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Protein Synthesis-Dependent but Bcl-2-Independent Cytochrome C Release in Zinc Depletion-Induced Neuronal Apoptosis

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Previously, we reported that chelation of intracellular zinc with *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN)-induced macromolecule synthesis-dependent apoptosis of cultured cortical neurons. According to the current theory of apoptosis, release of mitochondrial cytochrome C into the cytosol is required for caspase activation. In the present study, we examined whether cytochrome C release is dependent on macromolecule synthesis. Exposure of cortical cultures to 2 μM TPEN for 24 hr induced apoptosis as previously described. Fluorescence immunocytochemical staining as well as immunoblots of cell extracts revealed the release of cytochrome C into the cytosol 18–20 hr after the exposure onset. The cytochrome C release was completely blocked by the addition of cycloheximide or actinomycin D. Addition of the caspase inhibitor zVAD-fmk did not attenuate the cytochrome C release, whereas it blocked TPEN-induced apoptosis. Because Bcl-2 has been shown to block cytochrome C release potently, we exposed human neuroblastoma cells (SH-SY5Y) to TPEN. Whereas Bcl-2 overexpression completely blocked both cytochrome C release and apoptosis induced by staurosporine, it attenuated neither induced by TPEN. The present results suggest that, in neurons, macromolecule synthesis inhibitors act upstream of cytochrome C release to block apoptosis and that, in addition to the classical Bcl-2 sensitive pathway, there may exist a Bcl-2-insensitive pathway for cytochrome C release. J. Neurosci. Res. 61:508–514, 2000.

Key words: TPEN; neuronal death; cytochrome C; Bcl-2; cycloheximide

Chelation of intracellular zinc with *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) induces apoptosis of neurons in cortical culture (Ahn et al., 1998). As with other trophic factor deprivation-induced neuronal apoptosis (Martin et al., 1992; Pittman et al., 1993; Villa et al., 1994), macromolecule synthesis inhibitors and caspase inhibitors effectively block the TPEN-induced neuronal apoptosis. Insofar as zinc depletion has been implicated in Alzheimer's disease (AD; Constantinidis, 1991; Corrigan et al., 1993; Cuajungco and Lees, 1997) and age-related macular degeneration (ARMD; Newsome et al., 1988), this type of cell death may be a contributing mechanism for neuronal death under certain conditions.

Zinc has been shown to affect apoptosis in diverse ways. First, zinc activates PKC (Csermely et al., 1988; Noh et al., 1999) and tyrosine kinases (Park and Koh, 1999), which may attenuate apoptosis. Second, zinc inhibits endonucleases (Giannakis et al., 1991; Barbieri et al., 1992). Finally, zinc has been shown to inhibit caspases (Perry et al., 1997; Aiuchi et al., 1998). All these indicate that intracellular zinc may normally function to inhibit apoptosis. It seems, therefore, not very surprising that depletion of intracellular zinc with TPEN induces apoptosis in diverse cell types (McCabe et al., 1993; Jiang et al., 1995; Sakabe et al., 1998). However, the precise step(s) in the apoptosis cascade that is activated by TPEN remains unknown.

To elucidate the mechanism of zinc depletion-activated apoptosis in neurons, the present study attempted to find which step(s) is dependent on macromolecule synthesis. We, therefore, examined the release of cytochrome C from the mitochondria, which plays a crucial role in the activation of caspases (Hockenbery et al., 1990; Ellerby et al., 1997; Cai et al., 1998). Following various apoptosis-inducing stimuli, the mitochondrial membrane potential dissipates and cytochrome C release ensues, ultimately leading to caspase activation and apoptosis (McCabe et al., 1993; Liu et al., 1996; Li et al., 1997a,b; Heiskanen et al., 1999). Bcl-2 family proteins have been shown to regulate this release of cytochrome C. For example, Bcl-2 and Bcl-xL block the cytochrome C release (Adachi et al., 1997; Kharbanda et al., 1997; Yang et

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al., 1997; Parsadanian et al., 1998), whereas Bax and Bak promote it (Oltval et al., 1993; Finucane et al., 1999; Holinger et al., 1999). One mechanism for this block of cytochrome C release by Bcl-2-related factors has been demonstrated to occur through their interference with the opening of the voltage-dependent anion channel (VDAC; Shimizu et al., 1999).

In the present study, we examined in primary cortical cultures and Bcl-2-overexpressing SH-SY5Y cells whether TPEN induces cytochrome C release and whether this depends on the synthesis of macromolecules or on Bcl-2. Here we report that TPEN induces cytochrome C release in a macromolecule synthesis-dependent but Bcl-2-independent manner.

MATERIALS AND METHODS

Cell Culture

Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared as described previously (Choi, 1988) from fetal mice at 14–16 days of gestation. Briefly, dissociated cortical cells were plated onto a previously established glial cell monolayer at 2 hemispheres per 24-well plate (Nunc) or at 1 hemisphere per 60-mm dish (Falcon) in a plating medium consisting of Dulbecco's modified Eagle's medium (D-MEM; supplied glutamine-free) supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 2 mM glutamine, 5% fetal bovine serum, and 5% horse serum. Ten micromolar cytosine arabinoside was added 5–6 days after the plating to halt the growth of nonneuronal cells. Cultures were maintained at 37°C in a humidified CO_2 incubator (5%) and used for experiments between 7 and 10 days in vitro (DIV). Glial cultures were prepared from neocortices of newborn mice (postnatal day 1 or 2) and plated at 0.5 hemisphere per 24-well plate or 0.25 hemisphere per 60-mm dish in plating medium supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 2 mM glutamine, 10% fetal bovine serum, and 10% horse serum. Glial cultures were used for plating between DIV 14 and 28. SH-SY5Y neuroblastoma cells, pLXSN vector-transfected cells, and bcl-2-transfected SH-SY5Y cells (gifts of Dr. Se Nyun Kim, Seoul National University) were maintained in D-MEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Generation of Bcl-2-Overexpressing Cell Line

Bcl-2 overexpression construct was generated by inserting Bcl-2 cDNA into *Hpa*I cloning site of pLXSN (Lee et al., 1999). SH-SY5Y cells were transfected with the expression construct by calcium phosphate/DNA coprecipitation method. After selection against 500 μg/ml G418, high-expressor clonal cells were obtained by dilution cloning and screening with Western blot analysis.

Exposure to TPEN and Other Drugs

Exposures of cortical neurons to TPEN and other drugs were done via serum-free culture medium (Eagle's minimal essential medium, Earle's salts, supplied glutamine-free). Before the exposure, existing medium was washed out several times and replaced with the serum-free medium. Exposures to TPEN and other drugs were then accomplished by the addition of desired volumes of stock solutions to the serum-free exposure medium. In SH-SY5Y cells, appropriate volumes of drugs (less than 1% of total volume) were directly added into the medium.

Assessment of Cell Injury

Overall cell injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at ×100 or ×200. This examination was performed at various times (2–24 hr) after TPEN exposure. The process of cell death was completed after 24 hr of TPEN exposure. Overall neuronal cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released by damaged or destroyed cells into the bathing medium 24 hr after exposure to TPEN (Koh and Choi, 1987). A small amount of LDH was present in the media of control cultures with sham wash alone. The mean value of background LDH, determined on sister cultures within each experiment, was subtracted from values obtained in treated cultures.

The absolute value of the LDH efflux produced by TPEN exposure was quite consistent within sister cultures of a single plating, but differed somewhat between platings, largely as a function of neuronal density (which varied despite constant original plating densities, presumably reflecting small variations in cell preparation or serum characteristics). Therefore, each observed LDH value was scaled to the maximal neuronal LDH release (=100) produced by 24 hr exposure to 300 μM NMDA in sister cultures, in which near-complete neuronal death with no glial damage occurs (Choi, 1988).

In the experiments with SH-SY5Y neuroblastoma cell lines, 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium (MTT) assay was used to assess cell viability. After drug incubation for the desired times, MTT solution (2 mg/ml of MTT in PBS) was added to the media and further incubated at 37°C for 3 hr. After removal of the MTT solution, dimethyl sulfoxide was added to extract the MTT formazan crystal. The absorbances of samples were read at 570 nm using a microplate reader (Bio-Rad model 3550). Experiments examining cytotoxicity and cytochrome C release were done at least three times using cultures from different platings.

Detection of Cytochrome C Release

To detect cytochrome C release by immunocytochemistry, cultures, treated with appropriate drug, were fixed using 4% formaldehyde at 4°C for 1 hr and further incubated with PBS containing 0.3% Triton X-100 and 1% BSA at 4°C for 20 min. After the incubation with mouse anticytochrome C monoclonal antibody (clone 6H2.B4; Pharmingen) at 4°C for 1 hr, samples were rinsed and then incubated with FITC-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO) at 4°C for 1 hr. The samples were then observed under fluorescence microscope (Zeiss Axioplan 2).

Cytochrome C release was also examined by Western blot analysis. Cells were washed twice with PBS, and then the pellets were suspended in ice-cold hypotonic extraction buffer (HEB; 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiolthreitol, and protease inhibitors) containing 250 mM sucrose. After incubation on ice for 30 min, the cell lysates were resuspended by thorough swirling. The cell lysates

were then centrifuged at 16,000*g* (4°C) for 30 min. The supernatant was removed and used immediately as the cytosolic fraction. The pellet was further incubated with HEB plus 0.1% Nonidet P-40 on ice for 20 min to extract membrane protein. After centrifugation at 16,000*g* (4°C) for 10 min, the supernatant was used as the membrane fraction.

The extracts were electrophoresed on a 15% SDS/polyacrylamide gel and transferred onto nitrocellulose (NC) membrane. Protein-bound NC membrane was incubated with mouse anticytochrome C antibody (clone 7H8.2C12; Pharmingen), followed by horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham, Arlington Heights, IL). The relevant protein bands were then visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham).

Drugs and Chemicals

TPEN, staurosporine, cycloheximide, and actinomycin D were purchased from Sigma. zVAD-fmk was purchased from Enzyme Systems Products. All other chemicals used here were commercial products of analytical purity.

RESULTS

In our previous work (Ahn et al., 1998), we found that cultured cerebral cortical neurons treated with 2 μM of TPEN underwent cell death with characteristics of apoptosis such as cell body shrinkage, nuclear condensation, and DNA fragmentation (Kerr et al., 1972; Wyllie et al., 1984; Hockenbery et al., 1990; Martin et al., 1992; Sakabe et al., 1998). As in other experimental paradigms of apoptosis (Kharbanda et al., 1997; Finucane et al., 1999; Heiskanen et al., 1999; Martinou et al., 1999), the TPEN-induced neuronal apoptosis was accompanied by cytochrome C release into the cytosol, as demonstrated by immunocytochemical staining (Fig. 1A,B) and Western blot analysis (Fig. 1C) at about 18–20 hr after the exposure onset. In control sister cultures that underwent only sham wash, cytochrome C immunoreactivity was limited to the cytosol and showed a punctuated pattern, suggesting the association with mitochondria (Fig. 1A). However, in cultures treated with 2 μM of TPEN for 20 hr, the whole cytoplasm was homogeneously stained (Fig. 1B). The change in the staining pattern suggests that cytochrome C was released into the cytosol from mitochondria in the process of the TPEN-induced apoptosis. Western blot analysis of cell fractions further confirmed the release of cytochrome C into the cytosol, in that the cytosolic fraction of TPEN-treated cultures contained much more cytochrome C than that of control cultures. TPEN-induced cytochrome C release was not likely to be a nonspecific event of cell death, since exposure of cortical neurons to N-methyl-D-aspartate (NMDA) for 24 hr induced widespread neuronal death with features of necrosis, yet without any detectable cytochrome C release (data not shown).

As in many other cases of neuronal apoptosis (Oppenheim et al., 1990; Schwartz and Osborne, 1993; Koh et al., 1995), the TPEN-induced apoptosis was attenuated by inhibitors of macromolecule synthesis such as cycloheximide and actinomycin D (Fig. 2E). Whereas the effect is intriguing in that new protein synthesis seems to be required for apoptosis in central neuron, the current main model of apoptosis does not seem to encompass this possibility (Cai et al., 1998; Green and Reed, 1998; Susin et al., 1998). In case of TPEN-induced apoptosis, it seems that cytochrome C release or its upstream events are dependent on "macromolecule synthesis." Cytochrome C release induced by TPEN (2 μM for 20 hr) was completely blocked by the protein synthesis inhibitor cycloheximide (1 μg/ml; Fig. 2A,D) and the RNA synthesis inhibitor actinomycin D (2.5 μg/ml; Fig. 2B,D). Although treatment with the broad-spectrum caspases inhibitor carbobenzoxy-valyl-alanyl-aspartyl-fluoromethyl ketone (zVAD-fmk; 100 μM) completely blocked the TPEN-induced apoptosis, it failed to attenuate cytochrome C release by TPEN (Fig. 2C,D). This is consistent with the current model of apoptosis (Cai et al., 1998; Green and Reed, 1998).

To understand better the mechanism of TPEN-induced cytochrome C release, we used SH-SY5Y human neuroblastoma cell lines transfected with bcl-2, which

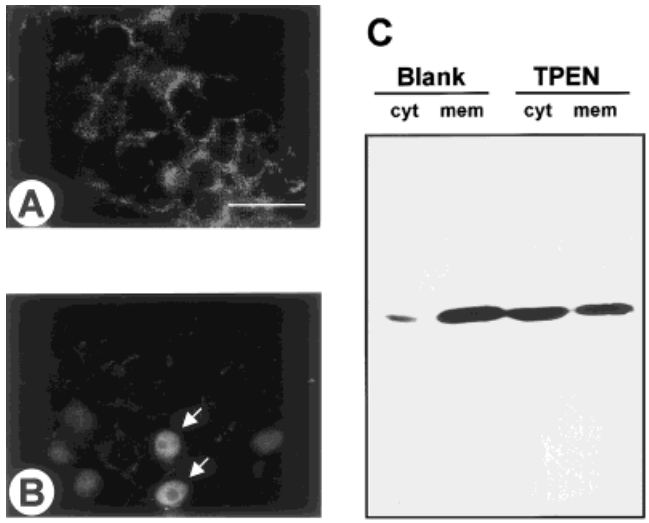

Fig. 1. Cytochrome C release during TPEN-induced apoptosis in mouse cortical neuron cultures. **A,B:** Fluorescence photomicrographs of cortical cultures that underwent sham wash alone (A) or a 20-hr exposure to 2 μM TPEN (B), stained with FITC-conjugated secondary antibody to mouse anticytochrome C antibody. In the control culture, punctuated staining pattern was seen in the cytosol, which likely represents the association of cytochrome C with mitochondria. However, in TPEN-treated culture, a diffuse, homogeneous pattern of staining throughout the cytoplasm was observed (arrows). Scale bar = 50 μm. **C:** Western blot analysis of cytochrome C release. Cortical cultures that underwent sham wash (Blank) or a 20-hr exposure to 2 μM TPEN (TPEN) were lysed, and the cytosolic (cyt) and membrane fractions (mem) were separated as described in Materials and Methods. These fractions were electrophoresed on SDS-PAGE and immunoblotted using anti-mouse cytochrome C antibody. The band signal in the cytosolic fraction was much denser in TPEN-treated cultures than in blank cultures. Conversely, the signal in the membrane fraction was greater in Blank than in TPEN. This is representative of at least three different results.

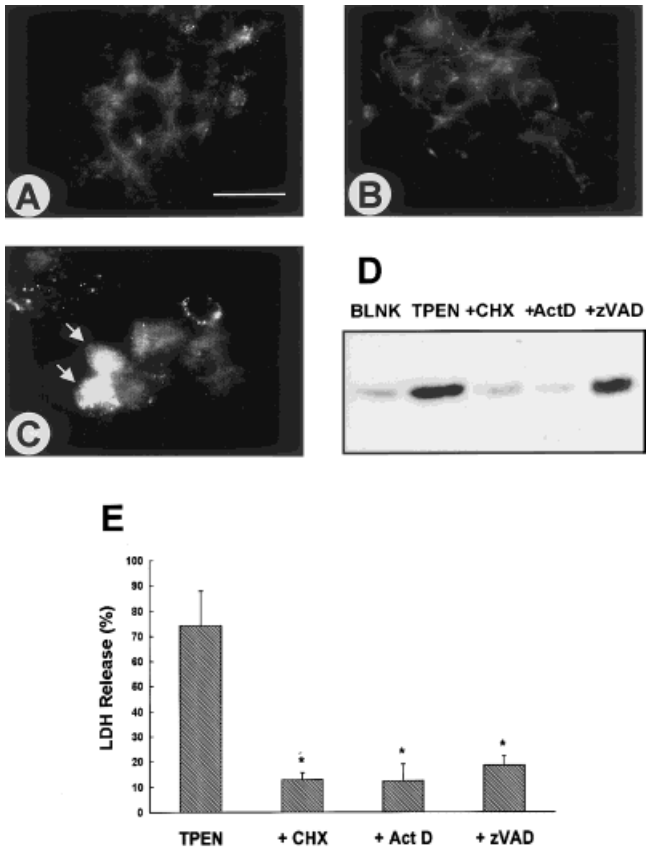

Fig. 2. Cytochrome C release is blocked by cycloheximide and actinomycin D but not by zVAD-fmk. **A–C:** Fluorescence photomicrographs of cortical cultures that underwent a 20-hr exposure to 2 μM TPEN in the presence of 1 μg/ml of cycloheximide (A), 2.5 μg/ml of actinomycin D (B), or 100 μM of zVAD-fmk (C), stained with FITC-conjugated secondary antibody to mouse anticytochrome C antibody. In cycloheximide- and actinomycin D-treated cultures, punctuated staining patterns were noted as seen in sham wash control culture (Fig. 1A), which likely represents little cytochrome C release. However, in cultures exposed to TPEN plus zVAD-fmk, the staining pattern appeared diffuse and homogeneous throughout the cytoplasm (arrows) as seen in cultures exposed to TPEN alone (Fig. 1B). Scale bar = 50 μm. **D:** Western blot analysis of the cytosolic fractions obtained from the cultures described above. Cytosolic cytochrome C was detected in cultures exposed to TPEN or TPEN plus zVAD-fmk (+zVAD), but not in sham wash control (BLNK), TPEN plus cycloheximide (+CHX), or TPEN plus actinomycin D (+ActD). This is representative of at least three different results. **E:** Bars denote LDH release (mean and SEM, n = 4) in cortical cultures incubated with 2 μM of TPEN alone for 24 hr (TPEN) or in the presence of 1 μg/ml of cycloheximide (+CHX), 2.5 μg/ml of actinomycin D (+Act D), or 100 μM of zVAD-fmk (+zVAD). All these drugs markedly attenuated TPEN-induced apoptosis. Asterisks denote difference from TPEN-treated group ($P < 0.01$, two-tailed *t*-test with Bonferroni correction for three comparisons).

blocks apoptosis in many but not all cases (Hockenbery et al., 1990; Garcia et al., 1992). The bcl-2-transfected cells contained about six times more Bcl-2 proteins than do parental cells or vector-transfected cells (Kim, 1999). In the SH-SY5Y cells (SY5Y) or those transfected with vector alone (LXSN), staurosporine, a commonly used apoptosis-inducing agent in a wide range of cells (Koh et al., 1995; Weil et al., 1996; Yang et al., 1997; Yu et al., 1997), induced cell death in a concentration-dependent manner. However, in bcl-2-transfected cells (Bcl-2), staurosporine-induced cell death was much less throughout the concentration range (Fig. 3A). We then examined whether cytochrome C release was also inhibited by the overexpression of Bcl-2. Whereas exposure to staurosporine (150 nM for 24 hr) induced cytochrome C release in cells transfected with vector alone (Fig. 3B), the same exposure failed to induce cytochrome C release in cells transfected with bcl-2. Using TPEN, we performed the same experiments. In contrast to staurosporine-induced cell death, TPEN-induced cell death (6–10 μM for 24 hr) was identical in SH-SY5Y cells regardless of the overexpression of bcl-2 (Fig. 3C). Analysis of the time course of TPEN-induced cell death in these cells revealed that Bcl-2 overexpression did not cause any shift in the rate of death (data not shown). Furthermore, cytochrome C release brought about by TPEN (8 μM for 24 hr) was virtually identical in bcl-2-overexpressing cells compared to that in vector alone (Fig. 3D). Cyclosporin A, the inhibitor of the membrane permeability transition pore (Bradham et al., 1998), could lessen the TPEN-induced cell death to some degree, nevertheless, which was statistically significant ($P < 0.01$), and the degrees of inhibition were similar between SH-SY5Y parental cells and Bcl-2-overexpressed cells (Fig. 3E,F). These results indicate that, in contrast to the staurosporine-induced apoptosis, TPEN-induced cytochrome C release and apoptosis are not under the inhibitory control of Bcl-2.

DISCUSSION

In our previous study, we demonstrated that intracellular zinc depletion by a cell-permeant zinc chelator, TPEN, induces neuronal apoptosis in cortical culture (Ahn et al., 1998). Here we show that the TPEN-induced neuronal apoptosis is accompanied by the release of cytochrome C into the cytosol, a few hours before the completion of cell death. This process appears to be a necessary event for caspase activation and apoptosis, as shown in other examples of apoptotic cell death (Li et al., 1997a; Green and Reed, 1998; Zhivotovsky et al., 1998; Brustugun et al., 1998; Cai et al., 1998; Finucane et al., 1999). In that sense, it is unlikely that TPEN-induced apoptosis occurs via the release of caspase activity from zinc-mediated inhibition, as suggested by Perry and colleagues (1997). Rather, it appears that TPEN triggers the classical pathway involving cytochrome C release and cytochrome C-mediated caspase activation.

Another interesting feature of TPEN-induced apoptosis in our previous work was its complete insensitivity to various neurotrophic factors or treatment with high K^+, conditions that have potent protective effects against diverse forms of neuronal apoptosis (Koh et al., 1995; Ahn et al., 1998). This suggests that TPEN acts downstream from neurotrophic factor receptor signaling, such as PI3

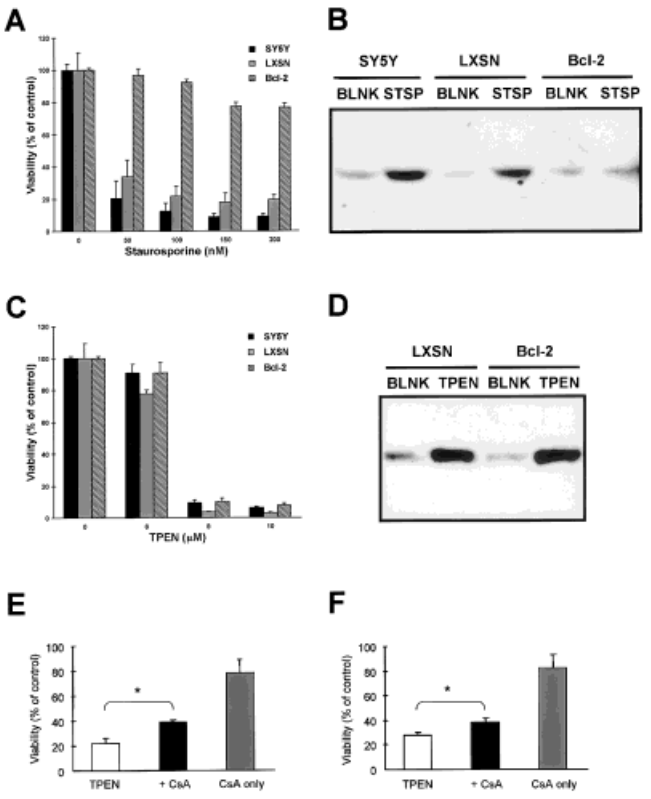

Fig. 3. Bcl-2 dose not inhibit TPEN-induced cytochrome C release in SH-SY5Y human neuroblastoma cells. **A:** Bars denote cell viability (mean and SEM, n = 4 cultures each) assessed by MTT assay. SH-SY5Y cells (SY5Y), pLXSN vector-transfected SH-SY5Y cells (LXSN), and bcl-2-transfected cells (Bcl-2) were exposed to the indicated concentrations of staurosporine (STSP) for 24 hr. Whereas SY5Y and LXSN cells were severely damaged by STSP, Bcl-2 transfectants exhibited remarkable resistance throughout STSP concentrations. **B:** The same cells as in A were incubated with 150 nM of staurosporine for 24 hr and then lysed. The cytosolic fractions were prepared as described in Materials and Methods. After electrophoresis on SDS-PAGE, cytochrome C in the cytosolic fraction was detected by Western blot. In SY5Y and LXSN, staurosporine (STSP) exposure induced cytochrome C release into the cytosol. In Bcl-2-overexpressing cells, however, no cytochrome C was detected in the cytosolic fraction. This is representative of at least three different results. **C:** Bars denote cell viability (mean and SEM, n = 4) of SY5Y, LXSN, or Bcl-2 after a 24-hr exposure to indicated concentrations of TPEN, as assessed by the MTT assay. All three cells showed similar responses to TPEN toxicity. **D:** Human neuroblastoma cells (LSXN and Bcl-2) were exposed to 8 μM TPEN for 24 hr. After the exposure, cytosolic fractions obtained from cell lysates were subjected to Western blot analysis to look at the cytochrome C release. Exposure to TPEN produced the release of cytochrome C into the cytosol in both cell lines. This is representative of at least three different results. **F:** Bars denote cell viability (mean and SEM, n = 4) of SY5Y (E) or Bcl-2 (F) after a 24-hr exposure to 8 μM TPEN in combination with 100 μM cyclosporin A (CsA). Asterisks denote difference from TPEN-treated group ($P < 0.01$, two-tailed *t*-test with Bonferroni correction for three comparisons).

kinase and Akt kinase activation (Skaper et al., 1998; Bhave et al., 1999; Dolcet et al., 1999; Hetman et al., 1999) but upstream from the cytochrome C release.

The TPEN-induced apoptosis, as with many other cases of neuronal apoptosis (Oppenheim et al., 1990; Schwartz and Osborne, 1993; Koh et al., 1995) is completely blocked by macromolecule synthesis inhibitors. Whereas this effect, known for long time, suggests the participation of "death effector genes," the identities of these genes remain unknown. Generally, possibilities include unidentified proteins that are necessary for the final caspase activation or recruitment of already known death effectors, such as caspases. In the case of TPEN-induced apoptosis, it appears that the step at which the inhibitors of macromolecule synthesis block lies upstream of the cytochrome C release. Insofar as almost all neuronal apoptosis in our cortical culture is blocked by macromolecule synthesis inhibitors (Koh et al., 1995; Ahn et al., 1998), it is likely that the induction of certain new proteins is a necessary step for the cytochrome C release in cortical neurons.

Finally, although cytochrome C release appears to be a common step in both TPEN- and staurosporine-induced apoptosis, both of which are sensitive to macromolecule synthesis inhibitors, it appears that two different pathways exist to activate this cytochrome C release. As depicted in many diagrams of the current model (Garcia et al., 1992; Kluck et al., 1997; Yang et al., 1997), Bcl-2 inhibits cytochrome C release in the case of staurosporine-induced apoptosis. The effect was so powerful that even 1 μM of staurosporine failed to induce cytochrome C release or apoptosis in HL60 cells (Yang et al., 1997). By contrast, TPEN-induced cytochrome C release was virtually insensitive to Bcl-2 overexpression. This case seems to be the first demonstration of Bcl-2-insensitive cytochrome C release that actively mediates apoptosis. Arguing against the possibility that the cytochrome C release in TPEN-induced neuronal death occurs merely as a result of cell death, inhibition of caspase activation blocks TPEN-induced apoptosis but fails to block cytochrome C release.

Insofar as zinc serves many essential normal roles in cells (Vallee and Auld, 1990, 1993; Choi and Koh, 1998), the fact that this intracellular depletion results in death may not appear surprising. However, the interesting aspect of zinc depletion-induced apoptosis is that it occurs by the classical apoptosis pathway involving cytochrome C release and caspase activation but bypassing the upstream neurotrophic factor signaling. Elucidation of the mechanism of zinc depletion-induced apoptosis may provide further molecular insights into incompletely characterized apoptotic cascades, especially in central neurons. In addition, the insight may help us to understand the pathophysiologic mechanisms of neuronal loss in AD and age-related macular degeneration (ARMD), in which zinc depletion has been suggested to play a role.

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