

# Evidence that 4-Hydroxynonenal Mediates Oxidative Stress-Induced Neuronal Apoptosis

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Oxidative stress is believed to play important roles in neuronal cell death associated with many different neurodegenerative conditions (e.g., Alzheimer's disease, Parkinson's disease, and cerebral ischemia), and it is believed also that apoptosis is an important mode of cell death in these disorders. Membrane lipid peroxidation has been documented in the brain regions affected in these disorders as well as in cell culture and *in vivo* models. We now provide evidence that 4-hydroxynonenal (HNE), an aldehydic product of membrane lipid peroxidation, is a key mediator of neuronal apoptosis induced by oxidative stress. HNE induced apoptosis in PC12 cells and primary rat hippocampal neurons. Oxidative insults (FeSO<sub>4</sub> and amyloid  $\beta$ -peptide) induced lipid peroxidation, cellular accumulation of HNE, and apoptosis. Bcl-2 prevented apoptosis of PC12 cells

induced by oxidative stress and HNE. Antioxidants that suppress lipid peroxidation protected against apoptosis induced by oxidative insults, but not that induced by HNE. Glutathione, which binds HNE, protected neurons against apoptosis induced by oxidative stress and HNE. PC12 cells expressing Bcl-2 exhibited higher levels of glutathione and lower levels of HNE after oxidative stress. Collectively, the data identify that HNE is a novel nonprotein mediator of oxidative stress-induced neuronal apoptosis and suggest that the antiapoptotic action of glutathione may involve detoxification of HNE.

**Key words:** Alzheimer's disease; amyloid  $\beta$ -peptide; Bcl-2; glutathione; hippocampal neurons; iron; lipid peroxidation; mitochondria; programmed cell death; reactive oxygen species; vitamin E

Oxidative stress may contribute to neuronal dysfunction and death in an array of disorders, including stroke, Alzheimer's disease (AD), and Parkinson's disease (for review, see Jesberger and Richardson, 1991; Mattson et al., 1996; Simonian and Coyle, 1996). Reactive oxygen species (ROS) are generated in several metabolic pathways, a major source being mitochondrially derived superoxide anion radical, which gives rise to hydrogen peroxide, which is converted further to hydroxyl radical, which induces membrane lipid peroxidation (Evans, 1993). Systems that detoxify ROS include the enzymes superoxide dismutase, catalase, and glutathione peroxidase and the thiol tripeptide glutathione (GSH). Oxidative stress occurs in neurons exposed to excitotoxins (Lafon-Cazal et al., 1993; Dugan et al., 1995; Mattson et al., 1995a), metabolic poisons (Beal et al., 1995), ischemia (Chan, 1996), and amyloid  $\beta$ -peptide (A $\beta$ ) (Behl et al., 1994; Goodman and Mattson, 1994). By damaging ion transport proteins and other regulatory systems in membranes, lipid peroxidation may be particularly detrimental to neurons (Mark et al., 1995, 1997).

Apoptotic cell death is characterized by cell shrinkage, cell surface blebbing, chromatin condensation, and DNA fragmentation with maintenance of membrane and organellar integrity;

macromolecular synthesis inhibitors can prevent apoptosis, suggesting an active or "programmed" mechanism of cell death (for review, see Bredesen, 1995; Johnson et al., 1995; Steller, 1995; Thompson, 1995). Necrosis is another form of cell death in which cells swell, mitochondria and other organelles are damaged, and the plasma membrane ruptures. Neuronal apoptosis may occur in stroke (Linnik et al., 1993; MacManus et al., 1993; Nitoro et al., 1995), AD (Loo et al., 1993; Su et al., 1994; Anderson et al., 1995), and Huntington's disease (Portera-Cailliau et al., 1995). The involvement of ROS in apoptosis is suggested by studies showing that apoptotic stimuli induce accumulation of ROS in neurons and that antioxidants can prevent apoptosis (Kane et al., 1993; Whittemore et al., 1994; Ferrari et al., 1995; Greenlund et al., 1995; Mark et al., 1995).

Proapoptotic gene products such as interleukin-1 $\beta$ -converting enzyme (ICE) (Troy et al., 1996a) and antiapoptotic gene products such as Bcl-2 (Hockenbery et al., 1993; Kane et al., 1993; Mah et al., 1993; Reed, 1994; Wiedau-Pazos et al., 1996) may play roles in oxidative stress-induced apoptosis. On the other hand, nonprotein mediators of apoptosis have not been identified. 4-Hydroxynonenal (HNE), an aldehydic product of lipid peroxidation, is cytotoxic to non-neuronal cells at concentrations reached when cells are exposed to various oxidative insults (Esterbauer et al., 1991). HNE forms covalent cross-links with proteins via Michael addition to lysine, cysteine, and histidine residues (Uchida and Stadtman, 1993; Uchida et al., 1994); HNE normally is detoxified by conjugation with GSH (Spitz et al., 1990; Grune et al., 1994; Ullrich et al., 1994). It is not known whether HNE induces apoptosis nor whether HNE is involved mechanistically in oxidative stress-induced apoptosis. We now report that HNE is generated in response to apoptotic oxidative insults and can induce neuronal apoptosis at pathophysiologically relevant concentrations.

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## MATERIALS AND METHODS

**Materials.** HNE, propanal, pentanal, heptanal, and nonenal were purchased from Cayman Chemical (Ann Arbor, MI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hexanal, malondialdehyde, vitamin E ( $\alpha$ -tocopherol), propyl gallate, glutathione (GSH)-ethyl ester, cycloheximide, actinomycin-D, ATA, and buthionine sulfoximine (BSO) were obtained from Sigma (St. Louis, MO). Nonaldehyde was from Aldrich (Milwaukee, WI), and *trans*-2-nonenal was from Wako Pure Chemicals (Osaka, Japan). Aldehydes were prepared as 200–500 $\times$  stocks in ethanol; ethanol vehicle (0.2–0.5% final concentration) was added to control cultures. A $\beta$ 25–35 was purchased from Bachem (Torrance, CA) and dissolved in water at a concentration of 2 mM 2–4 hr before experiments. Hoescht 33342, propidium iodide, rhodamine 123, and monochlorobimane were obtained from Molecular Probes (Eugene, OR). The antibody against HNE-conjugated proteins was generated and characterized as described in our previous study (Waeg et al., 1996). FITC-Annexin-V was obtained from Nexins Research.

**PC12 and rat hippocampal cell cultures and experimental treatments.** Control vector-transfected PC12 cells (PC12-V) and PC12 cells expressing high levels of human Bcl-2 (PC12-Bcl2) were established by methods described in our previous studies (Kane et al., 1993; Zhong et al., 1993a). Cultures were maintained at 37°C (5% CO<sub>2</sub> atmosphere) in RPMI-1640 medium supplemented 10% with heat-inactivated horse serum and 5% with heat-inactivated fetal bovine serum; immediately before experimental treatment the medium was replaced with RPMI-1640 containing 1% fetal calf serum. Rat hippocampal cell cultures were established from 18 d embryos, as described previously (Mattson et al., 1995b); experiments were performed in cells that had been in culture for 6–8 d. Immediately before experimental treatment the culture medium was replaced with Locke's solution containing (in mM): NaCl 154, KCl 5.6, CaCl<sub>2</sub> 2.3, MgCl<sub>2</sub> 1.0, NaHCO<sub>3</sub> 3.6, glucose 5, and HEPES 5, pH 7.2.

**Analyses of necrosis and apoptosis.** Numbers of trypan blue-positive (necrotic) and -negative cells were counted in four 20 $\times$  fields per culture (typically 70–100 cells/20 $\times$  field), and the percentage of trypan blue-positive cells in each culture was calculated. Under control conditions <5% of the cells were trypan blue-positive. Methods used to establish apoptotic cell death included Hoescht and propidium iodide staining of DNA, annexin-V binding, and suppression of cell death by macromolecular synthesis inhibitors. For Hoescht and propidium iodide staining, cells were fixed in 4% paraformaldehyde, membranes were permeabilized with 0.2% Triton X-100, and cells were stained with the fluorescent DNA-binding dyes Hoescht 33342 or propidium iodide, as described previously (Mark et al., 1995). Hoescht-stained cells were visualized and photographed under epifluorescence illumination (340 nm excitation and 510 nm barrier filter) with a 40 $\times$  oil immersion objective (200 cells per culture were counted, and counts were made in at least four separate cultures per treatment condition; analyses were performed without knowledge of the treatment history of the cultures). The percentage of apoptotic cells (cells with condensed and fragmented DNA) in each culture was determined. Images of propidium iodide-stained cells were acquired with a confocal laser scanning microscope (488 nm excitation and 510 nm barrier filter; Molecular Dynamics, Sunnyvale, CA) with a 60 $\times$  oil immersion objective. Alterations in plasma membrane phospholipids linked to apoptosis were detected by fluorescently labeled annexin-V, as described previously (Vermees et al., 1995; Van Engeland et al., 1996). After experimental treatment, cells were exposed for 10 min to FITC-annexin-V, using conditions recommended by the manufacturer (Nexins Research), and confocal images of annexin-V fluorescence were acquired.

**Quantification of lipid peroxidation and HNE levels.** The thiobarbituric acid reactive substances (TBARS) method was used to assess membrane lipid peroxidation, as described previously (Goodman et al., 1996); values were normalized to protein content. Levels of HNE were quantified by dot-blot analysis. After experimental treatment, culture medium was removed and stored at –80°C. Cells were washed three times with PBS, scraped in a lysis buffer containing 0.1% SDS, and sonicated. Samples (50  $\mu$ l of medium; or 10<sup>6</sup> cells, which corresponds to ~50  $\mu$ l cell volume) were blotted onto a nitrocellulose sheet with a dot-blot apparatus (Bio-Rad, Hercules, CA); a standard curve was generated by medium or cell homogenates containing HNE at concentrations ranging from 0.1–5  $\mu$ M. Then the membrane was washed with TTBS, blocked by incubation in the presence of 5% milk, exposed to HRP-conjugated anti-mouse secondary antibody, and detected by a chemiluminescence kit (Amersham, Arlington Heights, IL). Densitometric analysis of dots in images of the blots was used to calculate levels of HNE. It should be noted that this dot-blot

method is not truly quantitative and that the values reported are only an estimate of the actual HNE levels.

**Immunocytochemistry and Western blot analyses.** Methods for Western blot and immunocytochemical analyses of HNE-protein conjugates were similar to those described previously (Waeg et al., 1996; Mark et al., 1997). For immunostaining, cultured cells were fixed for 30 min in 4% paraformaldehyde/PBS, and membranes were permeabilized by incubation in 0.2% Triton X-100 in PBS. Cells were incubated for 1 hr in blocking serum (1% normal horse serum in PBS), for 3 hr in the presence of anti-HNE mouse monoclonal antibody (1:100 in PBS), for 1 hr in PBS containing biotinylated horse anti-mouse secondary antibody, and for 30 min in ABC reagent (Vector Laboratories, Burlingame, CA). Levels of cellular immunoreactivity were assessed by using a semiquantitative method described previously (Mattson, 1992); staining intensity of cell bodies was scored on a scale from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining). A total of 400 cells in four separate cultures per condition (100 cells/culture) were scored in a blinded manner without knowledge of their treatment history. For Western blot analysis, solubilized cell proteins were separated by electrophoresis in a 10% polyacrylamide gel, transferred to a nitrocellulose sheet, and immunoreacted with an antibody against HNE-conjugated proteins (clone 1 g4; Waeg et al., 1996). The nitrocellulose sheet was processed further with HRP-conjugated anti-mouse secondary antibody and a chemiluminescence detection method (Amersham).

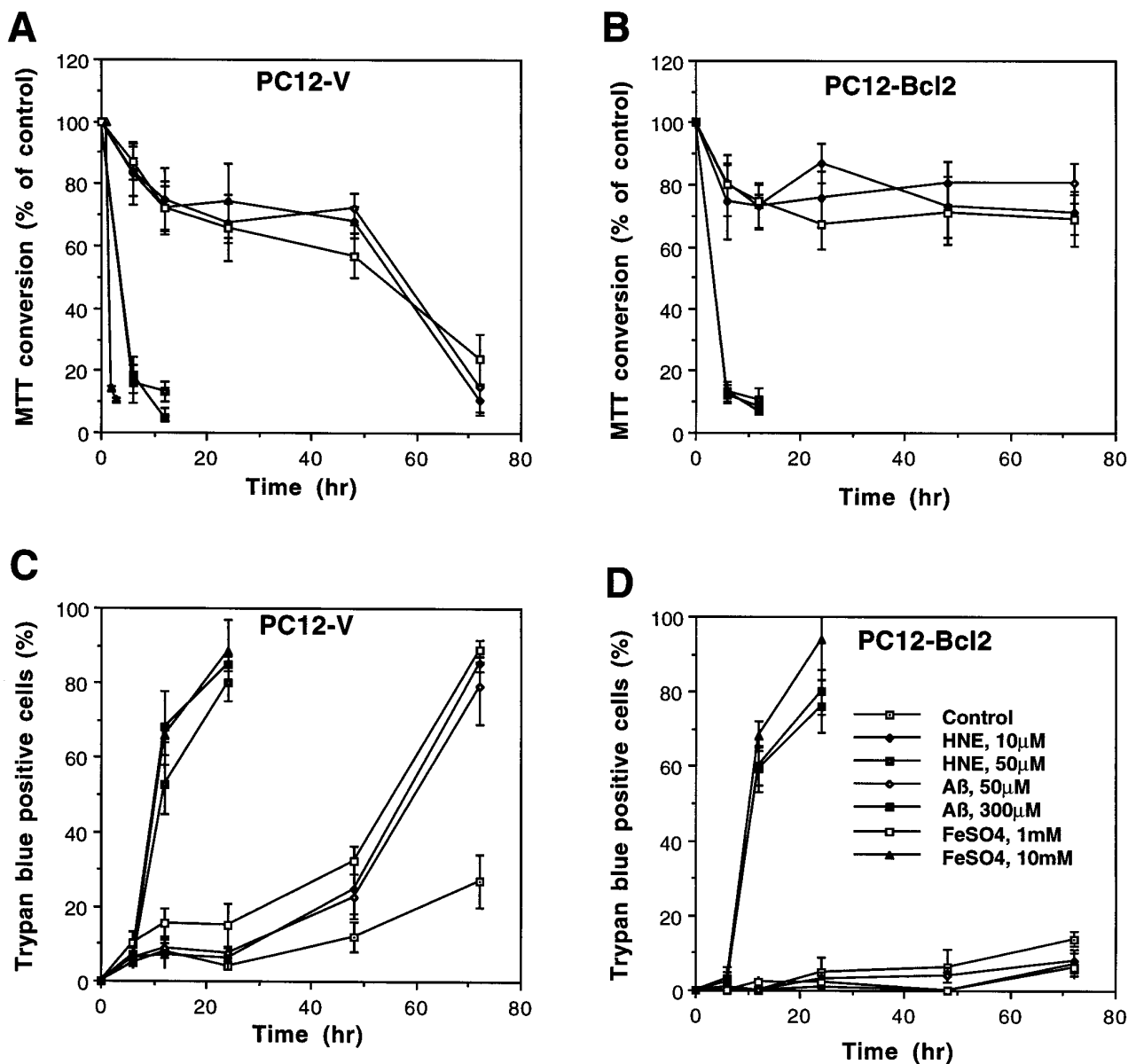
**Assessments of mitochondrial function.** The conversion of the dye MTT to formazan crystals in cells has been shown to be related to mitochondrial respiratory chain activity (Musser and Oseroff, 1994) and mitochondrial redox state (Shearman et al., 1995). Levels of cellular MTT reduction were quantified as described previously (Mattson et al., 1995b). The dye rhodamine 123 was used as a measure of mitochondrial transmembrane potential by methods described previously (Mattson et al., 1993).

**Quantification of GSH levels.** Two methods were used to quantify cellular GSH levels. The first method used monochlorobimane, a fluorescent probe for GSH (Barhoumi et al., 1995), and procedures described in our previous study (Kane et al., 1993). The second method was based on an enzymatic recycling procedure in which GSH is oxidized sequentially by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase and followed a protocol similar to that reported by Tietze (1969). A GSH standard curve was generated by analysis of serial dilutions of pure GSH. GSH levels were adjusted to sample protein content (determined with a Pierce BCA kit, Rockford, IL), and values were expressed as U/mg protein.

## RESULTS

### Oxidative insults and HNE induce delayed apoptosis in PC12 cells

Cultured control (PC12-V) and Bcl-2-expressing (PC12-Bcl2) PC12 cells were exposed to increasing concentrations of FeSO<sub>4</sub>, A $\beta$ , and HNE, and mitochondrial function and cell survival were quantified by MTT and trypan blue exclusion assays, respectively. FeSO<sub>4</sub> and A $\beta$  are known to induce lipid peroxidation and to kill cultured neurons (Poli et al., 1985; Zhang et al., 1993; Behl et al., 1994; Goodman and Mattson, 1994; Muller and Kriegstein, 1995; Goodman et al., 1996). Exposure of PC12-V or PC12-Bcl2 cells to high concentrations of FeSO<sub>4</sub>, A $\beta$ , and HNE resulted in rapid decreases in levels of MTT reduction to <20% of basal levels within 6 hr of exposure (Fig. 1A,B). Exposure of PC12-V cells to lower concentrations of each insult (1 mM FeSO<sub>4</sub>, 50  $\mu$ M A $\beta$ , or 10  $\mu$ M HNE) resulted in a moderate (20–30%) decrease in levels of MTT reduction during the first 12–24 hr; values stayed at this level through 48 hr of exposure and then decreased further (to 10–30% of basal levels) during a subsequent 24 hr period. In contrast, levels of MTT reduction were maintained at 80–90% of basal levels in PC12-Bcl2 cells exposed to the lower concentrations of FeSO<sub>4</sub>, A $\beta$ , and HNE throughout the entire 72 hr exposure period (Fig. 1A,B). Using rhodamine 123 fluorescence as an indicator of mitochondrial transmembrane potential (Mattson et al., 1993), we found that 10  $\mu$ M HNE caused a significant (>50%) decrease in rhodamine 123 fluorescence in PC12-V cells,

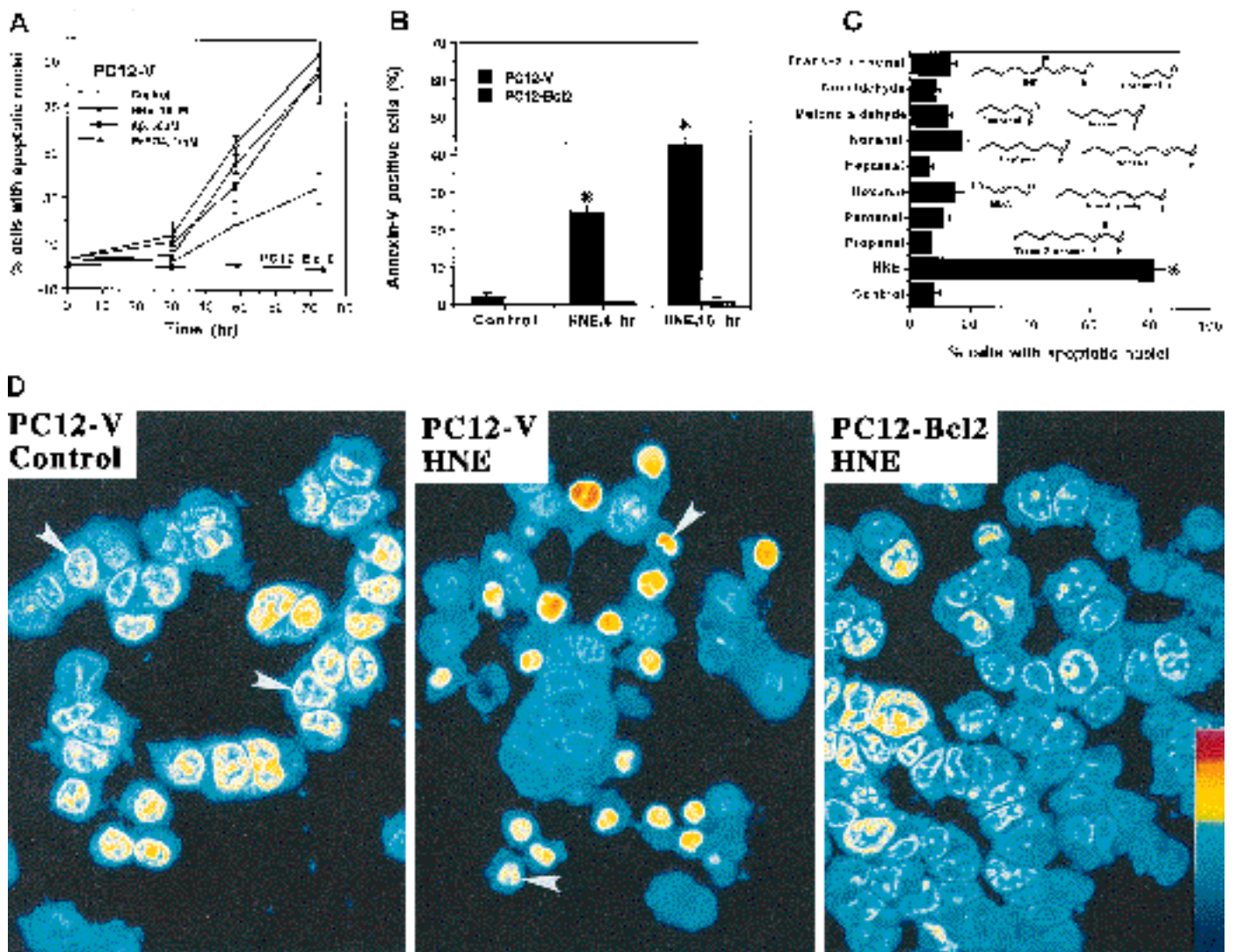


**Figure 1.** Oxidative insults and HNE induce rapid and delayed cell death in PC12 cells: Bcl-2 prevents delayed cell death. *A, B*, PC12-V (*A*) and PC12-Bcl2 (*B*) cells were exposed to the indicated concentrations of HNE, Aβ, or FeSO<sub>4</sub> for the indicated time periods, and levels of MTT reduction were quantified (mean and SEM of determinations made in four culture wells/condition). *C, D*, PC12-V cells (*C*) and PC12-Bcl2 cells (*D*) were exposed to vehicle (Control) or the indicated concentrations of HNE, Aβ, or FeSO<sub>4</sub>, and trypan blue-positive and nonstaining cells were counted. Values are the mean percentage of trypan blue-positive cells in four separate cultures.

but not in PC12-Bcl2 cells, during a 24 hr exposure period (data not shown). High concentrations of FeSO<sub>4</sub>, Aβ<sub>25–35</sub>, and HNE induced relatively rapid cell death in both PC12-V and PC12-Bcl2 cells that occurred within 12–24 hr of exposure (Fig. 1*C,D*). Lower concentrations of FeSO<sub>4</sub>, Aβ, and HNE induced delayed uptake of trypan blue in PC12-V cells that occurred 48–72 hr post-treatment; the delayed uptake of trypan blue likely was attributable to secondary necrosis (Slater et al., 1995). In contrast to PC12-V cells, PC12-Bcl2 cells were not killed by the lower concentrations of FeSO<sub>4</sub>, Aβ, or HNE (Fig. 1*C,D*), consistent with an apoptotic mechanism of cell death.

To establish whether the delayed cell death induced by oxidative insults and HNE in PC12-V cells was apoptotic, we examined nuclear condensation and fragmentation by using fluorescent

DNA-binding dyes Hoescht 33342 and propidium iodide (Fig. 2). Exposure of PC12-V cells to concentrations of FeSO<sub>4</sub>, Aβ, and HNE that caused delayed death resulted in the appearance of many cells exhibiting nuclear condensation and fragmentation, which occurred progressively beginning at ~30 hr post-treatment (Fig. 2*A,D*). In contrast, no PC12-Bcl2 cells exhibited nuclear signs of apoptosis during the 72 hr exposure period to FeSO<sub>4</sub>, Aβ, or HNE. An early event in apoptosis is a loss of plasma membrane asymmetry, resulting in the exposure of phosphatidylserine on the cell surface, which binds annexin-V (Martin et al., 1995). In control PC12-V cultures annexin-V-positive cells were rare (<5% of the cells). When PC12-V cells were exposed to 10 μM HNE, there was a relatively rapid and progressive appearance of annexin-V-positive cells such that ~25 and 45% of the cells were



**Figure 2.** Oxidative insults and HNE induce apoptotic nuclear and plasma membrane alterations in PC12 cells: prevention by Bcl-2. *A*, Cultures of PC12-V cells and PC12-Bcl2 cells were exposed to vehicle (*Control*) or the indicated concentrations of HNE, A $\beta$ , or FeSO<sub>4</sub>. At the indicated time points cells were stained with Hoescht dye, and the percentage of cells with condensed and fragmented (apoptotic) nuclei was determined (mean and SEM of determinations made in four separate cultures). No cells expressing Bcl-2 exhibited condensed and fragmented nuclei (PC12-Bcl2 line represents combined data from cells exposed to each treatment condition). *B*, Annexin-V-positive cells were counted in PC12-V and PC12-Bcl2 cultures exposed for 4 hr to 10  $\mu$ M HNE or for 16 hr to vehicle (*Control*) or 10  $\mu$ M HNE. Values are the mean and SEM of determinations made in four separate cultures. \* $p$  < 0.001 compared with values for control cultures and PC12-Bcl2 cultures (ANOVA with Scheffé's *post hoc* tests). *C*, PC12-V cell cultures were exposed for 48 hr to vehicle (*Control*) or the indicated aldehydes (10  $\mu$ M). Cells were stained with Hoescht dye, and the percentages of cells with condensed and fragmented nuclei were determined. Values are the mean and SEM of determinations made in four separate cultures. \* $p$  < 0.001 compared with each of the other values (ANOVA with Scheffé's *post hoc* tests). *D*, Confocal laser scanning microscope images of propidium iodide fluorescence in untreated control PC12-V cells (*left*), PC12-V cells exposed for 48 hr to 10  $\mu$ M HNE (*middle*), and PC12-Bcl2 cells exposed for 48 hr to 10  $\mu$ M HNE (*right*). Note that many PC12-V cells treated with HNE exhibit DNA condensation and fragmentation (*arrowheads*), whereas cells expressing Bcl-2 did not.

annexin-V-positive by 4 and 16 hr post-treatment, respectively (Fig. 2*B*). Confocal images suggested that the annexin-V was associated with the cell surface (data not shown). Because several aldehydes are liberated when membrane lipids are peroxidized, we determined whether other aldehydes also induce apoptosis. Among nine different aldehydes examined (HNE, propanal, pentanal, hexanal, heptanal, nonanal, malondialdehyde, nonaldehyde, and *trans*-2-nonenal), only HNE induced nuclear condensation and DNA fragmentation (Fig. 2*C*) and annexin-V binding (data not shown).

Macromolecular synthesis inhibitors and endonuclease inhibitors can prevent apoptosis (Bastiatou and Greene, 1991; Rukenstein et al., 1991).

Nuclear condensation and fragmentation induced by HNE, FeSO<sub>4</sub>, and A $\beta$  was prevented mainly in PC12-V cultures cotreated with either the protein synthesis inhibitor cycloheximide, the RNA synthesis inhibitor actinomycin-D, or the Ca<sup>2+</sup>-Mg<sup>2+</sup>-dependent endonuclease inhibitor aurintricarboxylic acid (ATA; Table 1).

#### Oxidative insults and HNE induce apoptosis in hippocampal neurons: protection by GSH

Although studies of PC12 cells have provided valuable insight into mechanisms of neural cell apoptosis (Bastiatou and Greene, 1991; Rukenstein et al., 1991; Ferrari et al., 1995; Troy et al.,

**Table 1. Effects of macromolecular synthesis inhibitors, an endonuclease inhibitor, and antioxidants on apoptotic cell death induced by oxidative insults and HNE in PC12 cells**

Insult	Cells with apoptotic nuclei (%)					
	Vehicle	Cyclohex	Act-D	ATA	VitE	PG
Cont (2 hr)	3 ± 1.2	1 ± 0.3	2 ± 0.6	1 ± 0.3	0 ± 0.3	1 ± 0.3
Cont (72 hr)	24 ± 4.7	11 ± 2.5	10 ± 3.3	9 ± 2.4	10 ± 2.1	13 ± 3.1
FeSO <sub>4</sub>	75 ± 3.2*	16 ± 2.7**	22 ± 3.3**	13 ± 3.3**	13 ± 2.8**	21 ± 2.1**
Aβ	72 ± 3.3*	21 ± 3.4**	23 ± 4.3**	12 ± 2.2**	11 ± 3.5**	14 ± 3.2**
HNE	71 ± 6.7*	29 ± 4.3**	29 ± 4.0**	26 ± 3.2**	59 ± 3.3	65 ± 3.8

Cultures were pretreated for 2 hr with the indicated agents: 0.2% ethanol (Vehicle), 10  $\mu$ M cycloheximide (Cyclohex), 5  $\mu$ M actinomycin-D (Act-D), 100  $\mu$ M aurintricarboxylic acid (ATA), 50  $\mu$ g/ml vitamin E (VitE), 10  $\mu$ M propyl gallate (PG), or 1 mM glutathione-ethyl ester (GSH). Some cultures were fixed at that point (Cont, 2 hr), while parallel cultures were exposed for 72 hr to 0.2% ethanol (Cont 72 hr), 1 mM FeSO<sub>4</sub>, 50  $\mu$ M Aβ, or 10  $\mu$ M HNE. Cells then were fixed and stained with Hoescht dye, and percentages of cells exhibiting nuclear condensation and fragmentation were determined. Values are the mean and SEM of determinations made in three or four separate cultures. \**p* < 0.001 compared with value for control vehicle-treated (72 hr) cultures; \*\**p* < 0.01 compared with value for vehicle-treated cultures exposed to the same insult. ANOVA with Scheffé's *post hoc* tests. Preliminary studies showed that 10  $\mu$ M cycloheximide reduced levels of protein synthesis by >90% during a 24 hr exposure period (data not shown).

1996a), PC12 cells do not exhibit several important features of primary neurons, including expression of glutamate receptors and synapse formation. We therefore turned to mature primary hippocampal cell cultures. Whereas <5% of hippocampal neurons exhibited apoptotic nuclei in vehicle-treated control cultures, 70–80% of the neurons exhibited nuclear condensation and fragmentation in cultures exposed to 2  $\mu$ M HNE (Fig. 3). Lower concentrations of HNE caused progressively less apoptotic neuronal death (0.5  $\mu$ M HNE, 18 ± 3.0%; 1  $\mu$ M HNE, 49 ± 4.1%; *n* = 4 cultures), whereas higher levels (5–10  $\mu$ M) induced rapid necrosis (data not shown). Eight other aldehydes (2  $\mu$ M) did not induce apoptosis (Fig. 3A) or necrosis (data not shown). Aβ and FeSO<sub>4</sub> also induced apoptosis in the cultured hippocampal neurons (Fig. 3B). Previous studies showed that GSH can conjugate with, and thereby detoxify, HNE in non-neuronal cells (Spitz et al., 1990). Pretreatment of hippocampal cultures with a membrane-permeant form of GSH (GSH-diethyl ester) afforded significant protection against apoptosis induced by each oxidative insult and HNE (Fig. 3B).

#### Oxidative insults induce lipid peroxidation and formation of HNE–protein conjugates in neural cells: suppression by GSH and Bcl-2

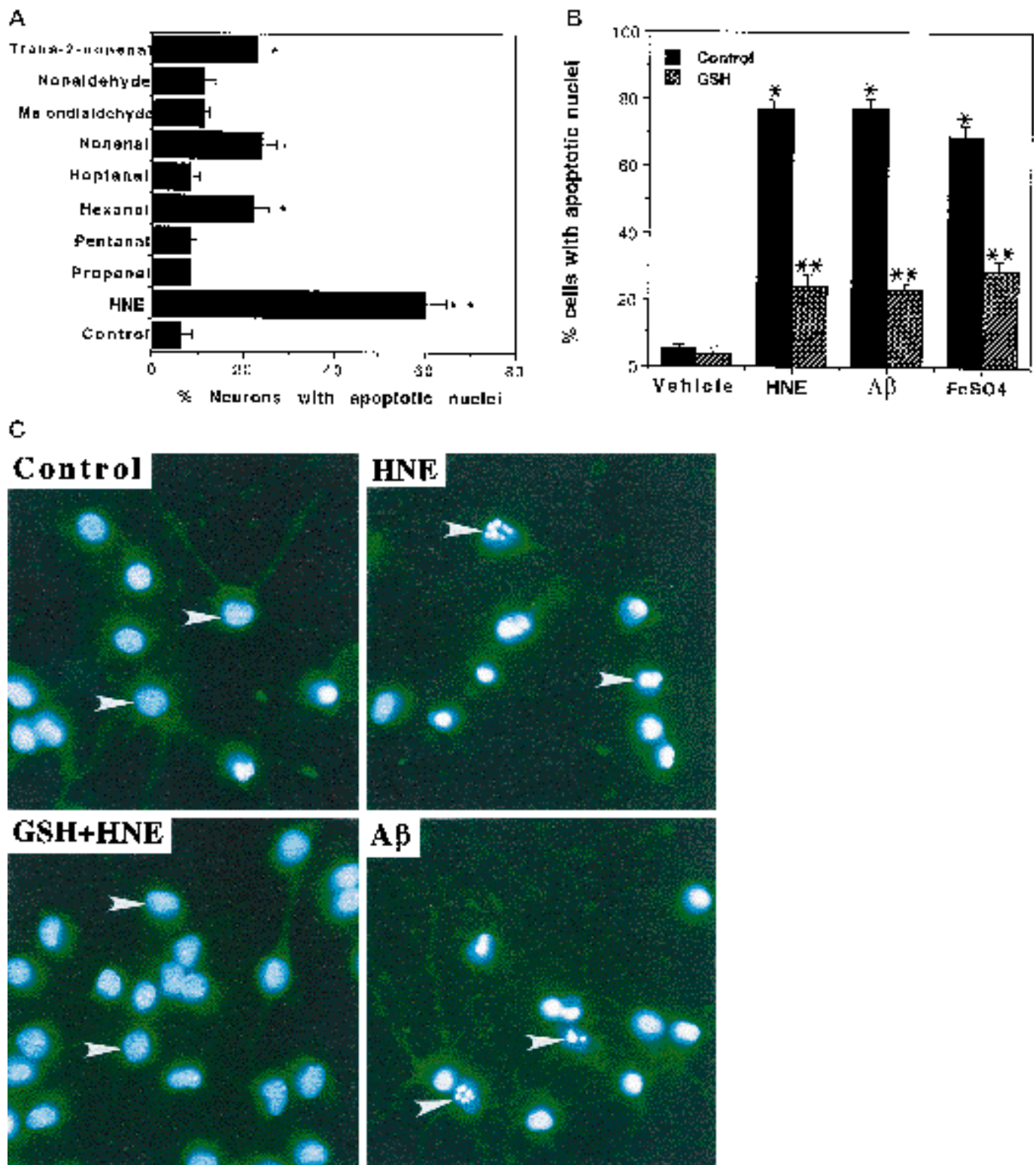
A TBARS fluorescence assay, which measures malondialdehyde levels (Kovachich and Mishra, 1980), was used to quantify membrane lipid peroxidation. Exposure of PC12-V cells or hippocampal neurons to apoptotic concentrations of FeSO<sub>4</sub> and Aβ resulted in marked increases in TBARS fluorescence (Fig. 4A). HNE did not affect TBARS levels in either cell type, consistent with its being a downstream effector of oxidative stress-induced apoptosis rather than an initiator of oxidative stress. If HNE mediates oxidative stress-induced apoptosis, then the oxidative insults should induce HNE accumulation in neurons to levels capable of inducing apoptosis. HNE levels were measured with an anti-HNE antibody (Waeg et al., 1996) in dot-blot, immunocytochemical, and Western blot assays. Hippocampal cells were exposed to 2  $\mu$ M FeSO<sub>4</sub> or 1  $\mu$ M HNE, and levels of HNE in the culture medium and cells were quantified at increasing time points after treatment. FeSO<sub>4</sub> induced a time-dependent increase in HNE levels in the cells; levels reached ~1  $\mu$ M 30 min after exposure, increased to nearly 3  $\mu$ M 6 hr post-treatment, and then decreased to ~2  $\mu$ M by 24 hr post-treatment (Fig. 4B). HNE was not detectable in the medium (<0.1  $\mu$ M) at any time point after exposure of cultures to FeSO<sub>4</sub>. When 1  $\mu$ M HNE was added to the cultures, HNE levels in cells increased to ~2  $\mu$ M during the first

2 hr and then decreased somewhat during the next 22 hr; HNE levels in the culture medium remained at ~1  $\mu$ M during the first 2 hr and then progressively decreased through 24 hr (Fig. 4B).

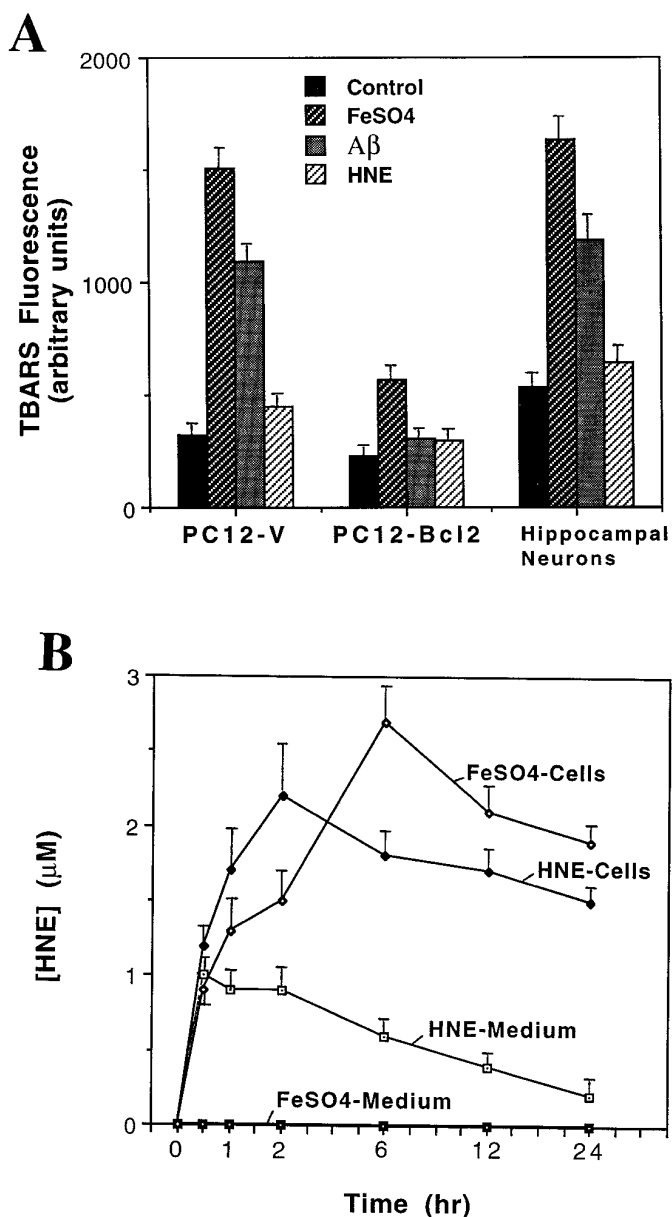
Immunocytochemical analyses showed that oxidative insults and HNE induced HNE immunoreactivity in cultured hippocampal neurons and PC12-V cells (Fig. 5). The levels of cellular HNE immunoreactivity after exposure to FeSO<sub>4</sub>, Aβ, and HNE were reduced greatly in hippocampal cells pretreated with GSH, as compared with control cells (Fig. 5A,D). Exposure of PC12-V cells to FeSO<sub>4</sub>, Aβ, and HNE for 12 hr resulted in the appearance of HNE immunoreactivity in essentially all cells; in most cells the HNE immunoreactivity appeared to be concentrated in perinuclear regions and at the cell periphery, suggesting plasma membrane and organellar localizations (Fig. 5B,D). In contrast, levels of HNE immunoreactivity in PC12-Bcl2 cells exposed to Aβ, FeSO<sub>4</sub>, and HNE were reduced markedly, as compared with levels in PC12-V cells exposed to the same insults (Fig. 5B,D). To determine whether HNE production occurs in all apoptotic paradigms or is specific for oxidative insults, we exposed PC12-V cells for 2, 6, 12, or 24 hr to an apoptotic concentration of staurosporine (500 nM) and then immunostained the cells with the HNE antibody. No detectable HNE immunoreactivity was observed at any time point after exposure to staurosporine (data not shown).

Western blot analysis of PC12-V and PC12-Bcl2 cells exposed for 12 hr to vehicle, FeSO<sub>4</sub>, or HNE showed that, whereas there were no detectable HNE–protein conjugates in control cultures, there were many proteins immunoreactive with the HNE antibody in PC12-V cells exposed to HNE or FeSO<sub>4</sub> (Fig. 5C). The pattern of HNE–protein conjugates in proteins from cells exposed directly to HNE were similar, but not identical, to the pattern seen in cells exposed to FeSO<sub>4</sub>. Although the basis for the differences in banding was not established, it seems likely that proteins located in the plasma membrane are exposed to locally higher concentrations of HNE in cells exposed to FeSO<sub>4</sub> (compared with cells exposed to exogenous HNE) because that is where the endogenous HNE comes from. In contrast to PC12-V cells, only very low levels of HNE–protein conjugates were present in Western blots of proteins from PC12-Bcl2 cells exposed to either FeSO<sub>4</sub> or HNE (Fig. 5C). In additional experiments we performed electron paramagnetic spectroscopy analyses with nitroxyl stearate spin labels to quantify lipid peroxidation; the results confirmed that Bcl-2 suppresses lipid peroxidation induced by iron and Aβ in PC12 cells (data not shown; cf. Bruce et al., 1995).

Pretreatment of PC12-V cultures with the antioxidants vitamin E and propyl gallate before exposure to FeSO<sub>4</sub> and Aβ resulted in



**Figure 3.** Oxidative insults and HNE induce apoptosis in primary hippocampal neurons: prevention by GSH. **A**, Cultures were exposed to 2  $\mu$ M of each aldehyde (see Fig. 2C for aldehyde structures) or 0.2% ethanol (Control) for 16 hr, and percentages of neurons with condensed and fragmented nuclei were quantified. Values are the mean and SEM of determinations made in four separate cultures. \* $p$  < 0.05, \*\* $p$  < 0.001 compared with control value (ANOVA with Scheffé's *post hoc* tests). **B**, Cultures were exposed for 16 hr to 0.2% ethanol (Control) or the indicated treatments, at which time cells were stained with Hoescht dye and the percentages of neurons with condensed and fragmented nuclei were quantified. Treatment concentrations were HNE, 10  $\mu$ M; A $\beta$ , 10  $\mu$ M; FeSO<sub>4</sub>, 2  $\mu$ M; GSH, 1 mM. Values are the mean and SEM of determinations made in four separate cultures. \* $p$  < 0.01 compared with control value; \*\* $p$  < 0.01, \*\*\* $p$  < 0.05 compared with corresponding values for cultures not cotreated with GSH (ANOVA with Scheffé's *post hoc* tests). **C**, Images of Hoescht dye fluorescence in hippocampal neurons from cultures exposed for 16 hr to 0.2% ethanol (Control), 2  $\mu$ M HNE, 1 mM GSH-ethyl-ester plus 2  $\mu$ M HNE (GSH+HNE), or 10  $\mu$ M A $\beta$ . HNE and A $\beta$  induced nuclear condensation and fragmentation in most neurons (arrowheads), whereas fluorescence remained uniformly distributed throughout the nucleus in neurons in control cultures and neurons in cultures cotreated with GSH and HNE (arrowheads).



**Figure 4.** Oxidative insults induce membrane lipid peroxidation in neural cells: suppression by Bcl-2. *A*, Levels of TBARS fluorescence were quantified in PC12-V cells, PC12-Bcl2 cells, and hippocampal neurons after 4 hr of exposure to vehicle (*Control*),  $FeSO_4$  (1 mM PC12 cells, 2  $\mu M$  hippocampal neurons),  $A\beta$  (50  $\mu M$  PC12 cells, 10  $\mu M$  hippocampal neurons), or *HNE* (10  $\mu M$  PC12 cells, 2  $\mu M$  hippocampal neurons). Values represent the mean and SEM of determinations made in four to six separate cultures. *B*, Hippocampal cell cultures were exposed to 1  $\mu M$  *HNE* or 2  $\mu M$   $FeSO_4$ , and *HNE* levels in the culture medium and cells were quantified at the indicated *time points*. Values represent the mean and SEM of determinations made in four separate cultures.

significant protection against apoptosis induced by these oxidative insults (Table 1). In contrast, vitamin E and propyl gallate did not protect PC12-V cells against *HNE*-induced apoptosis (Table 1), consistent with *HNE* acting as a downstream effector of lipid peroxidation-induced apoptosis.

**Evidence that endogenous GSH serves an antiapoptotic function**

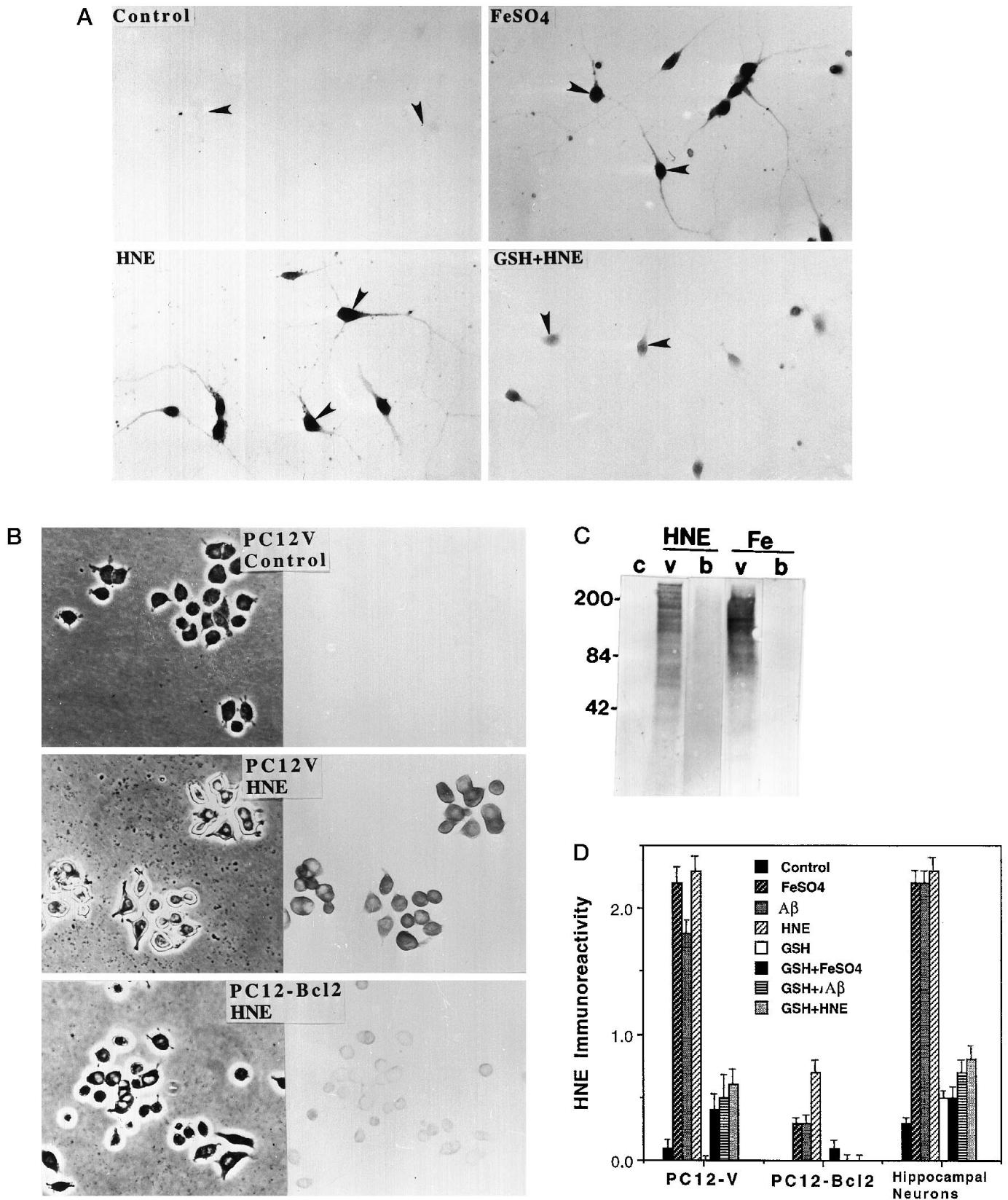
The ability of GSH to protect hippocampal neurons against apoptosis induced by oxidative stress and *HNE* prompted studies of

possible antiapoptotic actions of endogenous GSH. When PC12-V cells were pretreated with 1 mM GSH diethyl ester, they were resistant to apoptosis induced by  $FeSO_4$ ,  $A\beta$ , and *HNE* (Fig. 6*A*). Whereas ~80% of the cells exhibited apoptotic nuclei after exposure to  $FeSO_4$ ,  $A\beta$ , and *HNE*, only 15–40% of the cells were apoptotic in cultures cotreated with GSH. BSO, an agent that depletes endogenous GSH by inhibiting  $\gamma$ -glutamyl cysteine synthetase (Meister, 1995), induced apoptosis in PC12-V cells, suggesting that depletion of GSH was sufficient to induce apoptosis (Fig. 6*A*). Previous studies have shown that cell expressing Bcl-2 exhibit higher GSH levels than control cells and show less GSH depletion when exposed to oxidative apoptotic insults (Ellerby et al., 1996; Hedley and McCulloch, 1996). GSH levels quantified by the monochlorobimane method were 30–40% greater in PC12-Bcl2 cells, as compared with PC12-V cells (Fig. 6*B*). After exposure to  $A\beta$ ,  $FeSO_4$ , and *HNE*, levels of GSH were decreased significantly by 40–70% in PC12-V cells (Fig. 6*B*). The levels of GSH in PC12-Bcl2 cells after exposure to  $A\beta$  and  $FeSO_4$  were significantly greater than in PC12-V cells exposed to the same insults. Similar results were obtained with the enzymatic recycling procedure for quantification of GSH levels (data not shown). As expected, BSO caused a marked depletion of GSH levels in both PC12-V and PC12-Bcl2 cells; GSH levels remained at a higher level in PC12-Bcl2 cells than in PC12-V cells, although the difference was not statistically significant (Fig. 6*B*).

**DISCUSSION**

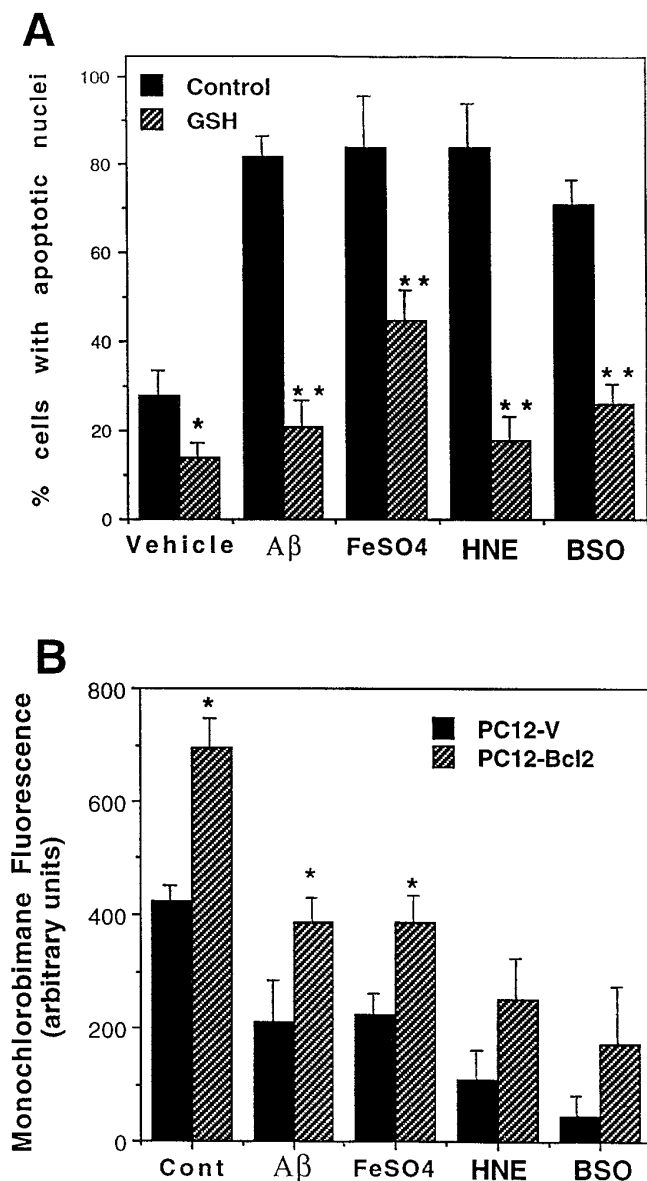
The present findings demonstrate that the lipid peroxidation product *HNE* can induce apoptosis in PC12 cells and primary rat hippocampal neurons and suggest that *HNE* is a mediator of oxidative stress-induced apoptosis. *HNE* induced loss of plasma membrane phosphatidylserine asymmetry (detected by annexin-V binding) and nuclear condensation and DNA fragmentation in PC12 cells and hippocampal neurons. *HNE*-induced cell death was prevented by cycloheximide, actinomycin-D, and aurintricarboxylic acid, consistent with a role for macromolecular synthesis and endonuclease activity in the cell death process. Although we recently reported that low concentrations of cycloheximide that do not cause sustained blockade of protein synthesis can protect neurons against excitotoxic and oxidative insults by a mechanism that apparently involves stimulation of antioxidant pathways (Furukawa et al., 1997), the concentration of cycloheximide used in the present study (10  $\mu M$ ) causes an essentially complete and sustained blockade of protein synthesis (Furukawa et al., 1997). In addition, PC12 cells expressing the antiapoptotic gene product Bcl-2 were resistant to apoptosis induced by *HNE*. The concentrations of *HNE* that induced apoptosis (2–10  $\mu M$ , estimated from dot-blot analyses) are within the range of concentrations (1–100  $\mu M$ ) known to be generated after exposure of non-neuronal cells (Esterbauer et al., 1991) and neurons (Mark et al., 1997) (present study) to oxidative insults. Among aldehydic products of lipid peroxidation, *HNE* seems to be a unique inducer of apoptosis, because eight other aldehydes did not induce apoptosis in PC12 cells or hippocampal neurons. These findings are consistent with a recent report showing that *HNE*, but not other aldehydes, is toxic to cultured human neuroblastoma cells (Mark et al., 1997).

Apoptotic concentrations of  $FeSO_4$  and  $A\beta$  induced the formation of *HNE*-protein conjugates, as detected by both Western blot and immunocytochemical analyses. Antioxidants that suppress lipid peroxidation protected PC12 cells against apoptosis induced by  $FeSO_4$  and  $A\beta$  but did not protect against apoptosis induced by *HNE*, consistent with *HNE* acting downstream of lipid peroxida-



**Figure 5.** Oxidative insults induce formation of HNE-protein conjugates in neural cells: attenuation by GSH and Bcl-2. *A*, Hippocampal cultures were exposed for 2 hr to 0.2% ethanol (*Control*), 2  $\mu$ M FeSO<sub>4</sub>, 2  $\mu$ M HNE, or 1 mM GSH plus 2  $\mu$ M HNE (*GSH+HNE*). Then cells were fixed and immunostained with HNE antibody. *Arrowheads* point to neuronal cell bodies. *B*, PC12-V cells and PC12-Bcl-2 cells were exposed for 2 hr to vehicle (0.2% ethanol) or 10  $\mu$ M HNE. Then cells were fixed and immunostained with anti-HNE antibody. Note the much greater level of HNE immunoreactivity in PC12-V cells exposed to HNE, as compared with PC12-Bcl-2 cells exposed to HNE. *C*, Cell homogenates from untreated (Figure legend continues)





**Figure 6.** GSH protects PC12 cells against apoptosis induced by oxidative insults and HNE. *A*, Cultures of PC12-V cells were exposed for 70 hr to Vehicle, 10 μM HNE, 50 μM Aβ, or 1 mM FeSO<sub>4</sub> in the absence (Control) or presence of 1 mM GSH. Then cultures were fixed and stained with Hoescht dye, and the numbers of cells with condensed and fragmented nuclei were counted. Values are the mean and SEM of determinations made in four separate cultures. \**p* < 0.01 compared with vehicle control value; \*\**p* < 0.01 compared with corresponding control value (ANOVA with Scheffé's *post hoc* tests). *B*, Cultures were exposed for 6 hr to vehicle (Cont), 50 μM Aβ, 1 mM FeSO<sub>4</sub>, 10 μM HNE, or 300 μM buthionine sulfoximine (BSO). Then relative levels of GSH were determined by using the monochlorobimane fluorescence method. Values are the mean and SEM of determinations made in four separate cultures. \**p* < 0.01 compared with corresponding value for PC12-V cells (ANOVA with Scheffé's *post hoc* tests).

tion in the apoptotic pathway induced by oxidative stress. GSH, which was shown previously to detoxify HNE (Spitz et al., 1990; Esterbauer et al., 1991), protected PC12 cells and hippocampal neurons against apoptosis induced by HNE and oxidative insults. Beaver and Waring (1995) reported that decreases in levels of intracellular GSH precede apoptosis in mouse thymocytes exposed to dexamethasone, thapsigargin, and gliotoxin; treatment with 1 mM GSH prevented apoptosis, suggesting a role for GSH depletion in the apoptotic process. Ratan et al. (1994) showed that macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in cultured cortical neurons by increasing GSH levels. We found that GSH levels were higher in PC12 cells expressing Bcl-2 than in PC12-V cells in untreated cultures and after exposure to Aβ, FeSO<sub>4</sub>, and HNE. Collectively, the data suggest that endogenous GSH may contribute to the resistance of cells expressing Bcl-2 to oxidative insults.

Levels of TBARS fluorescence and HNE-protein conjugates induced by FeSO<sub>4</sub> and Aβ were lower in PC12 cells expressing Bcl-2, consistent with data showing that Bcl-2 suppresses lipid peroxidation induced by oxidative insults in a hypothalamic tumor cell line (Kane et al., 1993; Myers et al., 1995) and PC12 cells (present study). On the other hand, levels of HNE-protein conjugates resulting from exposure of cells to HNE also were reduced in PC12 cells expressing Bcl-2, suggesting that Bcl-2 suppresses formation of HNE-protein conjugates. An increased level of GSH in cells expressing Bcl-2 could account for our results because GSH plays a central role in the cellular metabolism of HNE (Ishikawa et al., 1986; Spitz et al., 1990). In studies of spinal cord organotypic cultures, Rothstein et al. (1994) showed that downregulation of Cu/Zn-SOD resulted in neuronal apoptosis, which was inhibited by antioxidants. Troy et al. (1996b) reported that downregulation of Cu/Zn-SOD in PC12 cells induces apoptosis, which can be prevented with inhibitors of nitric oxide synthase, suggesting the involvement of peroxynitrite. When taken together with our data, the latter findings suggest that there may be at least two mechanisms whereby oxidative stress induces apoptosis, one involving nitric oxide production and peroxynitrite formation and the other involving hydroxyl radical formation, lipid peroxidation, and HNE production. However, the two pathways likely converge at some point because Bcl-2 prevents apoptosis in both cases. Because peroxynitrite is known to induce membrane lipid peroxidation, it will be of interest to determine whether HNE plays a role in apoptotic paradigms that involve nitric oxide production and peroxynitrite formation and, conversely, whether there is a role for peroxynitrite in HNE-induced apoptosis.

Loss of cellular ion homeostasis plays a central role in excitotoxic neuronal death and also may contribute to oxidative stress-induced apoptosis. HNE increases neuronal vulnerability to excitotoxicity (Mark et al., 1997), and loss of calcium homeostasis has been implicated in the apoptotic process in a number of paradigms (Nicotera et al., 1992; Takei and Endo, 1994; Le et al., 1995). Recent findings indicate that neuronal death induced by

control PC12-V cultures (*c*), PC12-V cultures exposed to 10 μM HNE or 1 mM FeSO<sub>4</sub> (*v*), and PC12-Bcl2 cultures exposed to 10 μM HNE or 1 mM FeSO<sub>4</sub> (*b*) were separated by SDS-PAGE (100 μg protein/lane). Then proteins were transferred to a nitrocellulose sheet and immunoreacted with an antibody against HNE-protein conjugates. Note that HNE and FeSO<sub>4</sub> induced the appearance of many HNE-protein conjugates in PC12-V cells, but not in the PC12-Bcl2 cells. *D*, PC12-V cells, PC12-Bcl2 cells, and primary hippocampal neurons were exposed for 2 hr to 0.2% ethanol (Control), FeSO<sub>4</sub> (1 mM for PC12 cells and 2 μM for hippocampal neurons), Aβ (50 μM for PC12 cells and 10 μM for hippocampal neurons), or HNE (10 μM for PC12 cells and 2 μM for hippocampal neurons). Then cells were fixed and immunostained with HNE antibody, and relative levels of HNE immunoreactivity were quantified (see Materials and Methods). Values represent the mean and SEM of determinations made in four separate cultures per condition (100 cells scored/culture).

excitotoxins or oxidative insults can manifest as either apoptosis or necrosis, depending on the severity of the insult (Ankarcrona et al., 1995; Bonfoco et al., 1995) (present study). Although not directly tested in the present study, disruption of calcium homeostasis may contribute to oxidative stress-induced apoptosis. FeSO<sub>4</sub>, A $\beta$ , and HNE each cause impairment of ion-motive ATPase activities in cultured hippocampal neurons, which is linked to subsequent elevation of [Ca<sup>2+</sup>]<sub>i</sub> and cell death (Mark et al., 1995, 1997). The ability of ATA to protect PC12 cells against HNE-induced apoptosis is consistent with a role for calcium in that ATA is an inhibitor of calcium-activated endonucleases (Zhitovitsky et al., 1994) and calpains (Posner et al., 1995), although it may have other actions, also (Zeevalk et al., 1995).

Mitochondrial alterations, including decreases in transmembrane potential and decreased levels of MTT reduction, occur at relatively early stages in the apoptotic process (Zamzami et al., 1996). We found that apoptotic concentrations of A $\beta$ , FeSO<sub>4</sub>, and A $\beta$  each caused a relatively rapid (2–6 hr) small decrease in levels of MTT reduction and mitochondrial transmembrane potential. Shearman et al. (1995) also found that A $\beta$  rapidly decreases levels of MTT reduction in PC12 cells. Interestingly, the initial modest decrease in levels of MTT reduction occurred in both PC12-V and PC12-Bcl2 cells exposed to oxidative insults and HNE, suggesting that this early mitochondrial alteration is not linked causally to subsequent apoptotic death. Indeed, previous data indicate that functioning mitochondria (and resultant ATP) are required for apoptosis (Slater et al., 1995). HNE also impairs mitochondrial function in synaptosomes (Keller et al., 1997), and GSH attenuates oxidative stress-induced disruption of mitochondrial transmembrane potential in lymphocytes (Pieri et al., 1995), suggesting that HNE contributes to oxidative stress-induced mitochondrial dysfunction.

HNE fulfills several criteria for a mediator of oxidative stress-induced apoptosis, including the following: HNE is produced in neurons exposed to apoptotic oxidative insults, HNE itself can induce apoptosis, and an inhibitor of HNE (GSH) protects neurons against oxidative stress- and HNE-induced apoptosis. Because essentially all vertebrate cells are capable of generating HNE in response to oxidative insults, HNE may serve a general role in apoptosis in many different organ systems. Because it can move readily across membranes and through cells, HNE has the ability to induce alterations in subcellular sites affected in cells undergoing apoptosis. Indeed, HNE has been reported to damage both proteins and DNA (Esterbauer et al., 1991; Martelli et al., 1994).

Finally, our data suggest possible roles for HNE in the pathogenesis of neurodegenerative disorders such as AD and Parkinson's disease, in which apoptosis may occur (Loo et al., 1993; Su et al., 1994; Stern, 1996). Very recent findings indicate that levels of protein-bound HNE are increased selectively in neurons in the substantia nigra of Parkinson's patients (Yoritaka et al., 1996) and that levels of free HNE are increased more than twofold in CSF of AD patients (M. A. Lovell and W. R. Markesbery, personal communication). Therefore, further studies of roles of HNE in apoptotic neuronal death are warranted to clarify its role in neurodegenerative disorders.

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