Staurosporine induces apoptosis in NG108-15 cells[1]

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ABSTRACT

AIM: To observe if staurosporine induced apoptosis in NG108-15 cells and its effect on protein expression level of several genes related to apoptosis. **METHODS:** Phase-contrast microscopy, fluorescence microscopy, and transmission electron microscopy were used to observe staurosporine-induced morphological changes. Agarose gel electrophoresis was used to detect DNA fragmentation. Western blots were used to measure protein expression level of several genes related to apoptosis. **RESULTS:** Cells treated with staurosporine 0.1 μmol/L showed typical morphological changes of apoptosis. A "ladder" pattern representing fragmentation of DNA into oligonucleosome length fragments was observed after 6 h of staurosporine treatment and sustained until 24 h. The Bax expression increased significantly at 6 h after exposure to staurosporine, peaked at 12 h compared with vehicle cultures, and decreased at 24 h. The Bcl-2 expression increased and reached the highest level at 3 h. It was then decreased gradually but still higher than normal expression level. There was an obvious caspase-3 cleavage at 6 h after exposing the cells to staurosporine. Treatment with staurosporine for 12 h markedly decreased the expression of p53 protein. Cdk5 protein expression did not have obvious changes after the cells were exposed to staurosporine. **CONCLUSION:** Staurosporine induced apoptotic death in NG108-15 cells. Cells might die via a pathway that is dependent on Bax expression but independent of p53, and caspase-3 cleavage was involved.

INTRODUCTION

There are two basic pathways or mechanisms of cell death: necrosis and apoptosis. Necrosis is characterized by swelling of the cytoplasm and mitochondria, rapid loss of cellular membrane integrity, followed by swelling and rupture of entire cell. In contrast, apoptosis, programmed cell death, is characterized morphologically and biochemically by cellular shrinkage, membrane blebbing, chromatin condensation and cellular DNA fragmentation. Neuronal death is normal during nervous system development but is abnormal in brain and spinal cord disease and injury. The re-emergence of apoptosis may contribute to the neuronal degeneration in chronic neurodegenerative disease, such as amyotrophic lateral sclerosis and Alzheimer's disease, and in neurological injury such as cerebral ischemia and trauma[1]. Therefore, understanding how neurons undergo apoptosis is important from both a development as well as a clinical perspective for developing rational therapies that may prevent neuronal death after injury or disease.

Staurosporine (STS), an indolo (2,3-alpha) carbazole, was initially discovered in the course of screening extracts of the bacterium *Streptomyces* species for constituent alkaloids with protein kinase C-

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inhibitory activity[2]. It has been shown to inhibit many different protein kinases and used extensively to induce apoptosis in a variety of cells. These include tumor cell lines, lymphocytes, neurons, and other primary cells. Despite its widespread use to study apoptosis, the mechanism by which STS induces cell death is largely unknown. Furthermore, a wide range of STS concentrations (100 nmol/L to 100 μmol/L) have been used to induce cell death in different cell types[3]. Therefore, the mechanism of STS-induced death may vary according to the cell type and the STS concentration used. The present study observed if STS induced apoptosis in NG108-15 cells and examined protein expression level of several genes related to apoptosis.

MATERIALS AND METHODS

Materials STS was purchased from Sigma Chemical Co and was dissolved as 1000×stock solutions in dimethyl sulfoxide. Chromatin dye bisbenzimide (Hoechst 33342) and Poly-*L*-lysine were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco/BRL. Primary antibodies included mouse monoclonal antibodies to human Bcl-2 (C-2, sc-7382), caspase-3 (E-8, sc-7272) and p53 (Pab 240, sc-99) and rabbit polyclonal antibodies to human Bax (P-19, sc-526) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody to human Cdk5 (Ab-2) was purchased from Calbiochem. An enhanced Chemiluminescent Detection System (ECL kit) from Amersham was used for immunodetection. All other reagents were of analytical reagent quality.

Cell culture The cell line NG108-15 (mouse neuroblastoma×rat glioma hybrid cell) obtained from ATCC (American Type Culture Collection) was used in the current experiments. The cells were cultured in DMEM supplemented with 10 % (v/v) FBS on Poly-*L*-lysine-coated dishes. Cultures were maintained at 37 °C, with an atmosphere of 5 % CO_2 and saturated humidity. All growth media contained streptomycin 100 mg/L, penicillin 100 kU/L. The cells were treated with STS 0.1 μmol/L for different time. Then biochemical and morphological assessments were made.

Hoechst 33342 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 % paraformaldyhyde with gentle agitation. After fixation at room temperature, the cells were washed with pre-chilled PBS for three times and then exposed to Hoechst 33342 2 mg/L in PBS at room temperature for 5 min. After washing, all samples were analyzed with a fluorescence microscope (Axiophot, Germany).

Transmission electron microscopy[4] Cultures were fixed at 12 and 24 h post-treatment of STS with 2.5 % glutaraldehyde, postfixed with 1 % osmium tetroxide, and then stained *en bloc* with 2 % aqueous uranyl acetate. Fixed cultures were then dehydrated. Silver-gold ultrathin sections were collected parallel to the culture monolayer and mounted on Formvar-coated carbon stabilized 1 mm×2 mm slot grids. Samples were counterstained with lead citrate and viewed with a Hitachi H 600 transmission electron microscope at 40-80 kV.

Assay of DNA fragmentation After treatment with vehicle or STS, the cells were scraped from the dishes using a rubber policeman and DNA were isolated according to the literature[5]. DNA fragments thus obtained were electrophoretically separated on a 1 % agarose gel at 100 V for 30 min. The gel was stained with ethidium bromide and photographed under UV transillumination.

Western blot analysis[6] Cell samples were harvested and added different volume lysis buffer. The sample was lysised on ice for 10 min and then was centrifuged at 10 000×*g* for 15 min. The resulting supernatants were removed and stored at -80 °C. Aliquots were taken for protein determinations using the Bio-Rad protein assay dye reagent. Cell extracts containing equivalent amount of protein were boiled in sample buffer for 5 min. Proteins (10 μg per lane) of the various supernatants were separated by SDS-polyacrylamide gel electrophoresis before being transferred to a polyvinylidene difluoride membrane in a trans-blotting buffer [Tris 20 mmol/L, glycine 200 mmol/L, 20 % (v/v) methanol]. Membranes were incubated with different antibodies (2 mg/L) in a blocking buffer containing 5 % BSA for 1 h followed by incubation with peroxidase-conjugated anti-mice IgG or anti-rabbit IgG in a blocking buffer containing 5 % skimmed milk at room temperature for 1 h. A signal was detected by ECL kit. Western blots were quantified by a Bio-Rad model GS-670 Imaging Densitometer.

Statistical analysis Data represent the means of at least three independent experiments. Statistical significance of results was assessed by one-way ANOVA followed by Duncan's multiple-range test. $P<0.05$ is considered significant.

RESULTS

STS-induced apoptotic death in NG108-15 cells In NG108-15 cells, exposure to STS resulted in a significant inhibition of MTT reduction in a time- and dose-dependent way (Tab 1). Visual inspection using phase-contrast microscopy indicated that normal NG108-15 cells were flat polygons grown in a monolayer, forming pseudopodia of various shapes (Fig 1A). After treatment with STS 0.1 μmol/L for 12 h, most cells lost neurites and their polyhedric shapes and became round in shape. Cell bodies showed obvious shrinkage and a few of cells were lysed or replaced by debris (Fig 1B). To test if apoptosis is involved, DNA-sensitive dye Hoechst 33342 staining and DNA fragmentation assay by agarose gel electrophoresis were used. The Hoechst 33342 staining was used to assess changes in nuclear morphology following STS treatments. As shown in Fig 1C, nuclei in normal NG108-15 cells were larger and exhibited diffused Hoechst 33342 staining of the chromatin. In contrast, nuclei in NG108-15 cells treated with STS (0.1 μmol/L for 12 h) were severally shrunken and compacted with condensed chromatin (Fig 1D). TEM analysis of untreated NG108-15 cells illustrated an organelle-rich electron-lucent cytoplasm with prominent granular endoplasmic reticulum, Golgi apparatus, mitochondria and polyribosomal rosettes. Nuclei morphology was characterized by dispersed euchromatin dominating the nucleoplasm in conjunction with promi-

Tab 1. Effects of staurosporine (STS) on the NG108-15 cells by colorimetric MTT assay. *n*=3 experiments. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* control group.

Concentration/nmol·L^{-1}		$A_{570\ nm}$ 24 h	48 h
Control		1.12±0.17	1.07±0.11
STS	10	0.81±0.04[b]	0.73±0.05[b]
	50	0.68±0.04[c]	0.55±0.03[c]
	100	0.40±0.06[c]	0.15±0.02[c]
	300	0.22±0.05[c]	0.09±0.01[c]

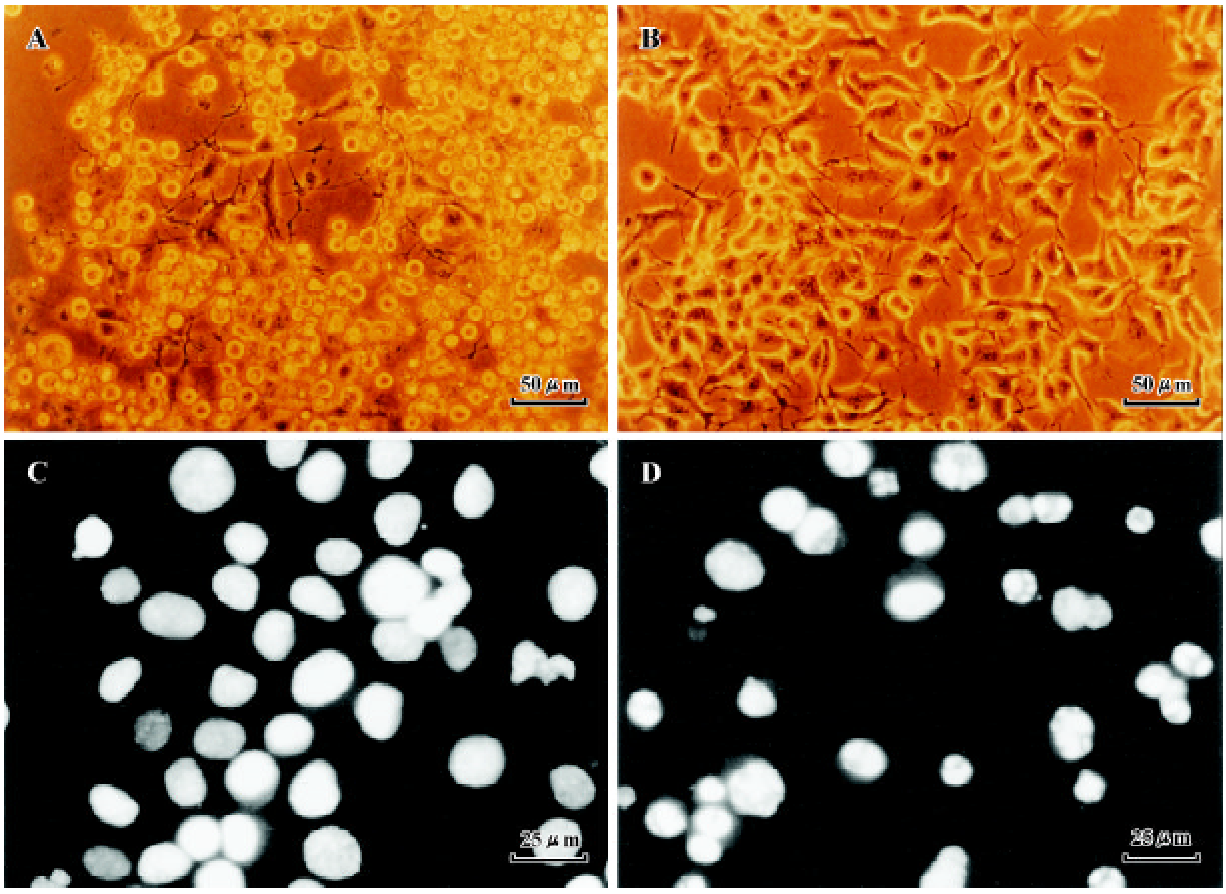

Fig 1. Morphological changes were observed in NG108-15 cells by phase-contrast microscopy (×200) and fluorescence microscopy (×400). A, C: untreated cells; B, D: cells treated with STS 0.1 μmol/L for 12 h.

nent nucleoli (Fig 2A). STS-treated NG108-15 cells revealed prominent chromatin condensation, nuclear pyknosis and fragmentation. Cytoplasmic alterations included disappearance of Golgi apparatus, dilation of the rough endoplasmic reticulum and mitochondria (Fig 2B). In addition, blebbing of the plasmalemma, a hallmark of apoptosis, was apparent (Fig 2B). As shown in Fig 3, a "ladder" pattern representing fragmentation of DNA into oligonucleosome length fragments was observed after 6 h of STS treatment and was sustained until 24 h. These biochemical and morphological changes are characteristics of apoptosis.

Modulations of Bax protein expression and caspase-3 cleavage are involved The Bcl-2 family of proteins, among which death agonist Bax and antagonist Bcl-2 are of particular significance, regulates the cellular commitment to apoptosis. Bax generally functions as a cell death agonist, and elevated levels of Bax have been shown to promote apoptosis in response to numerous cell death-inducing stimuli. In contrast, Bcl-2 has been shown to prevent both apoptotic and necrotic cell death induced by a variety of stimuli in several different systems. However, it is not clear whether Bax or Bcl-2 expression contributes to STS-induced neuronal cell apoptosis. To address this issue, we examined the correlation between STS-induced cell death and Bax and Bcl-2 expression in NG108-15 cells. The time course of changes in Bax protein expression after exposure to STS 0.1 μmol/L is shown in Fig 4A. The Bax expression increased significantly at 6 h after being exposed to STS, peaked at 12 h compared with vehicle cultures, and decreased at 24 h. The maximal increase of Bax protein expression is approximately 20-fold, which occurred in cultures exposed to STS 0.1 μmol/L for 6 to 12 h compared with vehicle-treated cultures. The Bcl-2 expression increased and reached the highest level at 3 h. It was then decreased gradually but still higher than normal expression level (Fig 4B).

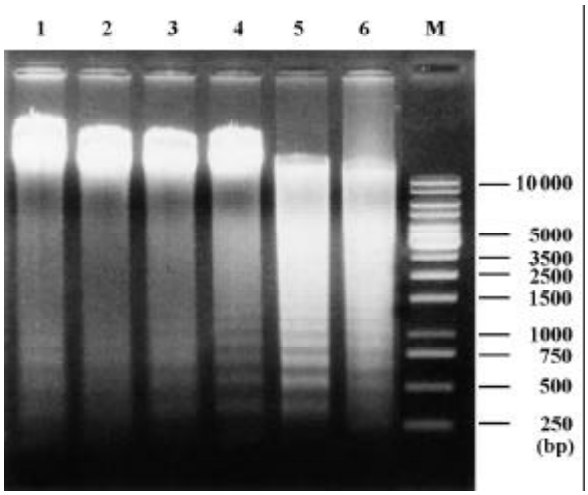

Fig 3. Agarose gel electrophoresis of DNA fragmentation. Lane 1, No treatment; Lane 2, STS exposure for 1 h; Lane 3, 3 h; Lane 4, 6 h; Lane 5, 12 h; Lane 6, 24 h; M, DNA marker.

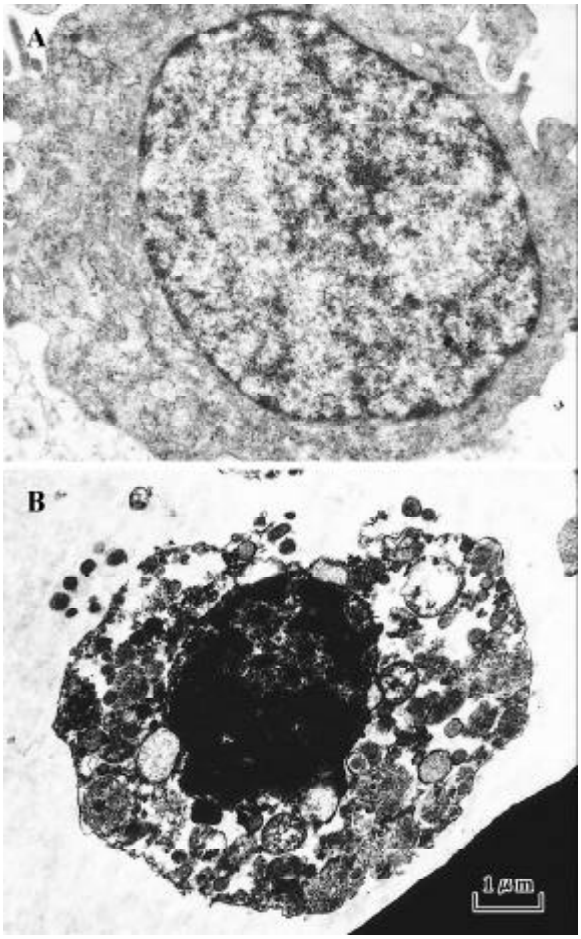

Fig 2. Morphological changes were observed in NG108-15 cells by transmission electron microscopy. A: untreated cells; B: cells treated with STS 0.1 μmol/L for 12 h. ×10 000.

Among the biochemical events involved in apoptosis, activation of members of the caspase family of cysteine proteases is believed to play a partially important role in coordinating the cell death process. Caspases cleave several different protein substrates that might play important roles in the execution of apoptosis[7]. In the present study, there was an obvious caspase-3 cleavage at 6 h after exposing the cells to STS (Fig 4C).

Effect of STS on p53 and Cdk5 protein expression level p53 was known as the tumour suppressor

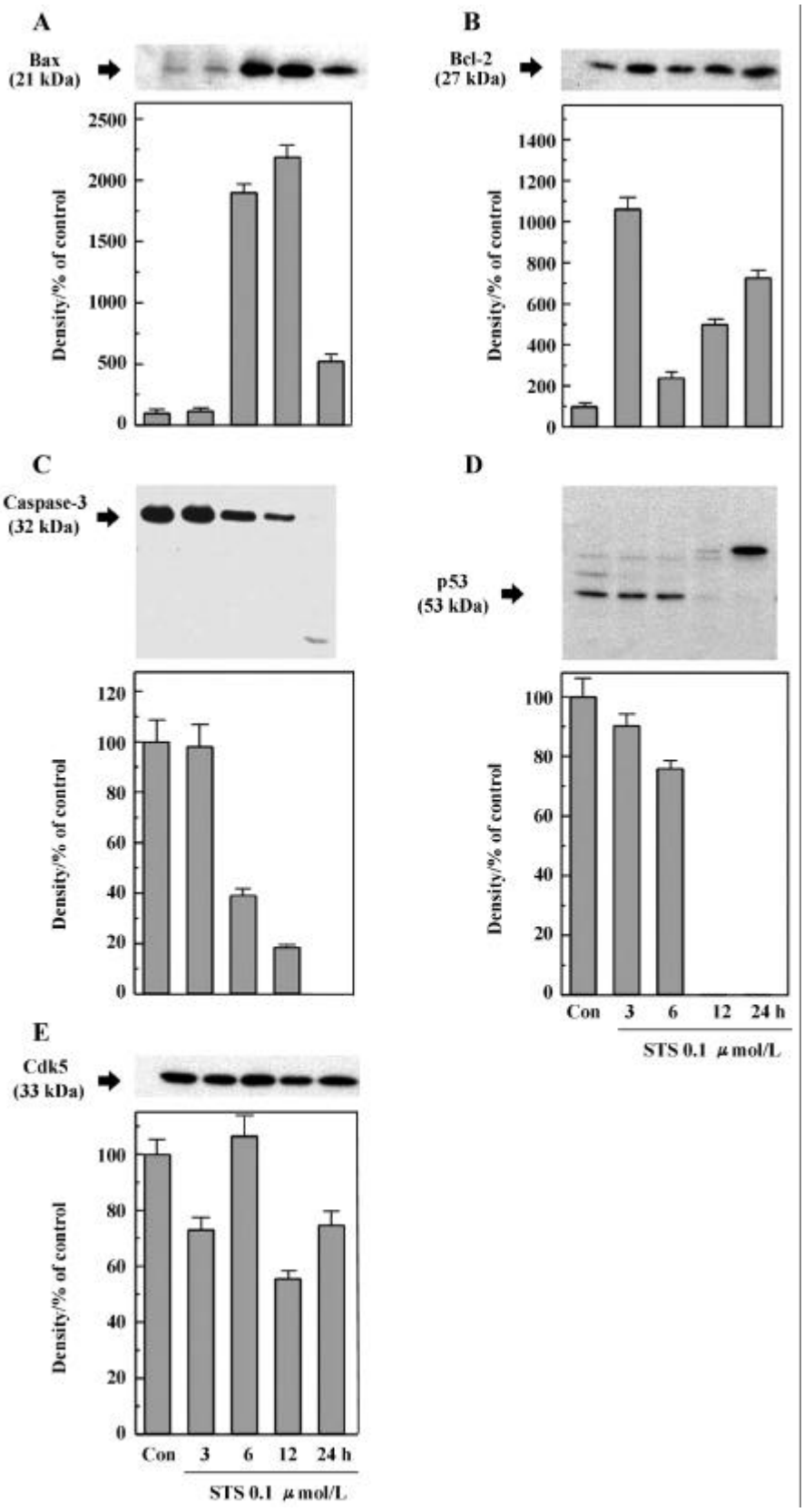

Fig 4. Western blot analysis for the protein expression of Bax (A), Bcl-2 (B), caspase-3 (C), p53 (D) and Cdk5 (E) in NG108-15 cells. Each value was measured by densitometric analysis of the immunoblots based on the density of the band in the vehicle control as 100 %.

gene. p53 protein, acting as a transcription factor, could induce cell apoptosis or growth arrest in cells depending on the cell type or physiological circumstances[8]. In the present study, treatment with STS markedly decreased the expression of p53 protein after the treatment for 12 h (Fig 4D).

Cyclin-dependent kinase 5 (Cdk5), a member of the CDK family important for neuronal maturation but not involved in cell cycle control, has been implicated in a number of physiological processes in nerve and muscle cells, including neurogenesis, neuritic outgrowth, axonal transport of membrane-bound organelles and myogenesis. Cdk5 and its neuron-specific activator p35 are required for neurite outgrowth and cortical lamination. Proteolytic cleavage of p35 produces p25, which accumulates in the brains of patients with Alzheimer's disease[9]. Conversion of p35 to p25 causes prolonged activation and mislocalization of Cdk5. Consequently, the p25/Cdk5 kinase hyperphosphorylates tau, disrupts the cytoskeleton and promotes the apoptosis of primary neurons. STS can affect a variety of kinases. To investigate whether Cdk5 was involved in STS-induced apoptosis of NG108-15 cells, we have investigated the expression of Cdk5 and its activator p35. Our data indicates that Cdk5 protein expression did not have obvious changes after the cells were exposed to STS (Fig 4E). We also did not see any significant conversion of p35 to p25 (data not shown).

DISCUSSION

In NG108-15 cells, STS induces cell death with the classic characteristics of apoptosis including cellular shrinkage, neurite degeneration, chromatin condensation, and DNA fragmentation. Several genes have been shown to be involved in both the positive and negative regulation of apoptotic cell death. Elevated protein level of Bax has been shown to promote apoptosis in response to numerous cell death-inducing stimuli[10]. In the present study, Bax protein expression markedly increased after the cells were exposed to STS for 6 h, which coincides with the appearance of DNA laddering in temporal sequences. This result implies that Bax was involved in the cascade of STS-induced NG108-15 cell apoptosis.

On the contrary, Bcl-2 has been shown to prevent both apoptotic and necrotic cell death induced by a variety of stimuli in several different systems[11]. The Bcl-2 protein level has been demonstrated to be up regulated in brains of patients with AD[12]. Such up-regulation of Bcl-2 protein might imply a compensatory response of remaining neurons to protect them from subsequent apoptosis. Therefore, neurons with lower Bcl-2 expression may be more vulnerable to apoptotic death. In the present study, Bcl-2 protein expression in NG108-15 cells was markedly increased after exposure to STS for 3 h and then decreased from 6 to 24 h. This increased expression of Bcl-2 should be a compensatory response of the cells against the STS insult.

In the mature nervous system, numerous studies indicate that p53 is essential for the neuronal death in response to a variety of insults, including DNA damage, ischemia and excitotoxicity[13]. However, some studies exclude a role for p53 in the death of NGF-deprived neurons[14]. Our results demonstrated that STS markedly decreased the expression of p53 protein after the treatment for 12 h. Although it is not clear whether suppression of p53 expression is a protective stress response, it implies that STS-induced NG108-15 cells might die via a pathway that is dependent on Bax expression but independent of p53.

Recently, increasing evidence has suggested a role for Cdk5 in neuronal apoptosis[15]. In addition, recent reports suggest that in cultured postmitotic neurons, activation of CDKs is a signal for death rather than cell division. For example, increases in Cdk4 and/or cyclin D1 have been reported in vulnerable brain regions of Alzheimer's disease patients[16], the kainic acid-evoked death of hippocampal neurons, and in strokes[17]. Cdk4/6/pRb pathway is also activated in a rat model of focal ischemia[18]. However, it is not clear if CDKs are also involved in STS-induced apoptosis. Although Cdk5 protein expression did not have obvious changes after the NG108-15 cells were exposed to STS, we cannot rule out the possibility that other CDKs may play an important role in apoptosis induced by STS. It needs further study for the effect of STS on Cdk2 and Cdk4, *etc*.

Accordingly, a better understanding of the molecular mechanisms of neuronal cell death in nervous system development, injury and disease can lead to new therapeutic approaches for the prevention of neurodegeneration and neurological disabilities and will expand the field of cell death biology.

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