi of [γ-^{32}P]dATP. The reaction was terminated by the addition of SDS-PAGE sample buffer, followed by 5 min of boiling. The samples were separated by 12 % SDS-PAGE, the gel was autoradiographed with X-ray film, and the bands were quantified by a Bio-Rad model GS-670 Imaging Densitometer.

Statistical analysis Data are expressed as mean±SD. Statistical significance was assessed with one-way ANOVA followed by Duncan's multiple-range test. $P<0.05$ was considered significant.

RESULTS

STS-induced apoptotic morphological alternations in cortical neuronal cultures In primary cerebral cortical neurons, STS induced significant cell injury as indicated by MTT assay (Tab 1). Visual inspection using phase-contrast microscopy indicated that control neurons were round and smooth and the dendritic trees were long and highly branched (Fig 1A). STS exposure (300 nmol/L for 12 h) caused significant changes in cellular morphology. The dendritic processes granulated and reduced in number and length and the complete dendritic network disappeared. Cell bodies showed an obvious shrinkage and a few of cells were lysed (Fig 1B). Nuclei in control cortical neurons exhibited diffused Hoechst 33258 staining of the chromatin (Fig 1C). On the contrary, nuclei in cortical neurons treated by STS were obviously shrunken with condensed chromatin (Fig 1D). TEM analysis (Fig 2) displayed that non-treated cortical neurons showed evenly dispersed euchromatin, polyribosomal rosettes, and mitochondria. STS-induced cell death was characterized by chromatin condensation, granular cytoplasmic inclusions, cytoplasmic vacuoles and dilation of the rough endoplasmic reticulum and mitochondria. Fig 3 indicated that DNA isolated from the cortical neurons showed the characteristic "ladder" pattern after 6 h of 300 nmol/L STS treatment and was sustained until 24 h. A comparison with molecular weight markers displayed that the fragments were multiples of approximately 180-200 bp. These biochemical and morphological changes are classical manifestations of apoptosis.

Tab 1. Effects of STS on the cortical neurons by colormetric MTT assay. *n*=3 experiments. Mean±SD. [a]*P*>0.05, [b]*P*< 0.05, [c]*P*<0.01 *vs* control group.

Groups	Dose/nmol·L^{-1}	$A_{570\ nm}$ 24 h	48 h
Control		1.048±0.9	0.98±0.13
STS	50	0.94±0.22[a]	0.81±0.05[a]
	100	0.88±0.11[a]	0.67±0.06[b]
	300	0.44±0.07[c]	0.24±0.06[c]
	500	0.43±0.11[c]	0.15±0.05[c]

Changes of protein levels of p35, Cdk5, and Cdk5 kinase activity We formerly examined the protein levels of Cdk5 and its activator p35 in STS-induced apoptosis of NG108-15 cells and obtained negative results. To explore whether Cdk5 was involved in STS-induced apoptosis of cortical neurons, the protein levels of Cdk5 and p35 were investigated. The time course of changes in Cdk5 protein level after exposure to 300 nmol/L STS was shown in Fig 4. The Cdk5 protein level increased markedly at 3 h and continued to 24 h. The p35 level increased at 3 h after being exposed to STS, and decreased at 12 h. Because the antibody against p35 (C-19, sc-820) used in this study is raised against a peptide mapping at the carboxyl terminus of p35, it can also react with p25 protein, the cleaved fragment of p35 protein which is composed of the C-terminus 280 amino acid residues of p35, the appearance of p25 signal as shown in Fig 4 suggested the conversion of p35 to p25. Protein level of p25 was detected at 12 h and increased at 24 h. There was no increase in Cdk5 kinase activity despite the increased cleavage of

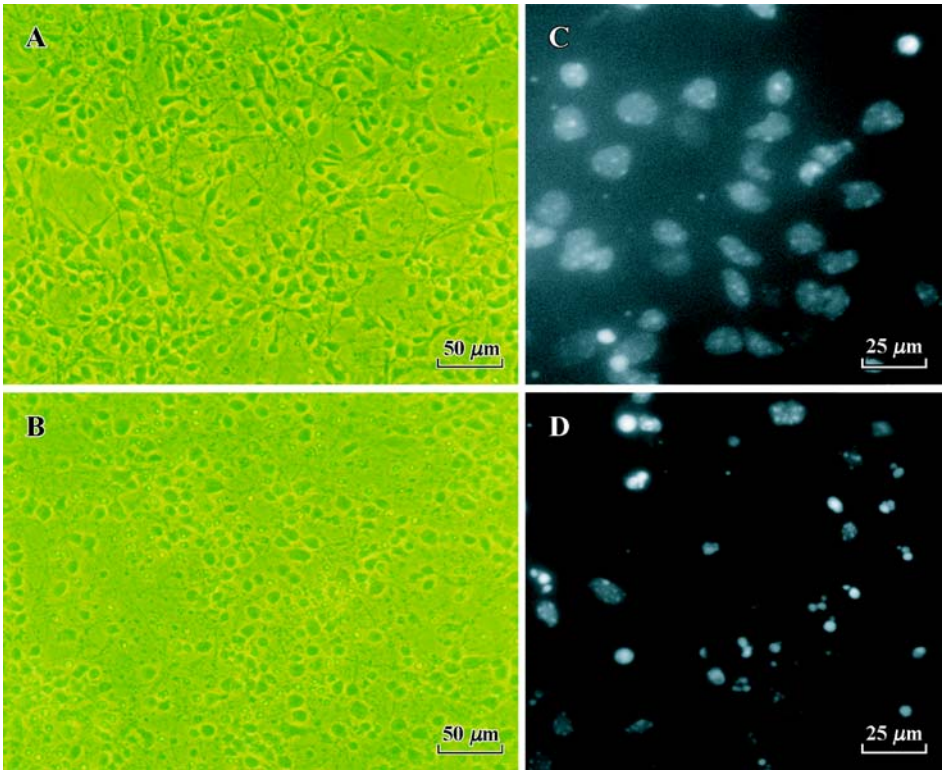

Fig 1. Cerebral cortical neurons were treated with vehicle (A, C) or 300 nmol/L STS (B, D) for 12 h. Nuclei were stained with Hoechst 33258. Morphological changes were observed by phase-contrast microscopy (×200) and fluorescence microscopy (×400). Numerous cells treated with STS showed apoptotic features.

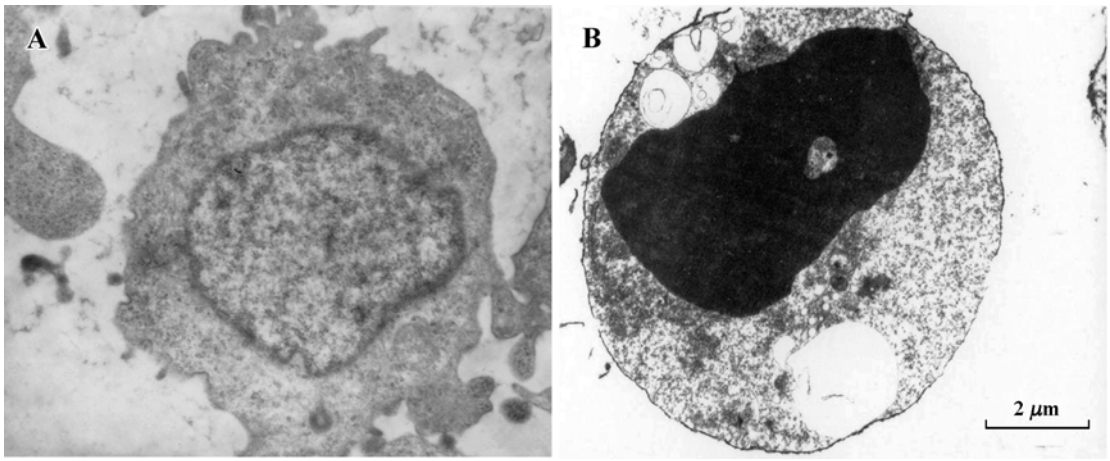

Fig 2. TEM analysis of morphological characteristics of cortical neuronal cultures following 300 nmol/L STS treatment. A: Non-treated intact control showing evenly dispersed euchromatin, polyribosomal rosettes, and mitochondria. B: At 12 h post-treatment, neurons were characterized by chromatin condensation, granular cytoplasmic inclusions, and cytoplasmic vacuoles. ×8000.

p35 (Fig 4).

Effect of STS on protein levels of Cdk4 and p53 Treatment with STS significantly increased the level of Cdk4 protein at 3 h and then decreased Cdk4 level gradually from 6 h, but Cdk4 level was still higher than that in the vehicle cultures at 12 h. It returned to normal expression level at 24 h (Fig 5A). The p53 level decreased obviously at 3 h after STS treatment and then seemed to increase at 12 h, but remained lower than that of vehicle cultures (Fig 5B).

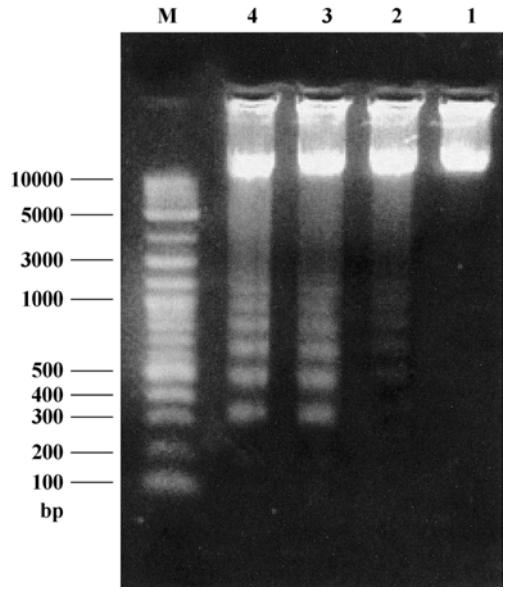

Fig 3. Agarose gel electrophoresis of cellular DNA following exposure of cortical neurons to STS. Bands were visualized by ethidium bromide. Lane 1, STS exposure for 3 h; Lane 2, STS exposure for 6 h; Lane 3, STS exposure for 12 h; Lane 4, STS exposure for 24 h; M, DNA size markers.

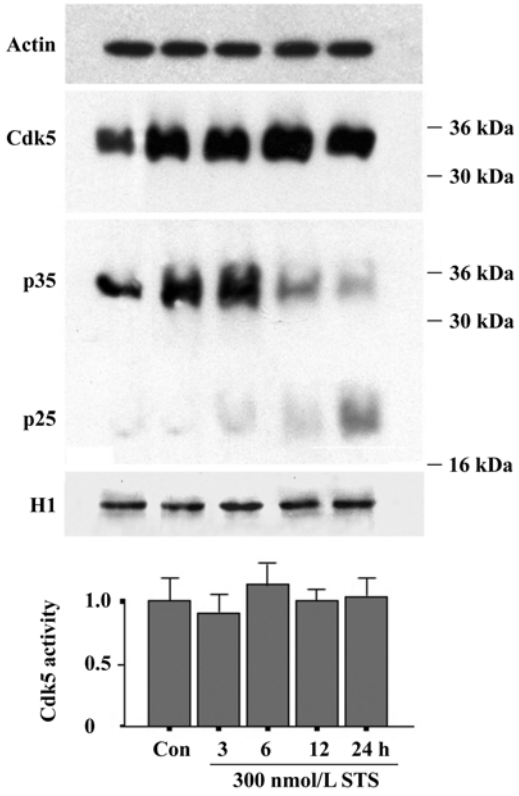

Fig 4. The protein levels of Cdk5 and p35 as well as Cdk5 activity in cortical neuronal cultures 3-24 h following 300 nmol/L STS treatment. The protein levels were measured by Western blotting, and an antibody against β-actin was used as a loading control. p35 and p25 were detected in the same Western blot but longer exposure time was needed to visualize the p25 band. The Cdk5 activity was assayed for histone H1 kinase activity as described under Materials and Methods. Each value was measured by densitometric analysis based on the density of the band, and Cdk5 activity in the vehicle control=1.0. The data were representative of two separate experiments.

DISCUSSION

Although Cdk5 was originally cloned by homology to other members of the CDK family, there were fascinating differences between Cdk5 and other CDKs. It is expressed predominately in mature neurons and is implicated in neurite extension, neuronal migration, and neuronal differentiation. More recently, increasing evidence suggested a role for Cdk5 in cellular apoptosis. However, the mechanisms involved are largely unknown. Previous studies on the protein levels of Cdk5 during apoptosis in neuronal cell cultures have resulted in conflicting data showing either increased, decreased, or unaltered Cdk5 levels[9-11]. Some findings have led to the speculation that the effects of some neurotoxic insults might be mediated by the Cdk5. Inhibition of Cdk5 activity by either pharmacological inhibitors or antisense oligonucleotides promotes survival of neurons challenged by fibrillary beta-amyloid peptide (Aβ)[12,13]. However, some studies showed that Cdk5 prevented neuronal apoptosis. Moreover, Cdk5-deficient cultured cortical neurons exhibit increased sensitivity to apoptotic stimuli[14]. These data suggest that the protein level and activity of Cdk5 are regulated in a complex manner which depends on cell type and context of cell death.

The kinase activity of Cdk5 is tightly regulated by p35 and p39 in brain development and survival. Apart from the developmental function of Cdk5/p35, it has recently been demonstrated that various neurotoxic insults induce the generation of p25 from p35, and the binding of p25 to Cdk5 can constitutively activate Cdk5[12,15]. Our present study showed that the Cdk5 protein level increased markedly at 3 h and continued to 24 h in cortical neuronal cultures treated with STS. In order to identify the role of Cdk5 activators, we examined the level of p35 regulatory proteins of Cdk5. The changes in p35 protein level suggested the conversion of p35 to p25. There was no evidence of increased activity of Cdk5 despite the increased cleavage of p35. Taken together, it might be possible that the cleavage of p35 does not affect Cdk5 activity, rather, it alters the properties of cdk5, results in the release of active Cdk5

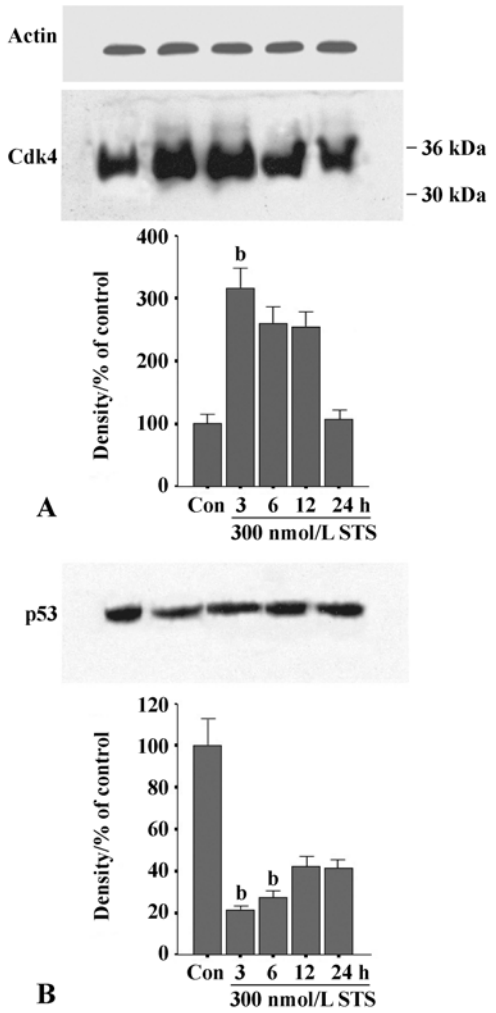

Fig 5. The protein levels of Cdk4 and p53 in cortical neuronal cultures 3-24 h following 300 nmol/L STS treatment. An antibody against β-actin was used as a loading control. Each value was measured by densitometric analysis of the Western blotting based on the density of the band in the vehicle control as 100 %. The data is representative of two separate experiments. $^{b}P<0.05$ *vs* control group.

from the particulate fraction to the free soluble form which is harmful for postmitotic neurons, and eventually causes cytoskeletal disruption and cell death. Recently, Lee *et al* showed that Aβ(1-42) caused conversion of p35 to p25, and the p25/cdk5 kinase induced cell death in cultured primary cortical neurons. Cdk5 inhibitor butyrolactone 10 mmol/L significantly inhibited neuronal cell death caused by Aβ(1-42)[12]. These results indicated that active Cdk5 was involved in Aβ-induced neuronal cell death. Our data also support the view that Cdk5 and its regulation may play a role in the execution of cell death induced by STS in cultured cortical neurons.

Recent evidence suggests a link between the aberrant re-expression of cell cycle proteins in the process of apoptotic neurodegeneration. Cyclins or CDKs, are re-expressed in neurons committed to death in response to a variety of insults, including excitotoxins, hypoxia and ischemia, loss of trophic support, or Aβ[1]. Our results indicate that the level of Cdk4 protein increased in the early phase of STS treatment. Then Cdk4 protein level decreased gradually from 6 h, but it was still higher than that in the vehicle cultures at 12 h. It needs further study if Cdk4 also contributes to STS-induced neuronal apoptosis.

Some study showed that the levels of p53 and Cdk5 were concomitantly increased during PC12 cell apoptosis induced by NGF withdrawal[16]. In contrast to it, many data support that cytokine withdrawal-induced apoptosis is p53-independent. As we know, post-translational modifications of p53 play an essential role in both stabilization and activation of the protein. Phosphorylation of Ser20[17], Thr81[18] can lead to p53 stabilization, and Ser46 seems to be related to UV-induced apoptosis[19]. There is also evidence that C-terminal phosphorylation of p53 may negatively regulate its function[20]. Recently, evidence is provided that p53 is a substrate of Cdk5. Cdk5/p25 can phosphorylate recombinant p53 *in vitro*, but the sites are still unknown[16]. It is uncertain yet if the phosphorylation of p53 by Cdk5/p25 is involved in apoptosis. Our experiment suggests that STS-induced neuronal cultures die via a pathway that is independent of p53. It is consistent with previous research[21].

In conclusion, our data clearly document alterations in the protein levels of Cdk4, p53, Cdk5, and its regulator p35 following STS treatment. Our current findings imply that STS-induced increase in Cdk5 protein level and the cleavage of p35 to p25 may contribute to neuronal apoptosis. Further study regarding the regulation of Cdk5 will provide valuable insight toward the understanding of neuronal apoptosis.

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