

Neuroprotective Action of Cycloheximide Involves Induction of Bcl-2 and Antioxidant Pathways

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Abstract. The ability of the protein synthesis inhibitor cycloheximide (CHX) to prevent neuronal death in different paradigms has been interpreted to indicate that the cell death process requires synthesis of “killer” proteins. On the other hand, data indicate that neurotrophic factors protect neurons in the same death paradigms by inducing expression of neuroprotective gene products. We now provide evidence that in embryonic rat hippocampal cell cultures, CHX protects neurons against oxidative insults by a mechanism involving induction of neuroprotective gene products including the antiapoptotic gene *bcl-2* and antioxidant enzymes. Neuronal survival after exposure to glutamate, FeSO₄, and amyloid β -peptide was increased in cultures pretreated with CHX at concentrations of 50–500 nM; higher and lower concentrations were ineffective. Neuroprotective concentrations of CHX caused only a moderate (20–40%) reduction in overall protein synthesis, and induced an increase in *c-fos*, *c-jun*, and *bcl-2* mRNAs and protein levels as determined by reverse transcription-PCR analysis and

immunocytochemistry, respectively. At neuroprotective CHX concentrations, levels of *c-fos* heteronuclear RNA increased in parallel with *c-fos* mRNA, indicating that CHX acts by inducing transcription. Neuroprotective concentrations of CHX suppressed accumulation of H₂O₂ induced by FeSO₄, suggesting activation of antioxidant pathways. Treatment of cultures with an antisense oligodeoxynucleotide directed against *bcl-2* mRNA decreased Bcl-2 protein levels and significantly reduced the neuroprotective action of CHX, suggesting that induction of Bcl-2 expression was mechanistically involved in the neuroprotective actions of CHX. In addition, activity levels of the antioxidant enzymes Cu/Zn-superoxide dismutase, Mn-superoxide dismutase, and catalase were significantly increased in cultures exposed to neuroprotective levels of CHX. Our data suggest that low concentrations of CHX can promote neuron survival by inducing increased levels of gene products that function in antioxidant pathways, a neuroprotective mechanism similar to that used by neurotrophic factors.

CYCLOHEXIMIDE (CHX)¹ is a protein synthesis inhibitor that has been widely used in studies of programmed cell death or apoptosis. Apoptosis and necrosis are two different forms of cell death whose distinguishing characteristics are based largely on morphological features (see Wyllie et al., 1980; for review see Steller, 1995). Cells dying by apoptosis undergo shrinkage, cell surface blebbing, and DNA condensation and fragmenta-

tion; their membranes remain intact as the cell dies. In contrast, cells dying by necrosis swell and lyse. Neuronal apoptosis has been most commonly studied in paradigms of natural developmental cell death in which withdrawal of trophic factor support initiates the cell death program (Deckwerth and Johnson, 1993). However, it is becoming increasingly recognized that apoptosis also occurs in both acute and chronic neurodegenerative conditions in the adult nervous system. For example, neurons may die by apoptosis in cerebral ischemia (MacManus et al., 1993; Linnik et al., 1993), epilepsy (Pollard et al., 1994), Huntington's disease (Portera-Cailliau et al., 1995), and Alzheimer's disease (for review see Cotman and Anderson, 1995). CHX delays or prevents the death of neurons subjected to a range of insults. For example, CHX prevents apoptosis of cultured sympathetic neurons induced by withdrawal of NGF (Martin et al., 1988, 1992) and also prevents trophic factor deprivation-induced death of PC12 cells (Pittman et al., 1993). In addition, CHX protects: cul-

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1. *Abbreviations used in this paper:* A β , amyloid β -peptide; BDNF, brain-derived neurotrophic factor; bFGF, basic FGF; BSO, buthionine sulfoximine; CHX, cycloheximide; DCF, 2,7-dichlorofluorescein diacetate; hnRNA, heteronuclear RNA; NMDA, *N*-methyl-D-aspartate; ODN, oligodeoxynucleotide; RT, reverse transcription; SOD, superoxide dismutase.

tured retinal ganglion cells against excitotoxicity (Dreyer et al., 1995); PC12 cells against glutamate toxicity (Serghini et al., 1994); adult cortical neurons against ischemic injury in vivo (Goto et al., 1990; Linnik et al., 1993; Tortosa et al., 1994); adult septal neurons against NGF withdrawal in vivo (Svendsen et al., 1994); cultured striatal and cortical neurons against the toxicity of 3-nitropropionic acid (Behrens et al., 1995); cultured cortical neurons against oxidative stress-induced death (Ratan et al., 1994a); and cultured cortical neurons against amyloid β -peptide (A β) toxicity (Takashima et al., 1993). One widely accepted interpretation of the ability of CHX to protect neurons is that it prevents the synthesis of "killer" gene products (for review see Schwartz and Osborne, 1993). However, that mechanism of CHX action has not been firmly established and alternative explanations exist, including the quite opposite possibility that CHX induces the expression of cytoprotective gene products. Indeed, CHX is well known to induce increases in levels of an array of immediate early gene mRNAs (Carter, 1993; Oguchi et al., 1994), and recent findings suggest that CHX can activate kinases such as mitogen-activated protein kinase (Zinck et al., 1995) known to be involved in neurotrophic factor signaling cascades (Seger and Krebs, 1995).

Data from a variety of cell death paradigms ranging from withdrawal of trophic factor support in sympathetic neurons (Greenlund et al., 1995a), excitotoxicity (Bondy and LeBel, 1993; Mattson et al., 1995), metabolic impairment (Beal, 1995), and A β toxicity (Mattson et al., 1993a; Behl et al., 1994; Goodman and Mattson, 1994) suggest that free radicals and disruption of calcium homeostasis are convergence points in the cell death process in both apoptosis and necrosis. Further evidence supporting common mechanisms of apoptotic and necrotic neuronal death comes from studies showing that the same neurotrophic factor can protect neurons against death induced by the range of insults mentioned above including: withdrawal of trophic support (Deckwerth and Johnson, 1993); excitotoxicity (Mattson et al., 1989, 1995); metabolic insults (Cheng and Mattson, 1991; Lindvall et al., 1994); cerebral ischemia (Koketsu et al., 1994); and A β toxicity (Mattson et al., 1993a; Goodman and Mattson, 1994). General mechanisms of neuroprotection appear to involve induction of the expression of proteins involved in suppressing free radical accumulation (e.g., antioxidant enzymes) and stabilizing ion homeostasis (e.g., calcium-binding proteins) (for review see Mattson et al., 1993b). For example, NGF, basic FGF (bFGF), and brain-derived neurotrophic factor (BDNF) increased to varying degrees activities of one or more antioxidant enzymes (Cu/Zn-superoxide dismutase [SOD], catalase, glutathione peroxidase, and glutathione reductase) in hippocampal cell cultures (Mattson et al., 1995). Data suggest that BDNF can also induce expression of the antiapoptotic gene product bcl-2 (Allsopp et al., 1995), which is believed to function in antioxidant pathways (Hockenbery et al., 1993). bcl-2 can delay cell death when overexpressed in a variety of cell paradigms of apoptotic and necrotic cell injury (Hockenbery et al., 1990; Martinou et al., 1994; Bredesen, 1995; Chen et al., 1995; Linnik et al., 1995; Lawrence et al., 1996). Sympathetic neurons from mice deficient in bcl-2 die more rapidly after NGF withdrawal than do sympathetic neurons from wild-

type mice (Greenlund et al., 1995b). These kinds of data led us to propose that cell death, whether apoptotic or necrotic, occurs when activation of cell life programs is not sufficient to overcome the level of stress (oxidative or ionic) imposed upon the neuron (Mattson and Barger, 1995). Because neurotrophic factors protect neurons against death in many of the same paradigms in which CHX is neuroprotective, we tested the hypothesis that CHX protects neurons by a mechanism similar to neurotrophic factors, namely, by inducing cytoprotective gene products.

Materials and Methods

Hippocampal Cell Cultures and Experimental Treatments

Dissociated cell cultures of fetal rat hippocampus were established from 18-d embryos and maintained as described previously (Mattson et al., 1989, 1995). Cells were plated in 35-mm-diam plastic or glass-well dishes on a polyethyleneimine substrate in 0.8 ml of medium consisting of MEM with Earle's salts supplemented with heat-inactivated FBS (5%; vol/vol), 1 mM L-glutamine, 1 mM pyruvate, 20 mM KCl, and 26 mM sodium bicarbonate (pH 7.2). Cells were grown at a density of 80–120 cells per mm² culture surface, and experiments were performed in 8–10-d-old cultures, a time period during which neurons express both *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors, and are vulnerable to excitotoxicity (Mattson et al., 1993c), A β toxicity (Mattson et al., 1993a), and FeSO₄ toxicity (Zhang et al., 1993). Cycloheximide, glutamate, buthionine sulfoximine, and FeSO₄ were purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as 200–500 \times stocks in saline (pH 7.2). A β 25–35 (lot ZM500) was purchased from Bachem California (Torrance, CA) and stored in lyophilized form, and 1 mM stocks were prepared by dissolving the peptide in sterile distilled water 2–4 h before use. Oligodeoxynucleotides (ODNs) were purchased from IDT Inc. (Coralville, IA). The sequence of the Bcl-2 antisense ODN was 5'-TCCCGGCTTGCGCCAT-3'. Three different control ODNs were used: sense ODN; missense ODN, 5'-TCGCGCATGCCCAT-3'; and nonsense ODN, 5'-CTGTCGCGCTCGACTC-3'. Immediately before experimental treatment, the culture maintenance medium was replaced with Locke's solution that had the following composition (mM): 154 NaCl; 5.6 KCl; 2.3 CaCl₂; 1.0 MgCl₂; 3.6 NaHCO₃; 5 Hepes; 10 glucose.

Quantification of Neuron Survival

These methods are detailed in our previous studies (Mattson et al., 1989, 1995). Briefly, viable neurons were counted in premarked microscope fields ($\times 10$ objective) before experimental treatment and 20–24 h after treatment. Most neurons that died during the exposure period to glutamate, FeSO₄, or A β were absent at the 20-h time point, and viability of the remaining neurons was assessed by morphological criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable. Neurons with fragmented neurites and a vacuolated and/or swollen soma were considered nonviable.

Protein Synthesis Assay

These methods were those described previously (Martin et al., 1992). Cultures (incubated in methionine- and cysteine-free medium) were pretreated with vehicle or CHX, and then [³⁵S]methionine/cysteine (ICN Radiochemicals, Irvine, CA; sp act >1,000 Ci/mmol) was added to a final concentration of 25 μ Ci/ml (in the continued presence of CHX). 30 min later, cultures were washed once with medium, and 2 ml of a solution containing 0.5% SDS, 1 mM EDTA, 10 mM Tris (pH 7.5) was added. 40 μ g of BSA was added to each sample, and protein was precipitated with 10% TCA on ice. Precipitated protein was collected by filtration onto 0.45- μ m nitrocellulose filters. Filters were washed twice with cold 10% TCA and dried, and radioactivity was quantified in a liquid scintillation counter.

PCR Analyses

Analyses of gene expression were performed as described previously (Es-

tus et al., 1994). Briefly, polyA+ RNA was isolated from cells after the indicated treatments by using an oligo-dT–cellulose-based purification kit (QuickPrep Micro kit; Pharmacia Fine Chemicals, Piscataway, NJ) and concentrated by coprecipitation with glycogen, all as directed by the manufacturer. Half of the mRNA was converted to cDNA by reverse transcription (RT) by using random hexamers (16 μ M) to prime Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies, Grand Island, NY). The 30- μ l reaction contained 50 mM Tris (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 1 mM dTT, 500 μ M each dATP, dTTP, dCTP, dGTP, and 20 U RNasin (Promega, Madison, WI). After 10 min at 20°C, the samples were incubated for 50 min at 42°C, and the reaction was then terminated by heating to 94°C for 5 min.

For PCR amplification of specific cDNAs, stock reactions (50 μ l) were prepared on ice and contained 50 μ M dCTP, 100 μ M each dGTP, dATP, and dTTP, 15 μ Ci α -[³²P]dCTP (3,000 Ci/mmol), 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, 1 μ M each primer, 1 U of Taq polymerase, and 3% of the cDNA synthesized in the RT reaction. The sequences of the primers used in this study were: *NFM* sense primer: 5' ACG CTG GAC TCG CTG GGC AA 3', *NFM* antisense primer: 5' GCG AGC GCG CTC CGC TTG TA 3' (156-bp product); *c-jun* sense primer: 5' ACT CAG TTG TTG TGC CCC AA 3', *c-jun* antisense primer: 5' CGC ACG AAG CCT TCG GCG AA 3' (64-bp product); *c-fos* sense primer: 5' AAT AAG ATG GTC GCA GCC AA, *c-fos* antisense primer: 5' TTG GCA ATC TCG GTC TGC AA 3' (116-bp product); *bcl-2* sense primer: 5'-CTGTACGGCCCCAGCATGCG-3', *bcl-2* antisense primer: 5'-GCTTTGTTTCATGGTACATC-3' (210-bp product). The stock solutions were separated into three equal aliquots that were covered with a drop of mineral oil and subjected to various numbers of PCR cycles to determine the minimum number of cycles necessary to detect the PCR product. The typical reaction conditions were 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. The results depicted here represent 16–22 cycles of amplification. After amplification, the cDNAs were separated by electrophoresis on 12% polyacrylamide gels, visualized by autoradiography of the dried gels, and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The identity of the PCR product amplified by each primer pair was confirmed typically either by direct sequencing (fmole sequencing kit; Promega), or by subcloning the amplified cDNAs into pBluescript (Stratagene, La Jolla, CA) and then sequencing the inserts.

Immunocytochemistry and Western Blot Analyses

These methods were similar to those described previously (Mattson et al., 1993c; Cheng et al., 1994). Briefly, cells were fixed in 4% paraformaldehyde, with membranes permeabilized by exposure for 5 min to 0.2% Triton X-100 in PBS, and placed in blocking serum (0.5% horse serum). Cells were then exposed to primary antibody overnight at 4°C, followed by incubation for 1 h with biotinylated anti-mouse IgG, a 30-min incubation in the presence of ABC reagent (Vector Labs, Inc., Burlingame, CA), and a 5-min exposure to DAB tetrahydrochloride. Parallel control cultures were processed identically except that the primary antibody was deleted from the procedure. The primary antibodies were affinity-purified rabbit anti-c-Fos (1:500) and anti-c-Jun (1:1,000) (Oncogene Science, Inc., Mineola, NY), and mouse monoclonal anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Immunostained cultures were visualized under bright-field optics, and relative levels of immunoreactivity of neuronal cell bodies were scored according to the following scale: 0, no immunoreactivity; 1, weak; 2, moderate; 3, intense; 4, very intense. 100 neurons were scored per culture. All cultures were scored without knowledge of their treatment history. For Western blot analysis, solubilized proteins were separated by SDS-PAGE (12% gel), transferred to a nitrocellulose sheet, and immunoreacted overnight with Bcl-2 antibody in the presence of blocking serum (1:500). The blot was then exposed for 1 h to HRP-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and immunoreactive protein was visualized using a chemiluminescence-based detection kit according to the manufacturer's protocol (ECL kit; Amersham Corp., Arlington Heights, IL).

Measurement of Hydrogen Peroxide Levels

These methods were similar to those described previously (Goodman and Mattson, 1994). Briefly, cells were incubated for 50 min in the presence of 50 μ M 2,7-dichlorofluorescein diacetate (DCF; Molecular Probes, Inc., Eugene, OR), and then were washed three times (2 ml per wash) in HBSS containing 10 mM Hepes and 10 mM glucose. Cells were imaged using a confocal laser scanning microscope (Sarastr 2000; Molecular Dynamics)

coupled to an inverted microscope (Nikon, Inc., Garden City, NY). Cells were located under visible light and a single image was acquired by scanning with the laser (488-nm excitation and 510-nm barrier filter). The laser intensity and photodetector gain were held constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Values of relative DCF fluorescence (average pixel intensity per cell) were obtained using the Imagespace software supplied by the manufacturer (Molecular Dynamics).

Antioxidant Enzyme Activity Assays

Activities of Cu/Zn-SOD, Mn-SOD, and catalase in cultured cells were quantified by methods described previously (Mattson et al., 1995). Cells from 60-mm culture dishes were pelleted by low speed centrifugation and homogenized in 2.5 ml of a nitrogen-purged buffer consisting of 10 mM Hepes, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 0.6 mM MgSO₄ (pH 7.4). The homogenate was centrifuged for 1 h at 100,000 g at 4°C, and the supernatant was used for enzyme assays. For the SOD activity assay, 200 μ l of the supernatant was added to 1.48 ml of 68 mM NaH₂PO₄ containing 1.35 mM EDTA (pH 7.8), 100 μ l of 4 mM xanthine, and 170 μ l of 3.53 mM epinephrine (pH 11.5). After a 5-min incubation at 30°C, 50 μ l of xanthine oxidase was added to the cuvette, and the absorbance was followed for 3 min at 320 nm. To control for variability among lots, xanthine oxidase was diluted until an assay mixture without SOD yielded 0.03 A change per min. Mn-SOD activity was taken as the SOD activity remaining after addition of potassium cyanide (200 mM). One unit of SOD activity was defined as the amount that reduced the absorbance change by 50%, and results were expressed as units per mg protein. For the catalase activity assay, cell homogenate supernatant (1.5 ml) was mixed with 18 μ l absolute ethanol and incubated on ice for 8 min, and 7.8 μ l of 10% Triton X-100 and 2.5 ml of 10 mM NaH₂PO₄ buffer (pH 7.4) were added. Aliquots (0.6 ml) of the solution were added to 20-ml test tubes along with 3.57 ml of cold 6 mM hydrogen peroxide and the tube was vortexed. After 3 min, 0.714 ml of 6 N H₂SO₄ was added and the mixture was vortexed. 5 ml of 0.01 N KMnO₄ was added to the solution, the tube was vortexed, and the absorbance was measured at 480 nm within 60 s using a Spec 21 UV-Vis spectrophotometer (Shimadzu, Inc., Kyoto, Japan). Quantitation was based on the comparison of the tissue samples with a calibration curve of known peroxide concentrations. The activity of the enzyme was expressed as units per mg protein with 1 unit = 1 μ mol of H₂O₂ catalyzed per min.

Results

CHX Protects Hippocampal Neurons against Excitotoxic and Oxidative Injuries in a Concentration-dependent Manner

Hippocampal neurons express NMDA and AMPA/kainate receptors and are vulnerable to glutamate-induced excitotoxicity mediated by calcium influx (Choi, 1987; Mattson et al., 1989) and generation of reactive oxygen species (Lafon-Cazal et al., 1993; Dugan et al., 1995; Mattson et al., 1995). Cultured hippocampal neurons are also vulnerable to killing by a variety of oxidative insults including exposure to FeSO₄ (Zhang et al., 1993) and to A β (see Yankner, 1996; for review Mark et al., 1996); A β -induced death manifests as apoptosis (Loo et al., 1993). Exposure of cultures to 10 μ M glutamate, 5 μ M FeSO₄, or 5 μ M A β resulted in killing 60–70% of the neurons (Fig. 1). Pretreatment of cultures with increasing concentrations of CHX for 16 h before exposure to glutamate, FeSO₄, or A β resulted in biphasic concentration-dependent increase in neuronal survival. CHX concentrations of 50–100 nM significantly increased neuronal survival such that 80–90% of the neurons survived exposure to each of the three insults (Fig. 1). Concentrations of CHX of \geq 1 μ M were ineffective in protecting neurons, and concentrations $>$ 1 μ M caused significant neuronal death during a 24-h exposure period (Fig. 1 D).

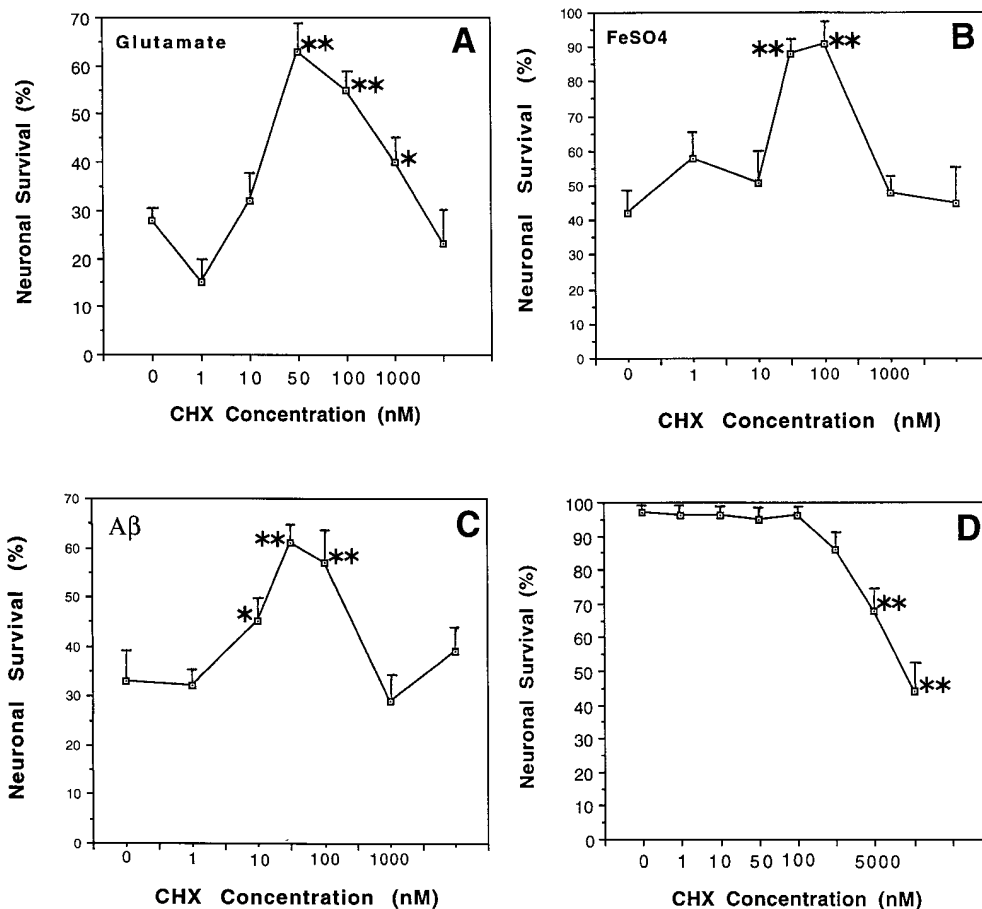


Figure 1. CHX protects cultured hippocampal neurons against excitotoxic and oxidative insults in a concentration-dependent manner. Cultures were preincubated for 16 h with vehicle or the indicated concentrations of CHX, and then were exposed to 10 μ M glutamate (A), 5 μ M FeSO₄ (B), 5 μ M A β (C), or vehicle control (D). Neuronal survival was quantified 20 h later. Values represent the mean and SEM ($n = 4$ separate cultures). * $P < 0.05$, ** $P < 0.01$ compared with 0 CHX value. ANOVA with Scheffe's post-hoc tests.

Neuroprotective Concentrations of CHX Do Not Inhibit Protein Synthesis

Since the concentrations of CHX that were neuroprotective were below those expected to inhibit protein synthesis maximally, we examined the time course and concentration dependence of CHX effects on incorporation of radiolabeled amino acids into TCA-precipitable protein (Fig. 2). Parallel cultures were exposed for increasing times (1, 4, 12, and 24 h) to either 100 nM or 10 μ M CHX. In cultures exposed to 100 nM CHX, levels of protein synthesis were reduced to \sim 60% of control levels within 1 h of exposure, recovered to \sim 80% of control levels by 4 and 12 h, and then decreased somewhat at 24 h (Fig. 2 A). In contrast, levels of protein synthesis in cultures exposed to 10 μ M CHX were reduced to 10% of control levels within 1 h of exposure and remained at this low level through 24 h (Fig. 2 A). In a separate experiment, the concentration dependence of inhibition of protein synthesis by CHX was examined in more detail. Levels of protein synthesis were reduced to 60–75% of control levels in cultures exposed to 10–300 nM CHX for 1 h. CHX concentrations of \geq 1 μ M caused a $>$ 90% inhibition of protein synthesis.

Neuroprotective Concentrations of CHX Induce Expression of mRNA and Protein for *bcl-2*, *c-fos*, and *c-jun*

A characteristic set of genes is rapidly induced when neurons are subjected to toxic insults. Perhaps the most widely

studied is the immediate early gene *c-fos*, which is induced at both the mRNA and protein levels in neural cells subjected to ischemia (Aden et al., 1994; Dragunow et al., 1994), excitotoxins (Ghosh et al., 1994), oxidative insults (Naveilhan et al., 1994; Goldstone et al., 1995), and trophic factor withdrawal (Mesner et al., 1995). We previously used RT-PCR approaches to examine expression of immediate early genes after NGF withdrawal from cultured sympathetic neurons (Estus et al., 1994), and after exposure to A β in cultured rat cortical neurons (Estus et al., 1995). To gain insight into the neuroprotective mechanism of CHX, we determined the effects of CHX on levels of mRNA encoding *c-Fos*, *c-Jun*, and the antiapoptotic gene *Bcl-2*. CHX induced a concentration-dependent increase in levels of *bcl-2*, *c-fos*, and *c-jun* mRNAs (Fig. 3). In the case of *bcl-2* mRNA, a twofold increase occurred in cells exposed to 30 nM CHX, and the peak increase of approximately fivefold occurred in cultures exposed to 100 nM CHX. Higher concentrations of CHX were ineffective in increasing *bcl-2* mRNA levels. In the cases of *c-fos* and *c-jun* mRNAs, the concentration-response curves were also biphasic and exhibited similar profiles with peak increases occurring in cultures exposed to 100–300 nM CHX (Fig. 3). Similar results were obtained in two additional concentration-response experiments using separate cultures. The time course of changes in *bcl-2*, *c-fos* and *c-jun* mRNAs after exposure to 100 nM CHX is shown in Fig. 3 C. The mRNA levels for each gene increased within 10 min of exposure to CHX, peaked by 30 min, and remained elevated for at least 8 h.

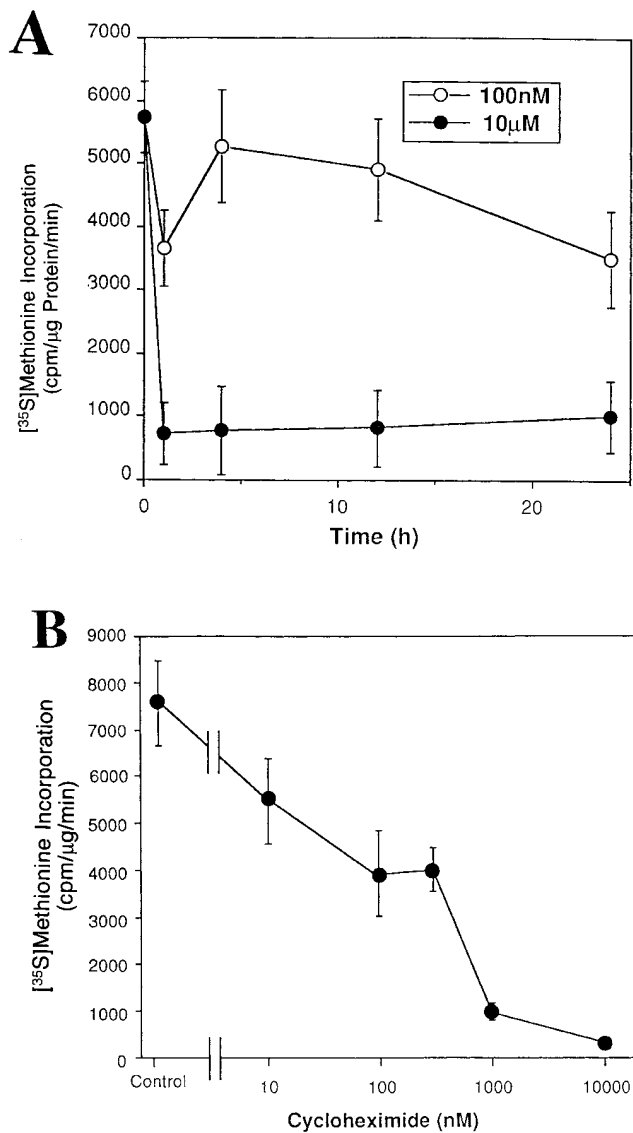


Figure 2. (A) Time course of effects of neuroprotective (100 nM) and high (10 μM) concentrations of CHX on protein synthesis. Cultures were exposed to 100 nM or 10 μM CHX for 1, 4, 12, or 24 h, and incorporation of [³⁵S]methionine into TCA-precipitable protein was quantified. Values are the mean and SEM of determinations made in three separate cultures. For 100 nM CHX, the 1 h value was significantly less than the 0 h value ($P < 0.05$). For 10 μM CHX, the 1, 4, 12, and 24 h values were each significantly less than the 0 h value and each value for cultures exposed to 100 nM CHX ($P < 0.001$). (B) Effect of increasing concentrations of CHX on protein synthesis. Cultures were exposed for 1 h to the indicated concentrations of CHX, and incorporation of [³⁵S]methionine into TCA-precipitable protein was quantified. Values are the mean and SEM of determinations made in three separate cultures. The values for cultures exposed to either 100 or 300 nM CHX were different than the values for cultures exposed to 0 ($P < 0.01$), 10 ($P < 0.05$), 1,000 ($P < 0.001$), and 10,000 ($P < 0.001$) nM CHX. Statistical comparisons used ANOVA with Scheffe's post-hoc tests.

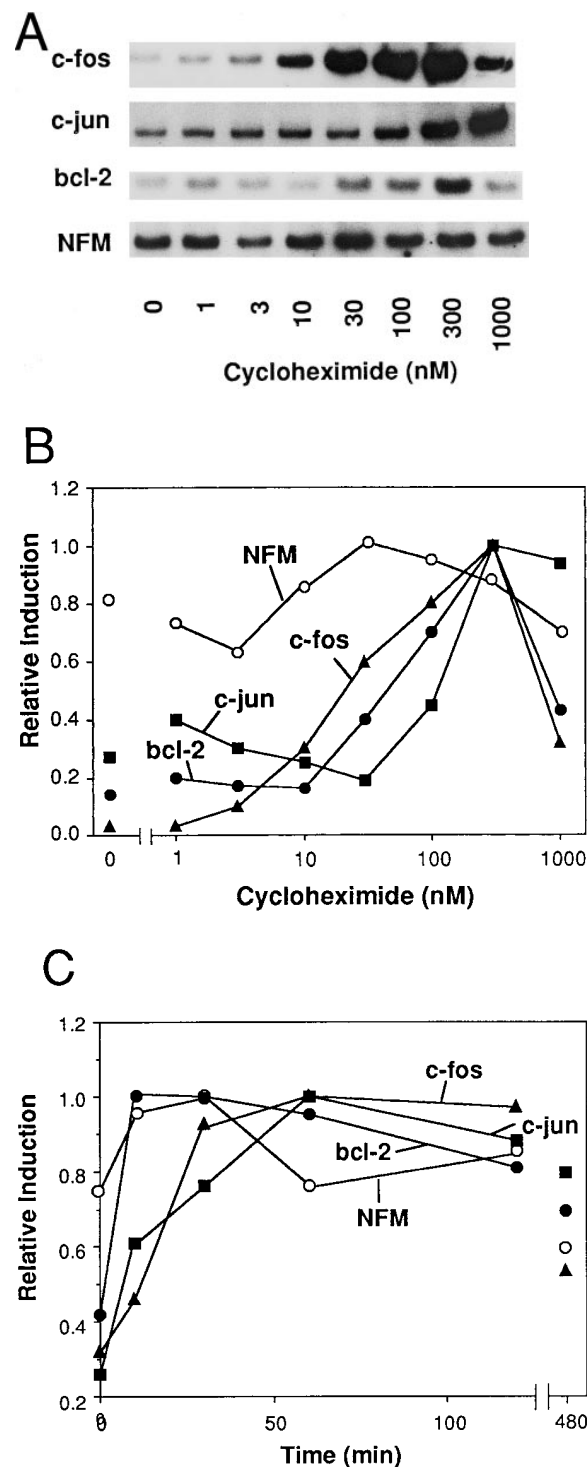


Figure 3. Neuroprotective concentrations of CHX induce expression of *c-fos*, *bcl-2*, and *c-jun* mRNAs. Hippocampal cultures were exposed for 1 h to the indicated concentrations of CHX (A and B), or were exposed to 100 nM CHX for the indicated time periods (C). RNA was isolated, and *c-fos*, *c-jun*, *bcl-2*, and neurofilament (*NFM*) mRNAs were amplified by RT-PCR. Data in B and C are representative of at least three independent experiments.

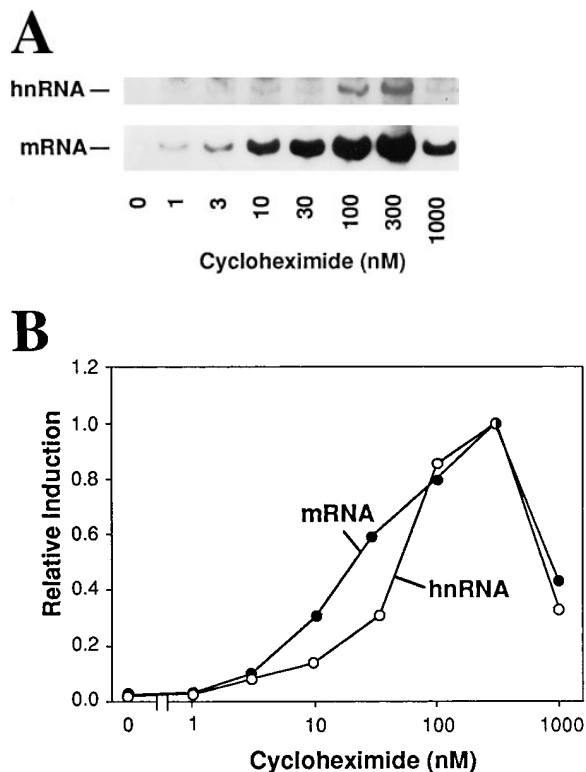


Figure 4. Neuroprotective concentrations of CHX induce *c-fos* transcription. Cultures were exposed for 1 h to the indicated concentrations of CHX and RNA was isolated. (A) RT-PCR analysis of RNA amplified with *c-fos*, *c-jun*, and NFM primers. Locations of *c-fos* heteronuclear RNA (*hnRNA*) and mRNA are indicated at the left. Levels of *c-fos* *hnRNA* were increased in cells exposed to 100 and 300 nM CHX, but not in cells exposed to 1 μ M CHX. (B) Relative levels of *c-fos* mRNA and *c-fos* *hnRNA* in cultures exposed for 1 h to the indicated concentrations of CHX. Similar results were obtained in three independent experiments.

To provide insight into whether the increase of immediate early gene mRNA levels resulted from increased transcription or stabilization of mRNA, we examined levels of *c-fos* heteronuclear RNA (*hnRNA*) in cultures exposed to increasing concentrations of CHX. At the CHX concentrations that were most effective in protecting neurons against excitotoxicity and oxidative insults (100–300 nM), CHX induced a clear increase in the level of *c-fos* *hnRNA* (Fig. 4). Lower and higher concentrations of CHX had little or no effect on *c-fos* *hnRNA* levels. Because the concentrations of CHX effective in protecting neurons against glutamate toxicity and oxidative insults were below concentrations that inhibit protein synthesis maximally, and because *c-fos* and *bcl-2* are induced by neurotrophic factors (Marsh et al., 1993; Downen et al., 1993) that can protect neurons against some of the same insults examined in the present study (Mattson et al., 1993b,c, 1995), we determined the effects of CHX on levels of Bcl-2, c-Fos, and c-Jun proteins. Cultures were exposed for 4 h to increasing concentrations of CHX, and then were immunostained with antibodies to Bcl-2, c-Fos, and c-Jun. Relative levels of neuronal immunoreactivity were quantified (see Materials and Methods). Photomicrographs of control and CHX-

treated cells immunostained with bcl-2 and *c-fos* antibodies are shown in Fig. 5 A. CHX induced concentration-dependent increases in levels of Bcl-2, c-Fos, and c-Jun, with maximum increases occurring in neurons exposed to 100 nM CHX (Fig. 5 B). Higher concentrations of CHX did not increase levels of any of the three proteins.

CHX Suppresses FeSO₄-induced Accumulation of Reactive Oxygen Species, and Depletion of an Endogenous Antioxidant Reduces the Neuroprotective Efficacy of CHX

Since neuroprotective concentrations of CHX induced increased levels of *bcl-2* mRNA and protein, and since *bcl-2* is known to have antioxidant functions (Hockenbery et al., 1993; Kane et al., 1993), we determined whether pretreatment of hippocampal cultures with CHX would reduce levels of oxidative stress in neurons exposed to FeSO₄. Cultures were pretreated with vehicle or 100 nM CHX, and then exposed to FeSO₄ for 20 min. Levels of peroxides were quantified using the DCF probe, and confocal laser scanning microscopy (Goodman and Mattson, 1994). FeSO₄ induced a large increase in peroxide levels (Fig. 6, A and B). The FeSO₄-induced increase of peroxide was attenuated in neurons pretreated with 100 nM CHX (Fig. 6 B), but not with higher or lower concentrations of CHX (10 nM and 1 μ M; data not shown), demonstrating that a neuroprotective concentration of CHX reduces oxidative stress.

If the mechanism whereby CHX protected neurons involved enhancement of antioxidant pathways, then depletion of antioxidants should prevent protection by CHX. Buthionine sulfoximine (BSO) is an agent that causes glutathione depletion by irreversibly inhibiting γ -glutamylcysteine synthase, an enzyme required for glutathione synthesis (Griffith and Meister, 1979). Hippocampal cultures were pretreated with vehicle or 100 nM CHX for 16 h, and then were exposed for 20 h to glutamate alone or in combination with 150 μ M BSO. BSO significantly reduced the excitoprotective effect of CHX (Fig. 6 C). BSO alone was not toxic during a 20-h exposure period.

Bcl-2 Antisense Oligodeoxynucleotides Abrogate the Neuroprotective Actions of CHX

Previous studies used antisense approaches to demonstrate roles for Bcl-2 in prevention of cell death in several cell death paradigms (Kitada et al., 1994; Allsopp et al., 1995; Keith et al., 1995). In preliminary studies we showed that exposure of cultured hippocampal neurons to 10–50 μ M of a Bcl-2 antisense ODN for 16 h resulted in a decrease in levels of Bcl-2 protein as determined by immunocytochemistry (Fig. 7 A) and Western blot analysis (Fig. 7 B). The ability of 100 nM CHX to protect neurons against glutamate toxicity in Bcl-2 antisense ODN-pretreated cultures and control (missense ODN- or vehicle-pretreated cultures) was then determined. Pretreatment with Bcl-2 antisense ODN significantly reduced the neuroprotective action of CHX, whereas the control ODN had no effect on the neuroprotective efficacy of CHX (Fig. 8). 20–35% of the neurons survived exposure to glutamate in the absence of CHX and in cultures pretreated with Bcl-2 antisense ODN plus CHX, whereas 50–65% of the neurons survived in

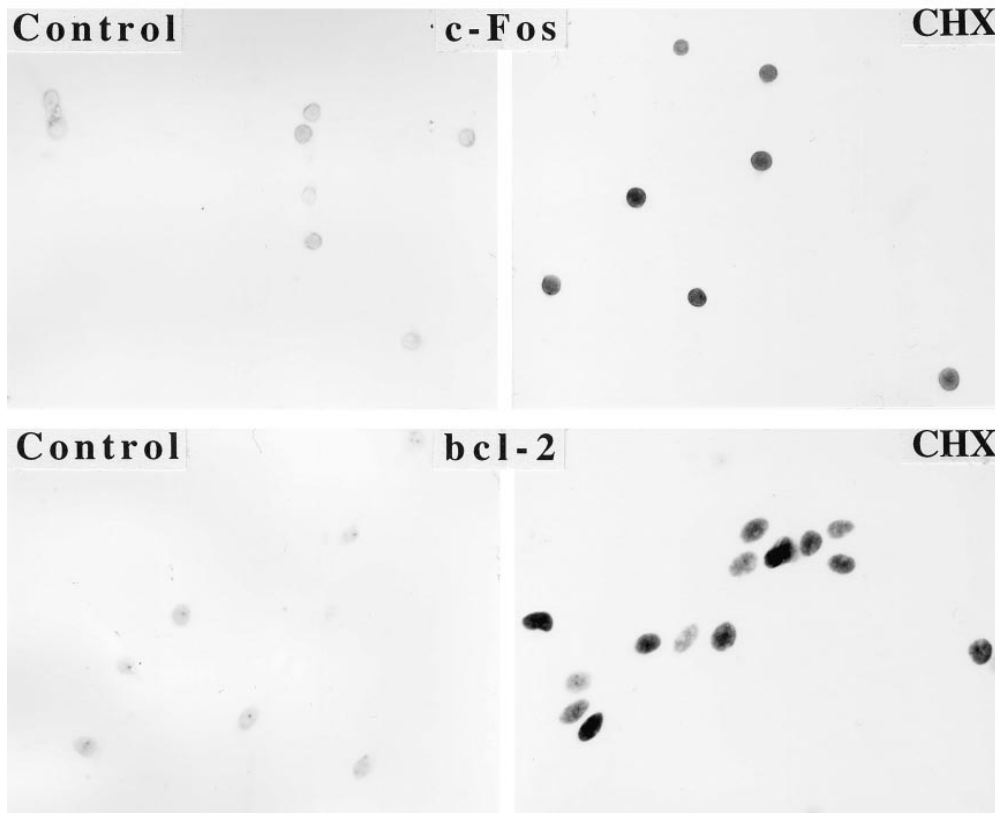
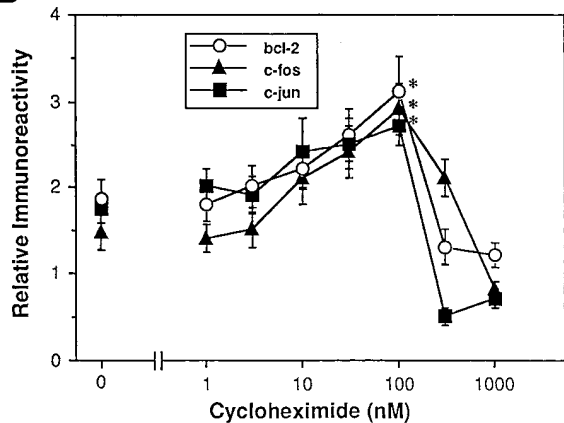
A**B**

Figure 5. CHX induces a concentration-dependent increase in levels of Bcl-2, c-Fos, and c-Jun protein in cultured hippocampal neurons. (A) Cultures were exposed for 5 h to vehicle or 100 nM CHX. Cultures were then immunostained with antibodies to c-Fos or Bcl-2. Note increased nuclear c-Fos and Bcl-2 immunoreactivities in neurons exposed to CHX compared with neurons in control cultures. (B) Relative levels of immunoreactivity of neurons with antibodies to c-Fos, Bcl-2, or c-Jun were determined in cultures exposed for 5 h to the indicated concentrations of CHX. Staining intensity was rated on a scale from 0 to 4 (0, no staining; 1, weak; 2, moderate; 3, strong; 4, very strong). Values are the mean and SEM of determinations made in three separate cultures (100 neurons scored per culture). * $P < 0.01$ compared with corresponding value for cultures not exposed to CHX; ANOVA with Scheffe's post-hoc tests.

cultures pretreated with CHX alone or CHX plus control ODN. Bcl-2 antisense and missense ODNs (Fig. 8), and sense and nonsense ODNs (data not shown), alone had no effect on neuronal survival during a 48-h exposure period.

CHX Increases Antioxidant Enzyme Activity Levels in Hippocampal Cultures

Several different neurotrophic factors that protect neurons against the kinds of oxidative insults examined in the present study have been shown to induce the expression of

one or more antioxidant enzymes in several types of neurons including hippocampal neurons (Jackson et al., 1994; Mattson et al., 1995). Since CHX suppressed accumulation of H_2O_2 and toxicity of oxidative insults, we quantified activities of the antioxidant enzymes catalase, Cu/Zn-SOD, and Mn-SOD in control hippocampal cultures and in cultures pretreated for 24 h with increasing concentrations of CHX. Activities of all three antioxidant enzymes were significantly increased in cultures treated with 100–500 nM CHX (Fig. 9). Lower or higher concentrations of CHX did not affect antioxidant enzyme activities.

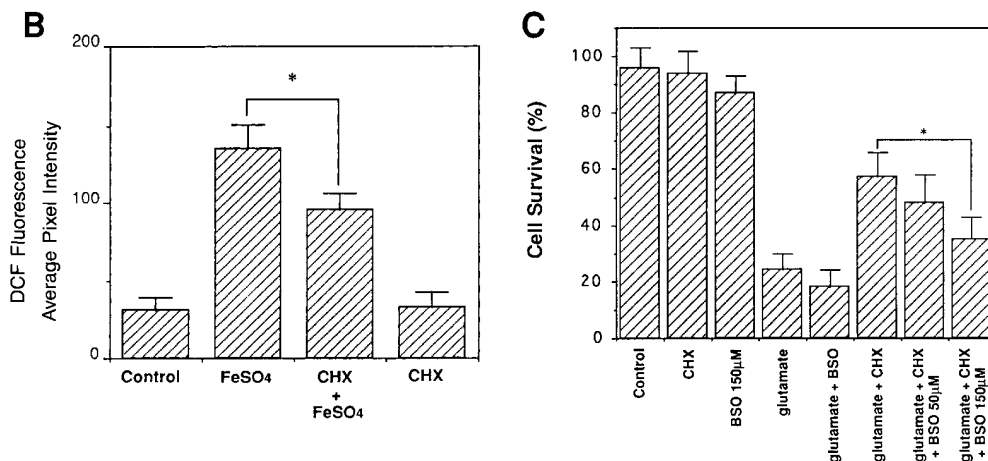
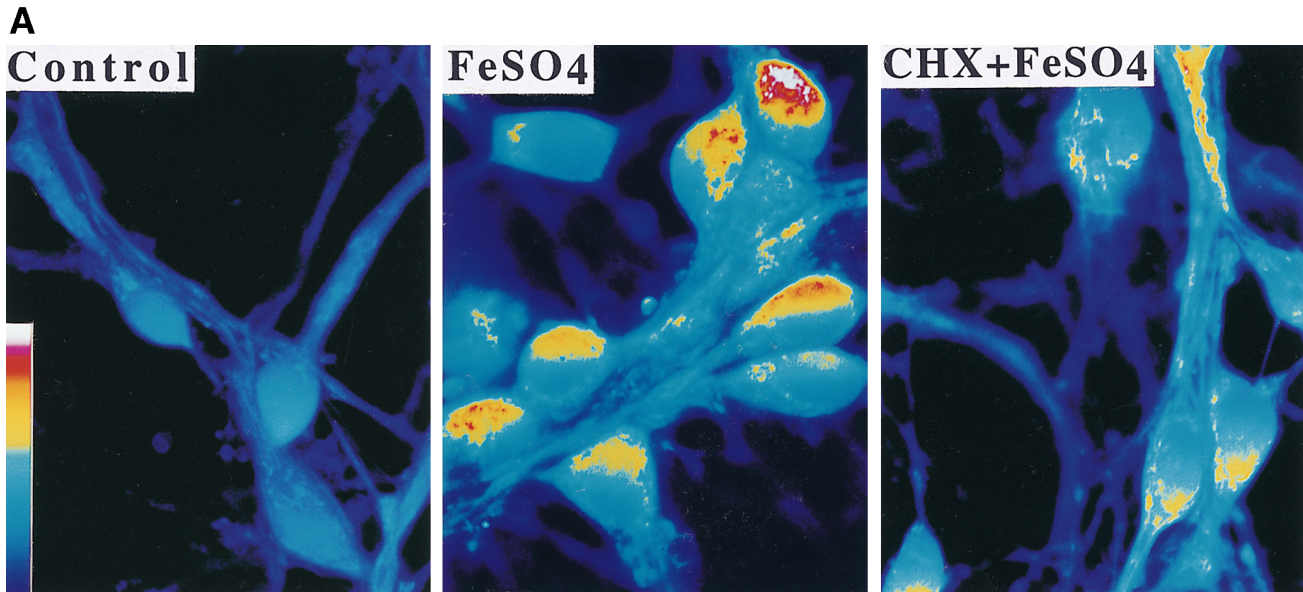


Figure 6. Neuroprotective concentrations of CHX suppress H₂O₂ accumulation induced by FeSO₄. (A) Representative confocal laser scanning microscope images of DCF fluorescence in neurons in a vehicle-treated control culture (left), a culture exposed to 10 µM FeSO₄ for 20 min (middle), and a culture that had been pretreated with 100 nM CHX for 16 h and then exposed to 10 µM FeSO₄ for 20 min (right). Fluorescence intensity is represented by the color scale at the lower left. (B) Cultures were preincubated for 16 h with

100 nM CHX or vehicle (*Control* and *FeSO₄*), and then were loaded with 2,7-dichlorofluorescein diacetate and exposed to vehicle or 10 µM FeSO₄ for 20 min. Average fluorescence in neuronal cell bodies was quantified (see Materials and Methods). Values are the mean and SEM of determinations made in a total of 30–48 neurons in three separate cultures. **P* < 0.02; ANOVA with Scheffe's post-hoc test. (C) Hippocampal cultures were pretreated with vehicle or 100 nM CHX for 16 h, and then were exposed for 20 h to glutamate alone or in combination with BSO as indicated. Values are the mean and SEM of determinations made in four separate cultures. **P* < 0.05; ANOVA with Scheffe's post-hoc test.

Discussion

The ability of CHX to protect cultured hippocampal neurons against the toxicities of glutamate, FeSO₄, and Aβ is in general agreement with previous reports in various cell culture models. CHX protected cultured retinal ganglion cells against NMDA toxicity (Dreyer et al., 1995), protected PC12 cells against glutamate toxicity (Serghini et al., 1994), and protected cultured cortical neurons against oxidative injury (Ratan et al., 1994b) and Aβ toxicity (Takashima et al., 1993). In such prior studies, a single concentration of CHX was usually used and concentration–effect analyses were not performed. Our data clearly show that the neuroprotective actions of CHX in hippocampal cultures are concentration dependent with significant effects being observed within the range of 50–500 nM CHX. It

was reported that only intermediate doses of CHX protect neurons against ischemic injury in adult rats in vivo (Tortosa et al., 1994), a finding consistent with our cell culture data. Moreover, Oguchi et al. (1994) directly demonstrated that low concentrations of CHX that have a negligible effect on protein synthesis can induce a large increase in inducible nitric oxide synthase mRNA levels in cultured macrophages. Several lines of evidence in the present study suggest that the neuroprotective actions of CHX were not the result of inhibition of protein synthesis. First, the most effective concentrations of CHX (50–100 nM) were considerably lower than the concentrations required to inhibit protein synthesis maximally in most cell types and, in our cultures, caused only a 40% reduction in incorporation of radiolabeled amino acids into TCA-precipitable protein. Second, higher micromolar concentrations of

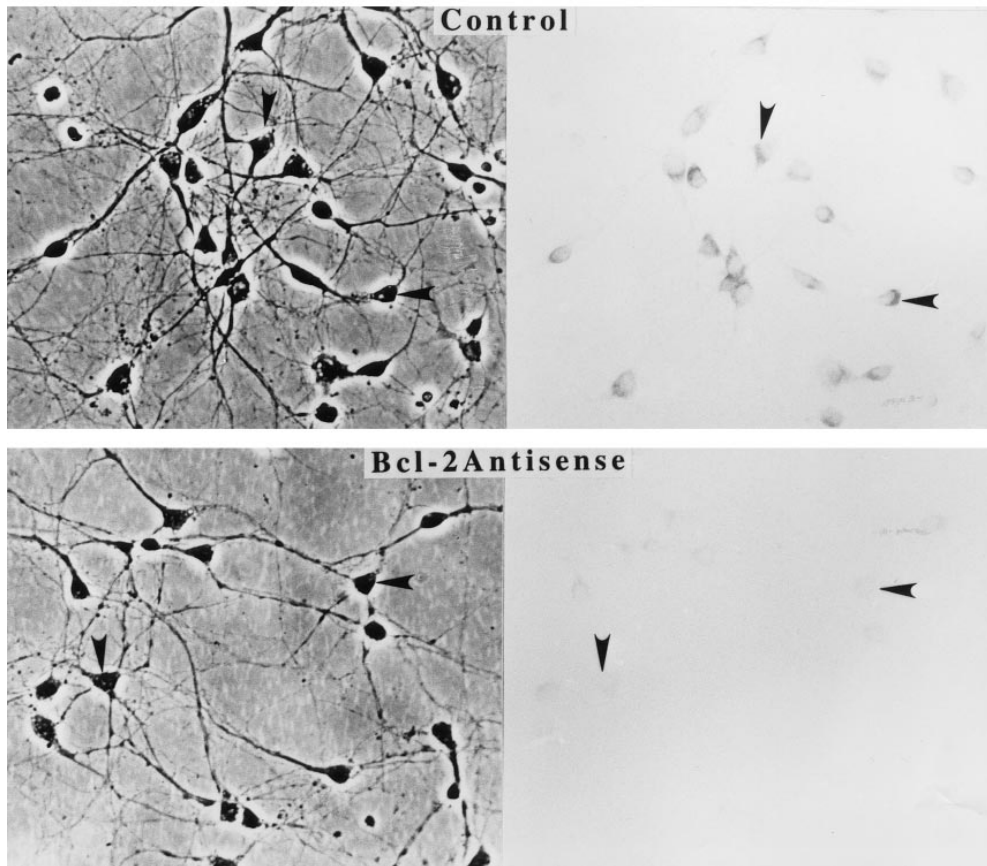
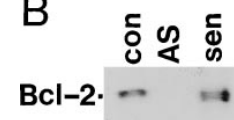
A**B**

Figure 7. Exposure of hippocampal cell cultures to Bcl-2 antisense oligodeoxynucleotide decreases levels of Bcl-2. (A) Parallel cultures were exposed for 16 h to 50 μ M control (sense) ODN or 50 μ M Bcl-2 antisense ODN. Cultures were then fixed and immunostained in parallel using an anti-Bcl-2 primary antibody. Shown are phase-contrast (*left*) and bright-field (*right*) micrographs. Note that levels of Bcl-2 immunoreactivity are greater in neurons in the control culture than in the culture exposed to Bcl-2 antisense ODN (*arrowheads* point to neuron cell bodies). (B) Cultures were exposed for 16 h to vehicle (water), 50 μ M Bcl-2 antisense ODN, or 50 μ M sense ODN. Solubilized proteins were electrophoretically separated, transferred to a nitrocellulose sheet, and immunoreacted with a Bcl-2 antibody. Levels of Bcl-2 were markedly decreased in the culture exposed to Bcl-2 antisense ODN compared with the control cultures.

CHX that inhibited protein synthesis maximally were ineffective in protecting neurons against the excitotoxic and oxidative insults. Third, neuroprotective concentrations of CHX induced increases in levels of Bcl-2, c-Fos, and c-Jun protein (and their encoding mRNAs); increased production of these proteins argues against a general inhibition of protein synthesis at those CHX concentrations. Finally, neuroprotective concentrations of CHX induced increased activity levels of the antioxidant enzymes catalase, Cu/Zn-SOD, and Mn-SOD.

It is increasingly recognized that apoptosis and necrosis often represent different manifestations of cell death that, nevertheless, share common underlying mechanisms. Indeed, the same initiator of cell death can kill the same cell type by either apoptosis or necrosis depending upon intensity/duration parameters of the insult (Ankarcrona et al., 1995). In the present study CHX protected hippocampal neurons against three different insults that are known to engender both apoptosis and necrosis. Our previous studies (Mattson et al., 1989; Mark et al., 1995) and unpublished data from studies of rat hippocampal cell cultures (M.P. Mattson and R.J. Mark) indicate that (at the concentrations used in the present study) glutamate and FeSO₄ kill neurons mainly by necrosis, whereas A β in-

duces mainly apoptotic death (see also Loo et al., 1993). The mechanisms of toxicities of these three insults have been well characterized in previous studies. Glutamate induces calcium influx through NMDA receptors and voltage-dependent channels, and the elevated calcium levels induce production of superoxide anion radical (Lafon-Cazal et al., 1993) and H₂O₂ (Mattson et al., 1995), probably by disrupting mitochondrial transmembrane potential (Mattson et al., 1993d). FeSO₄ kills neurons by inducing hydroxyl radical production and lipid peroxidation; activation of NMDA receptors also contributes to FeSO₄ neurotoxicity in hippocampal cell cultures (Zhang et al., 1993). A β induces membrane oxidation (Butterfield et al., 1994; Behl et al., 1994), which impairs function of ion-motive ATPases (Mark et al., 1995, 1997a) and glucose (Mark et al., 1997b) and glutamate (Keller et al., 1997) transporters, resulting in membrane depolarization and calcium influx; both free radicals and calcium appear to contribute to the neurotoxicity of A β (Mark et al., 1996). Our data indicate that the mechanism whereby CHX protects neurons against these different insults is by enhancing antioxidant defense systems. As evidence, we found that neuroprotective concentrations of CHX induce increased levels of Bcl-2. Moreover, exposure of hippocampal cul-

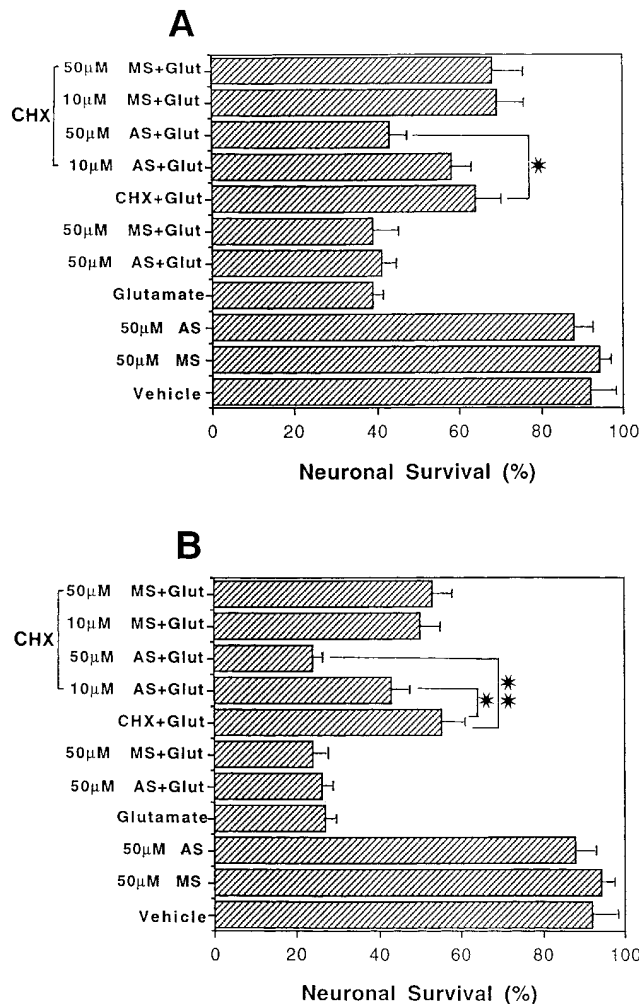


Figure 8. Blockade of the neuroprotective actions of CHX in cultures treated with *bcl-2* antisense oligodeoxynucleotide. Cultures were pretreated for 12 h with 10 or 50 μM *bcl-2* antisense ODN (*bcl-2 AS*) or control missense ODN (*MS*). Cultures were then exposed to vehicle (saline) or 100 nM CHX for 16 h, followed by a 20-h exposure to vehicle (saline) or 10 μM (A) or 50 μM (B) glutamate (*Glut*) in the continued presence of ODN. Neuronal survival was quantified and values (mean and SEM; $n = 3$ cultures) are expressed as a percentage of the initial number of neurons. * $P < 0.05$; ** $P < 0.01$.

tures to Bcl-2 antisense oligonucleotides reduced Bcl-2 levels and abrogated the neuroprotective effects of CHX. These findings suggest that the increase in Bcl-2 induced by neuroprotective concentrations of CHX was mechanistically linked to neuroprotection. Many different studies of both apoptotic and necrotic cell death paradigms have shown that Bcl-2 can protect cells against oxidative insults (Hockenbery et al., 1993; Fukunaga-Johnson et al., 1995; Kane et al., 1995). Measurements of levels of reactive oxygen species including H_2O_2 have shown that expression of Bcl-2 is correlated with reduced levels of oxidative stress in cells exposed to oxidative insults (Kane et al., 1993). Consistent with a role for Bcl-2 in the neuroprotective actions of CHX, we found that pretreatment of hippocampal neurons with neuroprotective concentrations of CHX resulted in a significant attenuation of $FeSO_4$ -induced accu-

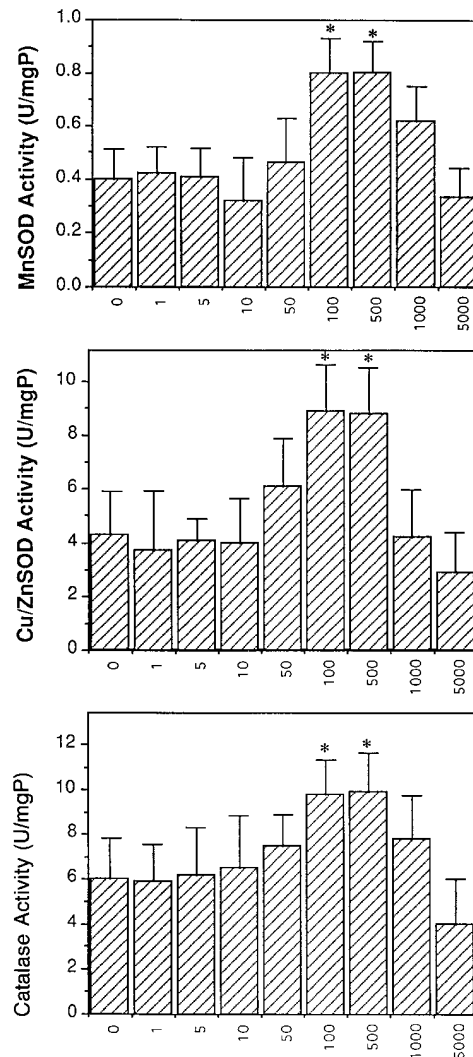


Figure 9. CHX induces increases in antioxidant enzyme activities in cultured hippocampal cells. Cultures were exposed to the indicated concentrations of CHX for 24 h. Activity levels of catalase, Cu/Zn-SOD, and Mn-SOD in cell homogenates were then quantified. Values are expressed in units of enzyme activity per mg protein and represent the mean and SEM of determinations made in three separate cultures. * $P < 0.01$ compared with corresponding cultures not exposed to CHX.

mulation of cellular H_2O_2 . In addition, we found that activity levels of three different antioxidant enzymes were increased in CHX-treated cultures. It is known that oxidative stress itself can induce expression of immediate early gene products and antioxidant enzymes (Goldstone et al., 1995). However, we did not detect an increase in levels of H_2O_2 in cultured hippocampal neurons exposed to neuroprotective concentrations of CHX, suggesting that CHX was likely acting by a more direct effect on expression of the antioxidant enzymes. Another potential mechanism by which CHX might increase resistance to oxidative insults is suggested by the work of Ratan et al. (1994b) who showed that CHX can enhance cellular antioxidant pathways by shunting cysteine from protein synthesis to glutathione. Consistent with the latter mechanism, we found that BSO, which depletes cellular glutathione levels, significantly re-

duced the excitoprotective efficacy of CHX. The cumulative data therefore suggest that there may be several mechanisms, each involving enhancement of antioxidant pathways, whereby CHX protects neurons against excitotoxic and oxidative insults. Our data suggest that, in the cell death paradigms examined in the present study, the neuroprotective mechanism of CHX involves induction of cytoprotective gene products involved in antioxidant pathways rather than suppression of killer gene products. However, because the neuroprotective concentrations of CHX did cause a modest 20% decrease in overall protein synthesis, we cannot completely rule out the possibility that a selective inhibition of production of death genes occurred under those conditions.

The similarities between the neuroprotective actions of CHX demonstrated in the present study and the neuroprotective actions of neurotrophic factors documented in prior studies (for review see Mattson et al., 1993b) are striking. Pretreatment of hippocampal cell cultures with bFGF, NGF, and BDNF protected neurons against glutamate toxicity and suppressed accumulation of reactive oxygen species (Mattson et al., 1989; Cheng and Mattson, 1994; Mattson et al., 1995). bFGF and NGF also protected cultured hippocampal neurons against FeSO₄ toxicity (Zhang et al., 1993). bFGF (Mattson et al., 1993b) and BDNF (Mattson, M.P., unpublished data) protected cultured hippocampal neurons against A β toxicity. Each of these neurotrophic factors has been shown to induce the expression of one or more gene products linked to resistance to oxidative insults. Examples include: bFGF induced Cu/Zn-SOD and glutathione reductase in hippocampal cell cultures and protected neurons against glutamate toxicity (Mattson et al., 1995); NGF induced catalase expression in PC12 cells and protected them against H₂O₂ toxicity (Jackson et al., 1994); BDNF induced glutathione peroxidase in striatal neurons and protected them against metabolic/excitotoxic insults (Spina et al., 1992); and PDGF induced a doubling of catalase and Cu/Zn-SOD activity levels in hippocampal cultures and protected neurons in those cultures against FeSO₄- and glucose deprivation-induced injury (Cheng et al., 1995). None of the latter studies examined the effects of the trophic factors on Bcl-2 levels. However, recent data suggest that BDNF can induce *bcl-2* expression in neurons that are dependent upon BDNF for survival, and that Bcl-2 may mediate the survival response to BDNF (Allsopp et al., 1995). Since we found that Bcl-2 antisense blocked the neuroprotective effects of CHX, the contribution of CHX-induced increases in antioxidant enzyme activities to the neuroprotective action of CHX remains to be established.

While it is well documented that CHX can increase levels of immediate early gene mRNAs (Greenberg and Ziff, 1984), our demonstrations of increased levels of immediate early gene proteins and antioxidant enzyme activities are novel findings. The mechanism whereby CHX induces increased levels of immediate early gene products and antioxidant enzymes may involve stimulation of transcription and/or stabilization of mRNAs. We found that neuroprotective concentrations of CHX induced an increase in *c-fos* hnRNA, indicating that, at least for this gene, CHX can induce transcription. Consistent with our findings are previous data showing that CHX can induce activation of transcription factors (Greenberg and Ziff, 1984; Subrama-

niam et al., 1989; Zinck et al., 1995). On the other hand, CHX has also been shown to reduce degradation of certain mRNAs (Edwards and Mahadevan, 1992) and could, in that way, increase mRNA levels. Such actions of CHX at concentrations below those that inhibit protein synthesis could, in theory, result in increased protein synthesis. Indeed, our data suggest that levels of *c-Fos*, *c-Jun*, and *Bcl-2* were increased in cultures exposed to neuroprotective concentrations of CHX.

Taken together with previous findings, the present data suggest that CHX can suppress cell death by two quite different mechanisms, one involving suppression of production of presumptive death genes, and the other involving induction of expression of cytoprotective gene products. Some prior studies have provided strong evidence that CHX can suppress cell death by inhibiting protein synthesis. For example, in sympathetic neurons where CHX acts at higher concentrations, CHX may suppress cell death by inhibiting synthesis of killer gene products (Martin et al., 1988, 1992). However, in most studies that have used CHX to block cell death, the extent to which the cytoprotective concentrations of CHX suppressed protein synthesis was not determined. Moreover, in *in vivo* studies of cerebral ischemia and other insults that have documented neuroprotective actions of CHX, no information was obtained concerning levels of protein synthesis in the "saved" neurons (e.g., Goto et al., 1990; Linnik et al., 1993; Svendsen et al., 1994; Tortosa et al., 1994). The collective data from the present study demonstrate that CHX protects cortical neurons against insults relevant to the pathogenesis of ischemic and excitotoxic brain injury by inducing production of antioxidant gene products. Therefore, we suggest that the fact that CHX protects neurons against a particular insult does not justify the conclusion that death genes are involved in the cell death process. It will be necessary to rigorously evaluate the mechanism of neuroprotection by CHX in each paradigm and, ultimately, to identify specific life gene products that are induced, or death gene products that are suppressed.

We thank J. Begley, S. Bose, and R. Pelphrey for technical assistance.

This work was supported by grants to M.P. Mattson from the National Institutes of Health (NIH) (NS29001 and NS30583), the Metropolitan Life Foundation, and the Kentucky Spinal Cord and Head Injury Trust, and by grants to S. Estus from the NIH and the Alzheimer's Association.

Received for publication 16 July 1996 and in revised form 2 December 1996.

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