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Depletion of Intracellular Zinc Induces Protein Synthesis-Dependent Neuronal Apoptosis in Mouse Cortical Culture

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The central nervous system (CNS) contains a large amount of zinc; a substantial fraction of it is located inside synaptic vesicles of glutamatergic terminals in chelatable forms and released in a calcium-dependent manner with intense neuronal activity. Recently, it has been shown that excessive zinc influx can kill neurons in rats subjected to transient forebrain ischemia. On the other hand, severe depletion of zinc has been also reported to induce cell death in certain nonneuronal cells. Since decreases in tissue zinc have been associated with Alzheimer's disease (AD) and senile macular degeneration, we examined whether depletion of intracellular zinc with a zinc chelator can directly induce neuronal death in mouse cortical cultures. Exposure of cortical cultures to a cell-permeant zinc-chelator, *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, 0.5–3.0 μM) induced gradually developing neuronal degeneration accompanied by various features of apoptosis: cell body shrinkage, nuclear condensation and fragmentation, and internucleosomal DNA breakage. At higher concentrations, TPEN induced additional glial cell death. TPEN-induced cell death was completely blocked by coaddition of zinc. Addition of a protein synthesis inhibitor cycloheximide as well as a caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-fluoromethyl ketone (zVAD-fmk) markedly attenuated TPEN-induced neuronal death. On the other hand, brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), phorbol 12-myristate 13-acetate (PMA), high K^+, or an antioxidant, trolox, did not show any protective effect. The present results demonstrated that depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in cortical culture. Combined with the findings that extracellular zinc may promote extracellular β-amyloid (Aβ) aggregation and that total tissue zinc is reduced in AD, present results suggest a possibility that redistribution of zinc from intracellular to extracellular space may synergistically contribute to neuronal apoptosis in AD. © 1998 Academic Press

Key Words: **TPEN; neuronal death; caspase; cycloheximide; metals; Alzheimer's disease; brain-derived neurotrophic factor.**

INTRODUCTION

Zinc is essential for the survival of all cells, as it is required for the normal function of many enzymes, zinc-finger transcription factors, and structural proteins (50, 51). The central nervous system (CNS) is not an exception and contains a large amount of zinc. One unique feature of the CNS zinc is that a substantial fraction of it is stored in chelatable forms inside synaptic vesicles of certain glutamatergic neuronal terminals (14, 15, 39). Furthermore, with neuronal excitation, the synaptic vesicle zinc, like other neurotransmitters, is released into the synaptic cleft in an extracellular calcium-dependent manner (1, 18). Recently, it has been shown that excessively released zinc may induce selective neuronal death after transient global ischemia (28) by accumulating in vulnerable neurons (14, 15, 48). Thus, too much zinc influx into neurons may play an important role in neuronal death in certain pathological conditions (5).

On the other hand, at least in nonneuronal cells, chelation of labile pools of cytoplasmic zinc has been shown to induce classical apoptosis (11, 20, 32, 33). Interestingly, some neurological disease states have been linked to decreased CNS zinc levels. For example, Alzheimer's disease (AD) brains have been reported to contain less total zinc than do normal brains (7–9). Although mechanisms, ultrastructural details, and pathologic significance of this finding have not yet been determined, deficiency of cytoplasmic labile pools of zinc in AD brains might well contribute to neuronal loss by triggering apoptosis. In addition to AD, zinc depletion has been suggested to play a pathogenic role in

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retinal diseases, including macular degeneration (34) and retinitis pigmentosa (21). Thus, in the present study, we examined the possibility that depletion of zinc directly induces apoptosis (programmed cell death) of central neurons, using mouse cortical cell cultures and a cell-permeant metal chelator, TPEN.

MATERIALS AND METHODS

Cortical Cell Cultures

Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared as described previously (5) from fetal mice at 14–16 days gestation. Briefly, dissociated cortical cells were plated onto a previously established glial cell monolayer at 2.5 hemispheres per 24-well plate (Nunc) in a plating medium consisting of Dulbecco's modified Eagle medium (DMEM, supplied glutamine-free) supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 2 mM glutamine, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside (10 μM) 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 (5%) incubator and used for experiments between 7 and 10 days *in vitro* (DIV). Glial cultures were prepared from neocortices of newborn mice (postnatal day 1–2) and plated at 0.5 hemispheres per 24-well plate, 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.

Near-pure neuronal cultures containing less than 0.5% of astrocytes were prepared as previously described (27). Cultures were plated and maintained in a serum-free defined medium (MEM supplemented with N-1 component). On DIV3, 10 μM cytosine arabinoside was added to halt the growth of nonneuronal cells. Cultures were used for experiments after DIV7.

To identify neurons and astrocytes, mixed or near-pure neuronal cultures were immunofluorescently stained with anti-microtubule associated protein-2 (MAP-2) or anti-glial acidic fibrillary protein (GFAP) antibodies.

Exposure to TPEN and Other Drugs

Exposures 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, preexisting medium was washed out several times and replaced with the serum-free medium. Then exposures to TPEN and other drugs were accomplished by the addition of desired volumes of stock solutions to the serum-free exposure medium.

Assessment of Neuronal Injury

Overall cell injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at ×100 and ×400. This examination was performed at various time points (2–24 h) after TPEN exposure. At 24 h of TPEN exposure, the process of cell death was complete. In some experiments, to verify that most degenerating cells in cultures after TPEN exposure were indeed neurons, cultures were fixed, permeabilized with Triton X-100, and stained immunofluorescently with anti-MAP-2 antibody and FITC-labeled secondary antibody (WB filter block, Olympus), and then with a fluorescent DNA dye, propidium iodide (10 μg/ml) to visualized nuclear fragmentation (WG filter block, Olympus). Since cells were permeabilized, nuclei in normal cells were also stained with PI, but did not show fragmentation or condensation. To identify astrocytes, cultures were stained with anti-glial acidic fibriallry protein (GFAP) antibody. In most experiments, overall neuronal cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) (24) released by damaged or destroyed cells into the bathing medium 24 h after exposure to TPEN. A small amount of LDH was present in the media of control cultures with sham wash alone. The mean value of background LDH, determined in sister cultures within each experiment, was subtracted from values obtained in treated cultures.

The absolute values of the LDH efflux produced by TPEN exposure was 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-h exposure to 300 μM NMDA in sister cultures, where near complete neuronal death with no glial damage occurs (6).

Transmission Electron Microscopy

Cultures were fixed in 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (pH 7.4). Cells were postfixed in 2% buffered osmium tetroxide. After staining *en bloc* in 0.4% uranyl acetate, cultures were dehydrated serially through increasing concentrations of ethanol and embedded in Epon resin (EMbed-812, Electron Microscopy Sciences). Ultrathin sections (70 nm) were prepared on Reichart–Jung Ultracut J, picked up on collodion-coated copper grids, and double-stained with 0.4% uranyl acetate and 2% lead citrate. After carbon coating, the samples were photographed on JEOL 1200EX-II electron microscope.

TUNEL Staining

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining of cultures was performed according to the procedure of *In Situ* Cell Death Detection kit (Boehringer-Mannheim). Briefly, cultures were fixed in 4% paraformaldehyde for 30 min at room temperature and then incubated with TUNEL reaction mixture containing TdT and fluorescein-labeled dNTP for 1 h at 37°C. Incorporated fluorescein was detected by anti-fluorescein antibody conjugated with horse-radish peroxidase. Afterwards, stained cells were analyzed under a light microscope.

DNA Agarose Gel Electrophoresis

DNA gel electrophoresis was performed as previously reported (17). Cultured cells were lysed in 0.5% Triton X-100, 5 mM Tris (pH 7.4), and 20 mM EDTA at 4°C for 30 min. After centrifugation, supernatants were extracted with phenol-chloroform and precipitated in ethanol. Isolated DNA samples were electrophoresed on a 1.5% agarose gel.

Drugs and Chemicals

TPEN, $ZnCl_2$, $CuCl_2$, $FeCl_2$, KCl, PMA, IGF-1, cycloheximide, N-1 supplement were purchased from Sigma. Trolox was obtained from Aldrich. BDNF was obtained from R & D. zVAD-fmk was purchased from Enzyme Systems Products and MK-801 from RBI. TUNEL staining kit (*In Situ* Cell Death Detection kit) was obtained from Boehringer-Mannheim. Antibodies to MAP-2 and GFAP and FITC-labeled secondary antibody were purchased from Chemicon.

RESULTS

Process-bearing cells with phase-bright rims around their cell bodies (Fig. 1A) in mixed cortical cultures were stained with anti-MAP-2 antibody, indicating their neuronal identity (Fig. 1C), whereas flat background cells (Fig. 1G) were stained with anti-GFAP antibody, indicating their astroglial identity (Fig. 1H). Addition of TPEN (2 μM) induced degeneration in a number of neurons within 18 h of exposure. Cell body shrinkage (Fig. 1B), a partial loss of MAP-2(+) neurites (Fig. 1D), and nuclear condensation/fragmentation visualized by propidium iodide staining (Fig. 1F; compare with 1E) were observed in degenerating neurons. In contrast to MAP-2(+) neurons, GFAP(+) astrocytes were not injured by 2 μM TPEN by phase-contrast microscopy (Fig. 1G) or propidium iodide fluorescence (Fig. 1H). Near-pure neuronal cultures maintained in N-1-supplemented serum-free medium also exhibited similar degenerative changes such as cell body shrinkage, nuclear fragmentation, and loss of membrane integrity as their counterparts in mixed cortical cultures after 24 h exposure to 2 μM TPEN (not shown).

The degenerative changes of neurons progressed gradually over the following day in a TPEN concentration-dependent manner; 24 h after the TPEN exposure, these neurons lost cytoplasmic membrane integrity and released lactate dehydrogenase (LDH) into the bathing medium. Estimated LD_{50} of TPEN with 24 h exposure was around 1.4 μM (Fig. 2A). At higher concentrations (>5 μM), TPEN induced additional glial cell death (not shown). To examine whether the mechanism of TPEN-induced neuronal death was due to depletion of cytoplasmic zinc but not other heavy metals, we added endogenous transition metals (zinc, iron, or copper) with TPEN; zinc as well as copper almost completely blocked neuronal death induced by TPEN (Fig. 2B). Since the stability constant of TPEN-Cu is higher than that of TPEN-Zn (33), this pattern of toxicity reversal indicates that chelation of zinc by TPEN is indeed responsible for the cell death. If the responsible metal were copper, addition of zinc would have failed to attenuate the cell death. And if the responsible metal were iron, addition of iron should have attenuated the cell death.

Next we examined whether TPEN-induced neuronal death occurred by apoptosis (programmed cell death). Under a phase-contrast microscope, we did not observe any cell body swelling, a hall mark of necrosis death, but cell body shrinkage and nuclear condensation/fragmentation, all typical for apoptosis death (Fig. 1) (22). Ultrastructural changes also supported that TPEN-induced neuronal death was apoptosis as it was accompanied by chromatin condensation and fragmentation, but little alterations in the morphological integrity of intracellular organelles, findings considered typical for apoptosis (Figs. 3A and 3B) (31, 47, 55). Furthermore, TUNEL staining (16, 44) and DNA agarose gel electrophoresis (17) revealed conspicuous internucleosomal DNA fragmentation (Figs. 3C and 3D).

As observed in other models of neuronal apoptosis in cortical cultures (26, 37, 45), addition of a protein synthesis inhibitor cycloheximide (1 μg/ml) almost completely blocked TPEN-induced neuronal death (Fig. 4). This protective effect was not reversed by an inhibitor of glutathione synthesis, buthionine sulfoximine (BSO), indicating that the protective effect of cycloheximide was not mediated by generating more glutathione (26). TPEN-induced neuronal apoptosis was also sensitive to caspase inhibition; addition of 100 μM zVAD-fmk almost completely blocked TPEN-induced neuronal death (Fig. 5). Brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), protein kinase C (PKC) activator (phorbol 12-myristate 13-acetate, PMA), or high K^+ medium (13, 26), while markedly protected against serum deprivation-induced neuronal apoptosis (27, 29), failed to attenuate TPEN-induced one (Fig. 5).

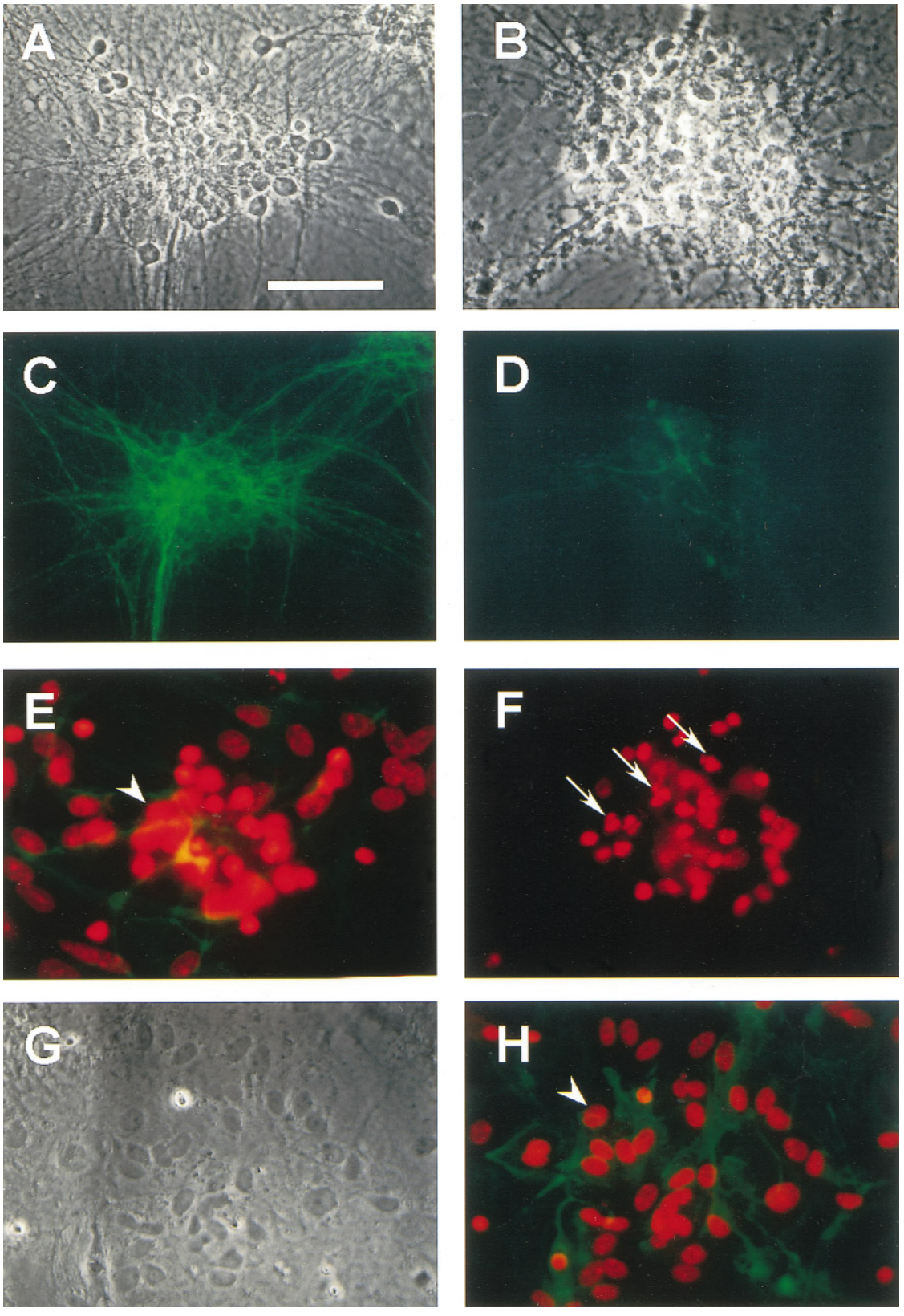


FIG. 1. TPEN induces neuronal degeneration. (A, B) Phase-contrast photomicrographs of sister cultures taken 18 h after sham wash (A) or the beginning of exposure to 2 μM TPEN (B). Note shrunken cell bodies of neurons in (B). (C, D) The same cultures as in (A) and (B) after immunofluorescence staining with anti-MAP2 antibody after fixation with paraformaldehyde and permeabilization with Triton X-100.

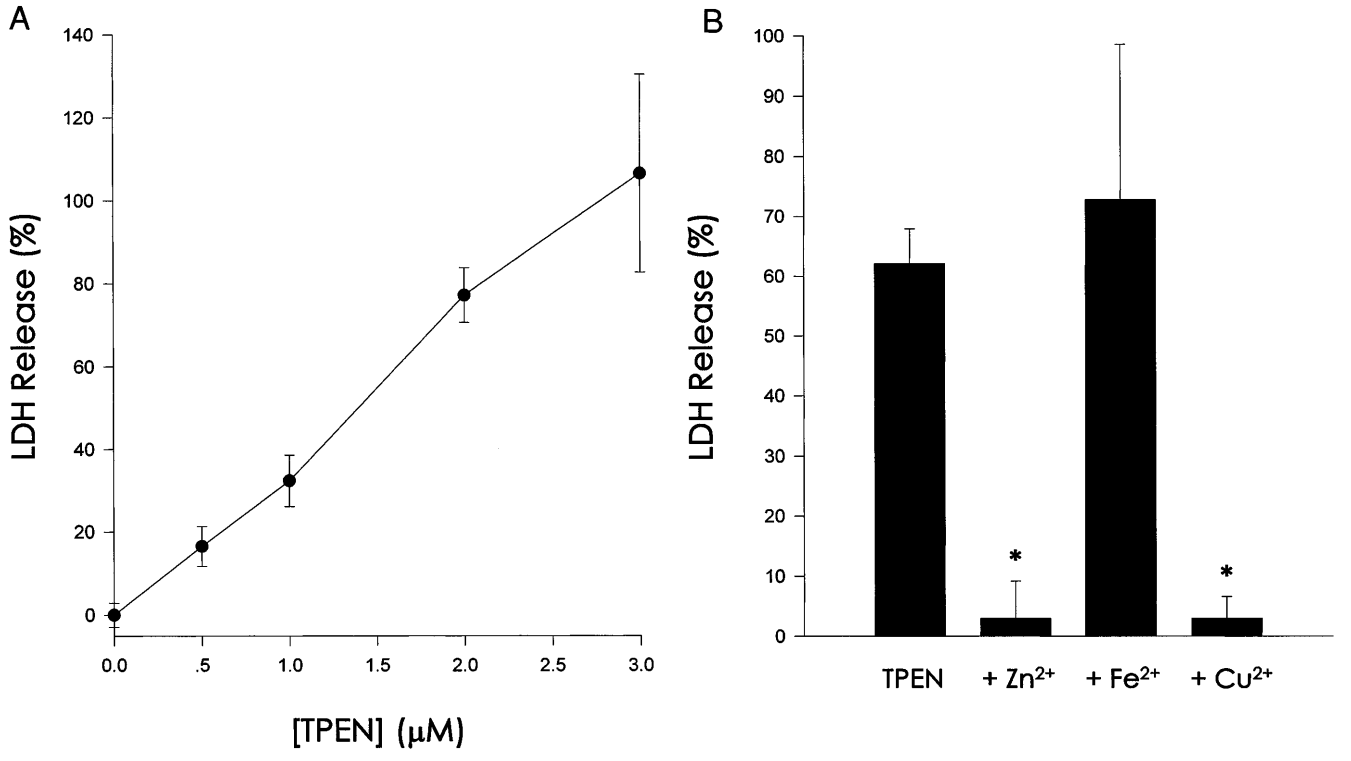


FIG. 2. TPEN-induced neuronal death: concentration–death relationship and the involvement of zinc chelation. (A) Bars represent LDH release (mean ± SEM, $n = 4$) in cortical cultures after 24 h exposure to indicated concentrations of TPEN. (B) LDH release (mean + SEM, $n = 4$) in cortical cultures after 24 h exposure to 2 μM TPEN alone or with the addition of 2 μM $ZnCl_2$, $FeCl_2$, or $CuCl_2$. Asterisks denote difference from TPEN ($P < 0.05$, two-tailed t test with Bonferroni correction for 3 comparisons).

As in other neuronal apoptosis models in our cortical culture, an antioxidant trolox and an NMDA antagonist MK-801 were ineffective in reducing TPEN-induced neuronal death (Fig. 5).

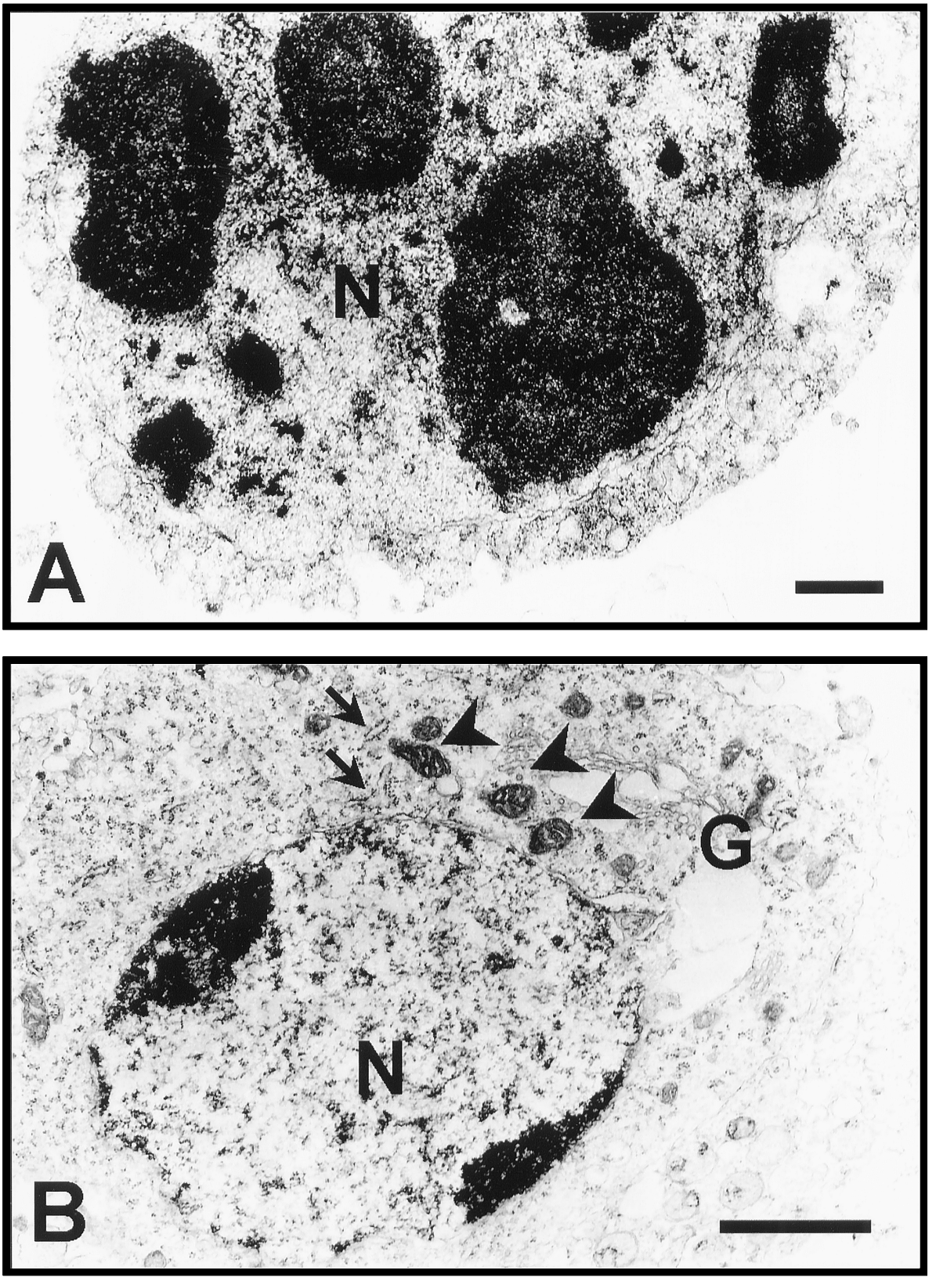
DISCUSSION

The main finding of the present study is that depletion of zinc with a membrane-permeant metal chelator TPEN triggers apoptosis of cultured cortical neurons. The identity of degenerating cells after low-intensity TPEN exposure in the mixed cortical cultures as neurons was demonstrated by their MAP-2 immunoreactivity. Further indicating the vulnerability of cortical neurons to depletion of cytoplasmic zinc, the majority of cells in near-pure neuronal cultures also underwent apoptosis upon TPEN exposure. The finding that TPEN exposure, but not the sham wash, induced neuronal apoptosis suggests that TPEN directly triggered apoptosis in neurons. Alternatively, since neurons in primary cultures die eventually, it is possible that TPEN may have enhanced the normally occurring neuronal apoptosis in culture condition. In either case, TPEN exposure appears to be potently proapoptotic.

The particular involvement of chelation of zinc, rather than other endogenous transition metals, in TPEN cytotoxicity could be deduced from differences in stability constants of TPEN-metal complexes (33). Since zinc and copper, but not iron, reversed TPEN-induced cell death, zinc must be the responsible metal. If copper had been responsible, addition of zinc that fails to displace copper from TPEN, would not have reversed TPEN-induced apoptosis. On the other hand, had iron been the responsible metal, iron as well as zinc and copper should have reversed the TPEN-induced cell death.

Since similar findings have been observed in other nonneuronal cell types, depletion of zinc may be univer-

Processes of the cells with phase-bright rims in (A) were stained. After TPEN exposure, shrunken neurons substantially lost neurites (B), which were demonstrated by decreases in MAP2 immunofluorescence (D). (E, F) Propidium iodide staining of cultures revealed normal nuclear morphology in sham wash control (arrowhead in E) and nuclear fragmentation in TPEN-exposed neurons (arrows in F). (G, H) Mixed cortical cultures after 18 h exposure to 2 μM TPEN. To better visualize astrocytes, neuron-free area was intentionally chosen. No degenerative changes in astrocytes were observed by phase-contrast microscopy (G) or by anti-GFAP immunofluorescnence (green) and propidium iodide fluorescence (red) (H). Arrowhead indicates a normal nucleus of an astrocyte. Scale bar, 100 μm.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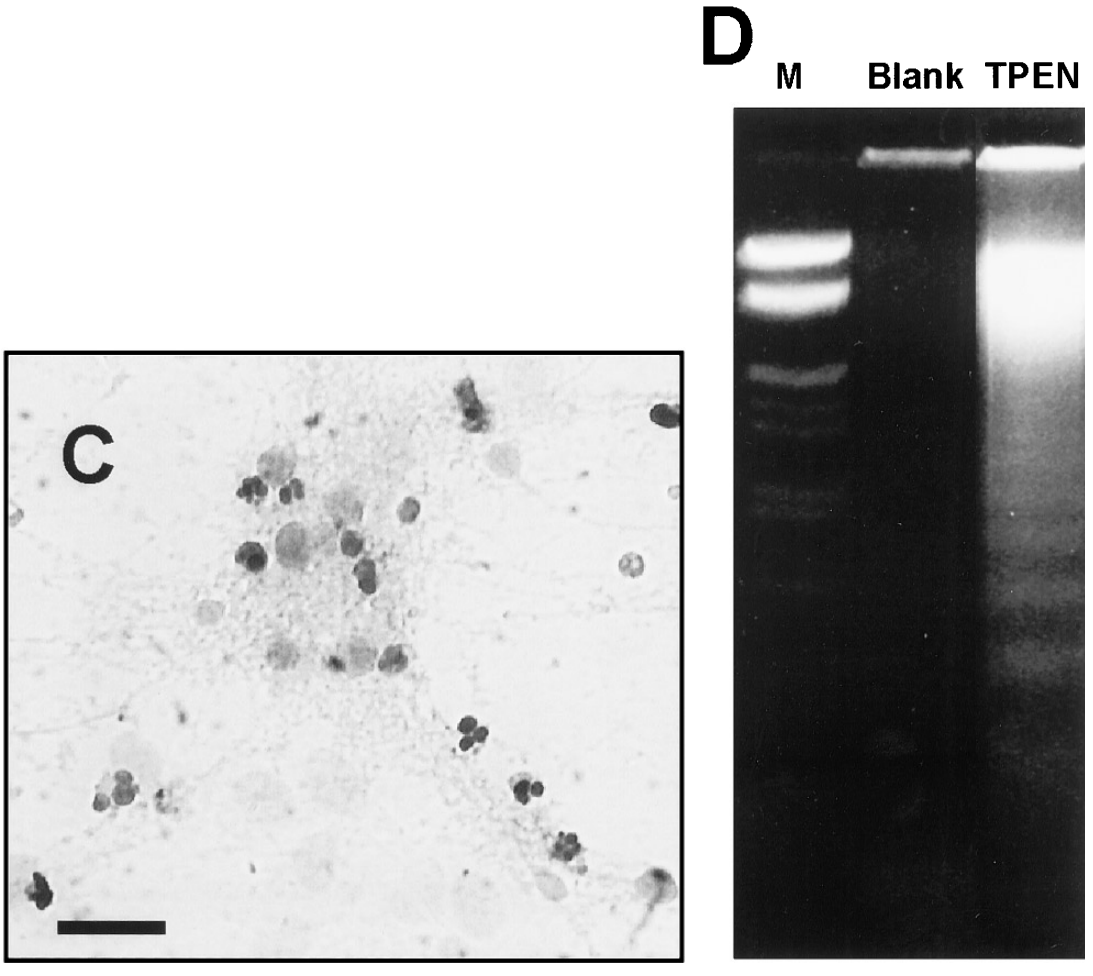

FIG. 3. Morphological and biochemical evidences for apoptosis. (A) Electron micrograph of cortical cultures exposed for 12 h to 2 μM TPEN. Note nuclear (N) condensation and fragmentation. Scale bar, 1 μm. (B) Another EM field showing relatively chromatin condensation at the margin of nucleus and relatively intact intracellular organelles (G, Golgi apparatus; arrowheads, mitochondria; arrows, endoplasmic reticulum). Scale bar, 1 μm. (C) TUNEL staining also revealed *in situ* DNA and nuclear fragmentation in cortical cultures after 24 h exposure to 2 μM TPEN. Scale bar, 50 μm. (D) DNA agarose gel electrophoresis revealed internucleosomal DNA fragmentation in cortical cultures exposed for 12 h to 2 μM TPEN (M, marker), but not in sister cultures with sham wash alone (Blank).

sally harmful to cells (11, 20, 32, 33). Considering many normal roles served by zinc (50, 51), this may be a highly expected outcome of severe intracellular zinc depletion. One intriguing fact, however, is that the zinc depletion-induced neuronal death in cortical cultures occurs by macromolecule synthesis-dependent apoptosis. This is different from a study in thymocytes where macromolecule synthesis inhibition did not block TPEN-induced cell death (33). This discrepancy again seems to support the widely held view that different cell types may activate different sets of apoptosis-executing machinery (52, 53). Although zinc depletion may enhance oxidative stress, for instance by decreasing the activity of Cu/Zn-SOD (43), this is unlikely the main mechanism of the TPEN-induced neuronal apoptosis, since a potent antioxidant trolox did not attenuate it.

With growing interests in mechanisms of programmed cell death/apoptosis, it may be important to examine diverse models to gain full insights into the molecular machinery of apoptosis. The present study provides another model for neuronal apoptosis in cortical cultures, in addition to serum deprivation (27, 31), staurosporine (26), voltage-gated calcium channel blocker (25, 27), mitochondrial enzyme inhibitor (2), and β-amyloid (30) models. Since cycloheximide and zVAD-fmk completely block, but other anti-apoptosis measures such as BDNF, IGF-1, PMA, and high K^+ medium fail to attenuate TPEN-induced neuronal death, zinc depletion is likely to trigger apoptosis by activating steps upstream to activation of putative death gene(s) and caspases, but downstream to activation of neurotrophic factor receptors, PKC, or increased intracellular calcium. One possibility is that caspases normally in an inhibited state by cytoplasmic zinc (40), may get activated by removal of zinc. Consistent with this scheme, over-expression of bcl-2, an anti-apoptosis

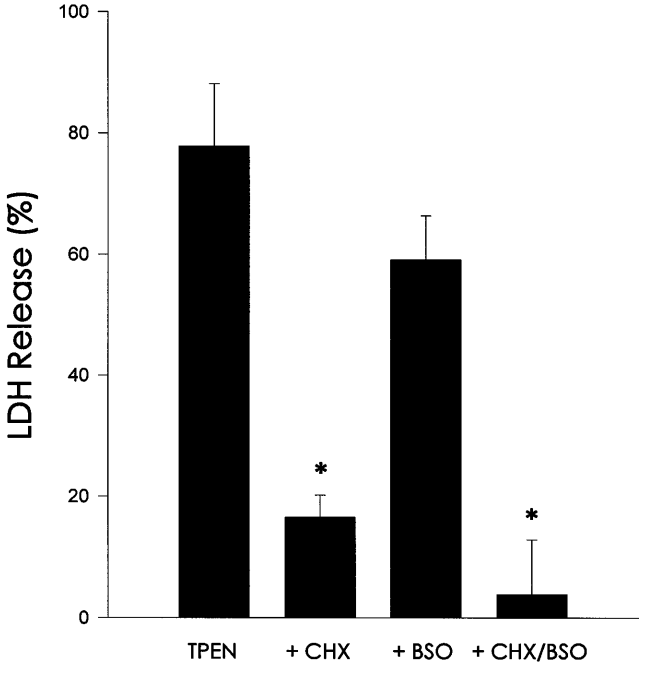

FIG. 4. Protein synthesis inhibition attenuates TPEN-induced neuronal apoptosis. Bars denote LDH release (mean + SEM, $n = 4$) in cortical cultures after 24 h exposure to TPEN alone (TPEN) or in the presence of 1 μg/ml cycloheximide (CHX) or CHX plus 500 μM buthionine sulfoximine (CHX/BSO). Asterisks denote difference from TPEN ($P < 0.01$, two-tailed t test with Bonferroni correction for 3 comparisons).

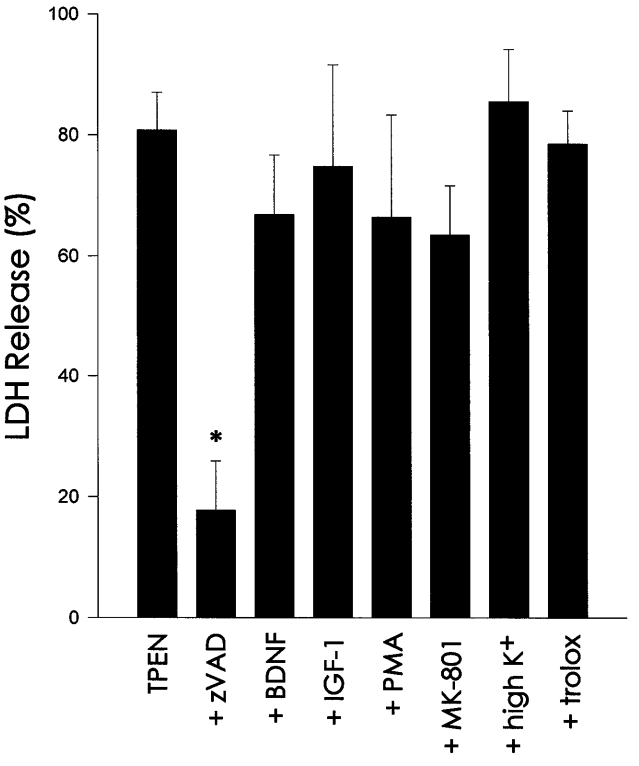

FIG. 5. Other pharmacology of TPEN-induced neuronal apoptosis. Bars denote LDH release (mean + SEM, $n = 4$) in cortical cultures after 24 h exposure to TPEN alone (2 μM) or in the presence of zVAD-fmk (100 μM), BDNF (50 ng/ml), IGF-1 (50 ng/ml), PMA (20 nM), MK-801 (10 μM), additional potassium (high K^+, total 30 mM), or trolox (100 μM).

protein probably acting upstream of the caspase cascade (23), fails to attenuate TPEN-induced apoptosis in the dopaminergic MN9D cell line (Oh and Koh, unpublished).

It is yet unclear whether intraneuronal zinc depletion has any relevance to pathological conditions in the human brain. Suggesting it might, it has been reported that zinc levels are reduced in AD brains (7–9). Several proposals have been made as to the significance of this change. For example, Constantinidis suggested that when intracellular zinc concentration is decreased, the zinc-dependent enzymes of neuronal detoxification, glutamate catabolism, and other neurotransmitter metabolism, may lose their functions, causing neuronal dysfunction (7). Present results provide additional support to the hypothesis that cytoplasmic zinc depletion may contribute to neuronal loss in AD. Recently, several studies reported that zinc promotes aggregation of β-amyloid (3, 4, 12). This finding suggested a key role of endogenous zinc in the development of the central pathology of AD, that is the formation of amyloid plaques in extracellular space (3, 12). Since the aggregation of β-amyloid in turn has been shown to be critical for the induction of neuronal death (30, 41), extracellularly released zinc may contribute to neuronal loss in AD by promoting β-amyloid aggregation. Hence, redistribution of cytoplasmic zinc to amyloid peptides would act as a double insult in AD brains. Further studies seem warranted to examine the possibility that the distribution pattern of the endogenous zinc is indeed altered in AD brains.

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