

Bcl-2 Protects Isolated Plasma and Mitochondrial Membranes Against Lipid Peroxidation Induced by Hydrogen Peroxide and Amyloid β -Peptide

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Abstract: The *bcl-2* protooncogene product possesses antiapoptotic properties in neuronal and nonneuronal cells. Recent data suggest that Bcl-2's potency as a survival factor hinges on its ability to suppress oxidative stress, but neither the subcellular site(s) nor the mechanism of its action is known. In this report electron paramagnetic resonance (EPR) spectroscopy analyses were used to investigate the local effects of Bcl-2 on membrane lipid peroxidation. Using hydrogen peroxide (H_2O_2) and amyloid β -peptide ($A\beta$) as lipoperoxidation initiators, we determined the loss of EPR-detectable paramagnetism of nitroxyl stearate (NS) spin labels 5-NS and 12-NS. In intact cell preparations and postnuclear membrane fractions, $A\beta$ and H_2O_2 induced significant loss of 5-NS and 12-NS signal amplitude in control PC12 cells, but not PC12 cells expressing Bcl-2. Cells were subjected to differential subcellular fractionation, yielding preparations of plasma membrane and mitochondria. In preparations derived from Bcl-2-expressing cells, both fractions contained Bcl-2 protein. 5-NS and 12-NS signals were significantly decreased following $A\beta$ and H_2O_2 exposure in control PC12 mitochondrial membranes, and Bcl-2 largely prevented these effects. Plasma membrane preparations containing Bcl-2 were also resistant to radical-induced loss of spin label. Collectively, our data suggest that Bcl-2 is localized to mitochondrial and plasma membranes where it can act locally to suppress oxidative damage induced by $A\beta$ and H_2O_2 , further highlighting the important role of lipid peroxidation in apoptosis. **Key Words:** Alzheimer's disease—Apoptosis—Electron paramagnetic resonance spectroscopy—Free radicals—Spin labeling.

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a variety of apoptotic paradigms including exposure of lymphocytes to glucocorticoids (Sentman et al., 1991), trophic factor withdrawal in neurons (Garcia et al., 1992; Srinivasan et al., 1996), and exposure of neural cells to a variety of oxidative insults (Hockenbery et al., 1993; Kane et al., 1993; Zhong et al., 1993; Myers et al., 1995; Ellerby et al., 1996; Kruman et al., 1997). The amino acid sequence of Bcl-2 predicts a transmembrane domain of 19 hydrophobic amino acids near the carboxy terminus, and mutagenesis studies have indicated that removal of this "anchor domain" prevents membrane targeting and compromises Bcl-2's ability to block cell death (Nguyen et al., 1994), although Bcl-2 may also possess antiapoptotic activity in the absence of membrane association (Borner et al., 1994). Confocal and electron microscope analyses indicate that Bcl-2 resides in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane (Hockenbery et al., 1993; Krafewski et al., 1993; Reed, 1994); however, localization to the plasma membrane has not been demonstrated. Localization of Bcl-2 to mitochondria has prompted speculation that Bcl-2 exerts its antiapoptotic effects through modulation of such mitochondrial processes as oxidative phosphorylation, oxygen consumption, or calcium regulation (Murphy et al., 1996; Petit et al., 1996; Richter et al., 1996). It has also been suggested that Bcl-2's survival-promoting actions involve modulation of nuclear transport or nuclear envelope assembly (Lithgow et al., 1994) or enhancement of endoplasmic reticulum

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Abbreviations used: $A\beta$, amyloid β -peptide; ct, control-transfected; EPR, electron paramagnetic resonance; GSH, glutathione; NS, nitroxyl stearate; PNM, postnuclear membrane; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance.

The *bcl-2* protooncogene was originally identified at the chromosomal translocation point in malignant human B-cell follicular lymphoma and has been shown to promote tumor formation by suppressing apoptosis (Reed, 1994). Cell culture studies have demonstrated that overexpression of Bcl-2 can prevent cell death in

calcium-buffering capacity (Lam et al., 1994). However, none of these mechanisms has been established.

Although considerable data suggest that Bcl-2's antiapoptotic mechanism involves suppression of the accumulation of reactive oxygen species (Hockenbery et al., 1993; Kane et al., 1993; Zhong et al., 1993; Myers et al., 1995; Ellerby et al., 1996; Kruman et al., 1997), its mechanism of action is unknown. Membrane lipid peroxidation is increased in cells undergoing apoptosis in response to a range of insults including exposure to iron, hydrogen peroxide, and amyloid β -peptide ($A\beta$) (Behl et al., 1994; Whittemore et al., 1994; Goodman et al., 1996; Kruman et al., 1997). PC12 cells expressing Bcl-2 are resistant to apoptosis induced by oxidative insults and the lipid peroxidation product 4-hydroxynonenal (Kane et al., 1993; Kruman et al., 1997). Moreover, accumulation of 4-hydroxynonenal was greatly reduced in cells expressing Bcl-2 (Kruman et al., 1997), suggesting the possibility that Bcl-2 acts within membranes to prevent their peroxidation. In the present study, we used electron paramagnetic resonance (EPR) spectroscopy analyses of isolated cellular membrane fractions to test the hypothesis that Bcl-2 acts locally in mitochondrial and plasma membranes to suppress lipid peroxidation. This approach uses two different membrane-associated, oxidation-sensitive, paramagnetic nitroxyl stearate (NS) probes: 5-NS, whose paramagnetic center resides near the lipid-water interface, and 12-NS, in which the nitroxide moiety localizes deep in the lipid bilayer. This protocol has been used to measure *in vitro* oxidation of synaptosomes (Zaleska et al., 1989; Butterfield et al., 1994), and we now use this novel assay of membrane oxidation to characterize the direct protective mechanisms of Bcl-2. The acute effects of $A\beta$ and H_2O_2 on 12-NS and 5-NS spin labeling were determined in intact cell preparations and in mitochondrial and plasma membrane fractions from Bcl-2-transfected (Bcl-2-PC12) and control-transfected (ct-PC12) PC12 cells. The results demonstrate the sensitivity and applicability of the spin label technique for measuring lipid peroxidation and provide novel insight into the mechanism of Bcl-2-mediated cytoprotection.

MATERIALS AND METHODS

Generation and maintenance of PC12 cell lines

PC12 cells were transfected using a pBabepuro retroviral expression vector (H. Land, Imperial Cancer Research Fund, London), as previously described (Mah et al., 1993). Cells were maintained and grown to confluence in 75-mm flasks under standard incubator conditions (37°C in 6% CO_2) in RPMI medium containing 10% heat-inactivated horse serum and 5% fetal bovine serum.

Subcellular fractionation

After Bcl-2-PC12 and ct-PC12 cells were removed from flasks by trypsinization, the resulting suspensions were pelleted, washed, and either incubated directly with 12-NS or 5-NS in phosphate-buffered saline or subjected to subcellular

fractionation. Cells were homogenized in ice-cold breaking buffer (0.6 M mannitol/20 mM HEPES-KOH, pH 7.5) using a Dounce homogenizer, and nuclei were removed by low-speed centrifugation. Crude postnuclear membrane (PNM) fragments were then isolated from the supernatant by high-speed centrifugation (100,000 g, 45 min), or alternatively, mitochondrial fractions were isolated from supernatants (in breaking buffer with 1 mM phenylmethylsulfonyl fluoride) by centrifugation at 10,000 g for 10 min. Plasma membrane-enriched fractions were then isolated from the resultant supernatant by high-speed centrifugation (100,000 g, 45 min). Isolated fractions were washed twice in phosphate-buffered saline, and protein content was determined using a bicinchoninic acid/Pierce protein determination kit (Rockford, IL, U.S.A.).

Spin labeling techniques

These methods were similar to those described previously (Butterfield, 1982; Butterfield et al., 1994; Hensley et al., 1994). In brief, the spin labels 5-NS or 12-NS (Sigma) were dissolved in chloroform, separated into 10- μ g aliquots in borosilicate glass tubes, and evaporated under N_2 . Freshly prepared cell suspensions or membrane fractions (20–80 μ g of protein) were incubated in these spin label-coated tubes with gentle agitation for 20 min at room temperature. Membrane fractions thus labeled were then exposed to either water, $A\beta$ 25–35 (400 μ M), or H_2O_2 (3 mM) for 15 min at room temperature with gentle agitation. All spectra were acquired immediately at the end of the exposure period using a Bruker model 300 D spectrometer equipped with computerized data acquisition and analysis capabilities. Instrumental parameters were as follows: receiver gain = 1×10^5 , conversion time = 10.28 ms, time constant = 1.28 ms, incident microwave power = 19 mW, modulation amplitude = 0.39 G, scan time = 10 s. All data (peak height) were analyzed using one-way ANOVA, followed by least significant difference post hoc analysis to determine statistical significance. *p* values of ≤ 0.05 are designated as statistically significant.

Thiobarbituric acid-reactive substance (TBARS) assay

Relative levels of malondialdehyde, a lipid released from oxidized membranes, were determined using the TBARS assay as previously described (Goodman et al., 1996). In brief, purified mitochondrial and plasma membrane fractions were incubated with either water, $A\beta$ 25–35 (400 μ M), or H_2O_2 (3 mM) for 15 min at room temperature with gentle agitation, after which proteins were immediately precipitated with trichloroacetic acid (final concentration 5%). Precipitates were then incubated at 95°C for 30 min in the presence of 2-thiobarbituric acid (0.335% in 50% glacial acetic acid). The solutions were cooled, and fluorescence in the butanol-extractable lipid fraction was determined using a Cytofluor 2350 (Millipore) fluorescent plate reader (518-nm excitation and 588-nm emission). Values were determined on a per microgram of protein basis and then converted into percent control (percentage of water-treated value).

Western blot analysis

Proteins were separated by polyacrylamide gel electrophoresis (10% gel) and transferred electrophoretically to nitrocellulose paper. After blocking of nonspecific sites (5% milk), immunoreactive proteins were visualized by sequential reaction with a primary monoclonal or polyclonal anti-

bodies, alkaline phosphatase-linked secondary antibodies, and BPIC/NBT color developer (Vector). Primary antibodies used were against Bcl-2 (mouse monoclonal; Santa Cruz; 1:500 dilution), F₁/F₀-ATPase (rabbit polyclonal; 1:1,000 dilution) (see Zhang et al., 1995), GLUT3 (polyclonal antibody directed against a carboxy-terminal peptide of GLUT3; Chemicon; 1:500 dilution), and Na⁺,K⁺-ATPase (Upstate Biotechnology; 1:500 dilution).

Quantification of antioxidant enzyme activities and glutathione (GSH) levels

Catalase activity was measured by the method of Beers and Siter (Carrillo et al., 1991), in which the disappearance of H₂O₂ is followed spectrophotometrically at 240 nm. The reaction mixture contained 15 mM H₂O₂ in potassium phosphate buffer (pH 7.0) and was started by the addition of an aliquot of supernatant. The change in optical density was measured for 2.5 min, and standard curves were established using known amounts of purified bovine catalase (Sigma Chemical, St. Louis, MO, U.S.A.) under the same conditions. Total superoxide dismutase (SOD) activity was measured according to the method of Kostyuk and Potapovich (1989). This method spectrophotometrically follows the superoxide-driven autooxidation of quercetin at pH 10 in the presence of TMEDA (*N,N,N',N'*-tetramethylethylenediamine) and EDTA. The standard assay mixture contained 0.8 mM TMEDA and 0.08 mM EDTA in 0.02 M potassium phosphate buffer (pH 10) with or without sample (10–35 µg protein). The reaction was started by addition of 0.1 ml of quercetin (0.44 mM in dimethylformamide), and the oxidation of quercetin was followed for 9.5 min at 406 nm. The percent inhibition of the autooxidation of quercetin by SOD present in the tissue sample was determined, and standard curves using known amounts of purified SOD (Sigma Chemical) under identical conditions were established. Mn-SOD was determined in the same manner except that all cuvettes contained 2 mM KCN to inhibit Cu/Zn-SOD activity. Glutathione peroxidase activity was measured by the method of Paglia and Valentine (1967). The incubation mixture contained 1 mM GSH, 0.2 mM NADPH, and 1.4 IU of glutathione reductase in 50 mM potassium phosphate buffer (pH 7.0). The assay reaction was initiated by the simultaneous addition of sample (100–500 µg protein) and 0.25 mM H₂O₂. The change in absorbance at 340 nm was followed for 4.5 min, and standard curves were drawn using purified glutathione peroxidase (Sigma Chemicals) under identical conditions. Total GSH was quantified by the Tietze recycling assay, as modified by Eady et al. (1995). In brief, samples were homogenized in 5-sulfosalicylic acid (final concentration 5%) and centrifuged at 10,000 *g* for 10 min, and the cytosolic fraction (150–250 µg of protein) was incubated for another 25 min at 37°C with 0.21 mM NADPH and 0.6 mM DTNB (2-dinitrobenzoic acid) in 143 mM sodium phosphate/6.3 mM EDTA buffer (pH 7.5). The GSH recycling assay was initiated by the addition of 0.5 unit of glutathione reductase (type III, from *S. cerevisiae*), and the increase in absorbance at 412 nm was measured spectrophotometrically for 45 s. Standards of known GSH concentration were assessed in an identical manner.

RESULTS

Bcl-2 protects intact cells and PNM preparations against loss of spin label induced by peroxidative insults

To determine if Bcl-2 expression modified levels of membrane oxidation in intact cells, ct-PC12 cells and

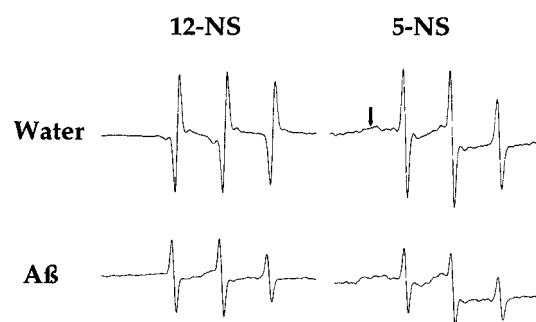


FIG. 1. EPR analysis of 5-NS and 12-NS signals in PNM preparations exposed to vehicle (water) or A β . 12-NS and 5-NS were incorporated into membranes, which were then exposed to 400 μ M A β for 15 min. A β caused large decreases in the magnitude of both the 12-NS and the 5-NS signals (peak height).

Bcl-2-PC12 cells were labeled with the NS spin probes 5-NS and 12-NS and were then exposed to H₂O₂, A β , or vehicle (water). A β -associated free radicals or H₂O₂-derived hydroxyl radicals (via reaction with endogenous Fe²⁺), if reacted with the unpaired electron on the nitroxide spin probes, result in loss of paramagnetism and hence loss of EPR signal amplitude of the spin probe (Butterfield et al., 1994). 5-NS and 12-NS intercalate into the lipid bilayer with the long axis of the spin probe aligned along the acyl chains of phospholipids and the charged carboxylic acid near the polar head groups of the phospholipids (Butterfield, 1982). The paramagnetic centers of 5-NS and 12-NS reside, respectively, in proximal (near the lipid–H₂O interface) and deep lipid layers (Butterfield, 1982). Incorporation of spin probes into whole-cell or membrane preparations results in characteristic oxidation-sensitive EPR spectra (Fig. 1) (Butterfield et al., 1994; Hensley et al., 1994). The spectrum of 5-NS, as expected, shows more immobilization (Fig. 1, arrow) than 12-NS, which measures ordered motion deeper in a more fluid region of the bilayer. These two probes were very sensitive to the membrane oxidative insults, which caused rapid and irreversible loss of spin probe intensity, manifested by a decrease in peak amplitude (Fig. 1). Treatment of intact Bcl-2-PC12 cells with either A β or H₂O₂ for 15 min did not decrease 5-NS signal amplitude, while the same treatments in ct-PC12 cells caused significant 39 and 29% decreases, respectively, in 5-NS incorporation (Figs. 1 and 2a). Decreases in signal amplitude were not reversible and remained unchanged over a subsequent 60-min incubation period (data not shown). 12-NS incorporation in intact cells was not decreased in either cell type by A β or H₂O₂.

Spin label signal amplitude in PNMs, which contain moderate to light organellar and plasma membrane constituents, was assessed to determine if the specific antioxidant effects of Bcl-2 depended on interactions with cytosolic factors. Treatment of 5-NS-labeled PNMs from Bcl-2-PC12 cells with A β for 15 min did not significantly decrease signal amplitude, while the same treatment resulted in a dramatic 48% decrease in the signal in ct-

PC12 cells (Fig. 3a). This decrease was significantly different from both the water-treated ct-PC12 levels and the $A\beta$ -treated Bcl-2-PC12 levels, indicating that Bcl-2 confers direct protection against lipid peroxidation in isolated membranes. H_2O_2 did not significantly alter 5-NS signal amplitude in PNMs isolated from either Bcl-2-PC12 or ct-PC12 cells (Fig. 3a). In the case of the 12-NS signal, PNMs from Bcl-2-PC12 cells were resistant to $A\beta$ - or H_2O_2 -mediated oxidation, whereas ct-PC12 cell membranes demonstrated significant decreases in the 12-NS signal amplitude (40 and 29%) after $A\beta$ and H_2O_2 treatment (Fig. 3b).

Characterization of subcellular fractions from control and Bcl-2-expressing cells

We next prepared subcellular fractions containing mitochondria or plasma membranes and examined both their content of Bcl-2 and their purity. Western

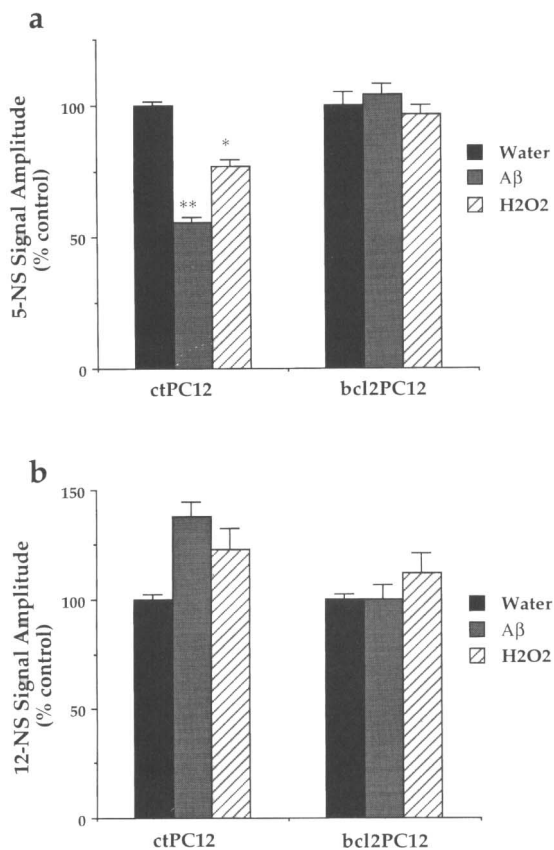


FIG. 2. H_2O_2 and $A\beta$ induce decreases in 5-NS signal amplitude in intact PC12 cells: protection by Bcl-2. 5-NS (a) and 12-NS (b) were incorporated into intact cell suspensions and were then exposed to water (vehicle), $400 \mu M$ $A\beta$, or $3 mM$ H_2O_2 for 15 min. EPR signals were recorded and peak amplitudes quantified. Values are the means \pm SEM of four to six separate experiments. * $p < 0.05$, compared with water value in ct-PC12 cells and $A\beta$ value in Bcl-2-PC12 cells; ** $p < 0.01$, compared with water value in ct-PC12 cells and H_2O_2 value in Bcl-2-PC12 cells. Basal rates of incorporation were as follows: 5-NS ct-PC12, $26,257 \pm 525$; 5-NS Bcl-2-PC12, $22,813 \pm 1,163$; 12-NS ct-PC12, $24,963 \pm 624$; 12-NS Bcl-2-PC12, $30,875 \pm 833$.

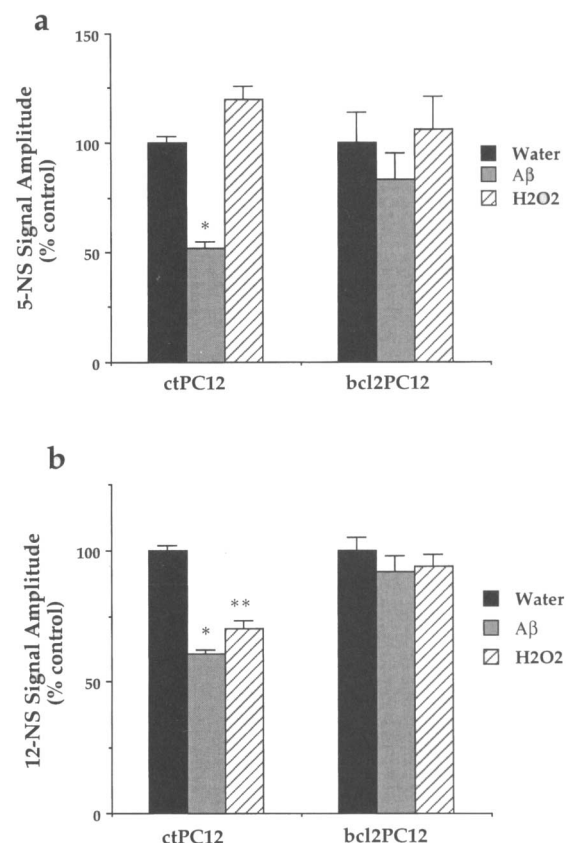


FIG. 3. Effects of $A\beta$ and H_2O_2 on 5-NS and 12-NS signal amplitudes in PNM fractions from ct-PC12 and Bcl-2-PC12 cells. 5-NS (a) and 12-NS (b) were incorporated into PNM fractions, which were then exposed to water (vehicle), $400 \mu M$ $A\beta$, or $3 mM$ H_2O_2 for 15 min. EPR signals were recorded and peak amplitudes quantified. There was no difference in basal rates of incorporation between ct-PC12 and Bcl-2-PC12 cells, and values are the means \pm SEM of four to six separate experiments. * $p < 0.01$, compared with water value in ct-PC12 cells and $A\beta$ value in Bcl-2-PC12 cells; ** $p < 0.01$, compared with water value in ct-PC12 cells and H_2O_2 value in Bcl-2-PC12 cells.

blot analysis of proteins from intact cell preparations showed that Bcl-2-PC12 cells contained high levels of Bcl-2, whereas Bcl-2 immunoreactivity was not detected in ct-PC12 cells (Fig. 4a). Bcl-2 was present in both the mitochondrial and the plasma membrane fractions of Bcl-2-PC12 cells but was absent from these fractions of ct-PC12 cells (Fig. 4a). To verify the purity of the mitochondrial and plasma membrane fractions, proteins from each fraction were subjected to western blot analyses using antibodies that recognize established mitochondrial (F_1/F_0 -ATPase) or plasma membrane (Na^+, K^+ -ATPase and glucose transporter GLUT3) proteins. High levels of F_1/F_0 -ATPase immunoreactivity were present in the mitochondrial fraction but were not detected in the plasma membrane fraction (Fig. 4b). Conversely, Na^+, K^+ -ATPase and GLUT3 were present in the plasma membrane fraction but not in the mitochondrial fraction (Fig. 4b).

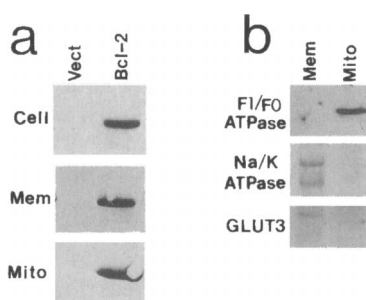


FIG. 4. Characterization of subcellular fractions of control and Bcl-2-expressing PC12 cells. **a:** Western blot analysis of Bcl-2 protein levels in intact cells (cell), isolated plasma membranes (Mem), and isolated mitochondria (Mito). **b:** Western blot analysis of F₁/F₀-ATPase, Na⁺,K⁺-ATPase, and GLUT3 in plasma membrane (Mem) and mitochondrial (Mito) fractions from ct-PC12 cells. Similar results were obtained in analyses of fractions from Bcl-2-PC12 cells.

Bcl-2 protects isolated mitochondrial and plasma membranes against loss of spin label induced by peroxidative insults

Isolated mitochondrial membranes were loaded with 5-NS or 12-NS and exposed to A β or H₂O₂. Treatment of Bcl-2-PC12 mitochondria with A β caused a 48% decrease in 5-NS signal amplitude, whereas H₂O₂ exposure resulted in only a 10% decrease in the 5-NS signal (Fig. 5a). Mitochondria from ct-PC12 cells, however, demonstrated 68 and 29% decreases in the 5-NS signal amplitude in response to A β and H₂O₂ (Fig. 5a). These decreases were significantly different from both the vehicle-treated ct-PC12 levels and the A β - and H₂O₂-treated Bcl-2-PC12 levels. A β administration to mitochondrial fractions from Bcl-2-PC12 cells did not affect 12-NS signal amplitude, whereas H₂O₂ caused a 20% decrease in the 12-NS signal amplitude (Fig. 5b). Exposure of ct-PC12 mitochondria to A β or H₂O₂ resulted in 42 or 36% decreases in 12-NS label, values that were significantly different from both the vehicle-treated ct-PC12 levels and the A β - and H₂O₂-treated Bcl-2-PC12 levels.

Treatment of Bcl-2-PC12 plasma membranes with A β caused a 35% decrease in 5-NS signal amplitude, whereas H₂O₂ exposure resulted in a 17% decrease (Fig. 6a). Plasma membranes from ct-PC12 cells, however, showed 48 and 28% decreases in 5-NS signal amplitude in response to A β and H₂O₂ (Fig. 6a). These decreases were significantly different from both the vehicle-treated ct-PC12 levels and the A β - and H₂O₂-treated Bcl-2-PC12 levels. Neither A β nor H₂O₂ affected 12-NS signal amplitude in Bcl-2-PC12 cells (Fig. 6b). In contrast, exposure of ct-PC12 plasma membranes to A β resulted in a 45% decrease in 12-NS signal amplitude, which was significantly different from both the water-treated ct-PC12 level and the A β -treated Bcl-2-PC12 level (Fig. 6b).

To determine if the data obtained using spin label technology could be replicated using more conventional lipid peroxidation methodologies, lipid peroxi-

dation in purified mitochondrial and plasma membrane fractions exposed to water, A β , or H₂O₂ was determined using the TBARS assay, as described previously (Goodman et al., 1996). TBARS levels in the plasma membrane preparation were below the threshold of detection of this assay, and hence no data could be obtained from this fraction (data not shown). In the mitochondrial fraction from ct-PC12 cells, both A β and H₂O₂ caused significant increases in TBARS levels (260–360% increase); the increase in TBARS fluorescence was significantly reduced in mitochondria from Bcl-2-PC12 cells (Fig. 7).

Levels of antioxidant enzyme activities and GSH associated with plasma and mitochondrial membranes are low and are unaffected by Bcl-2 overexpression

Although the data above suggested a rather direct antiperoxidative action of Bcl-2 in plasma and mito-

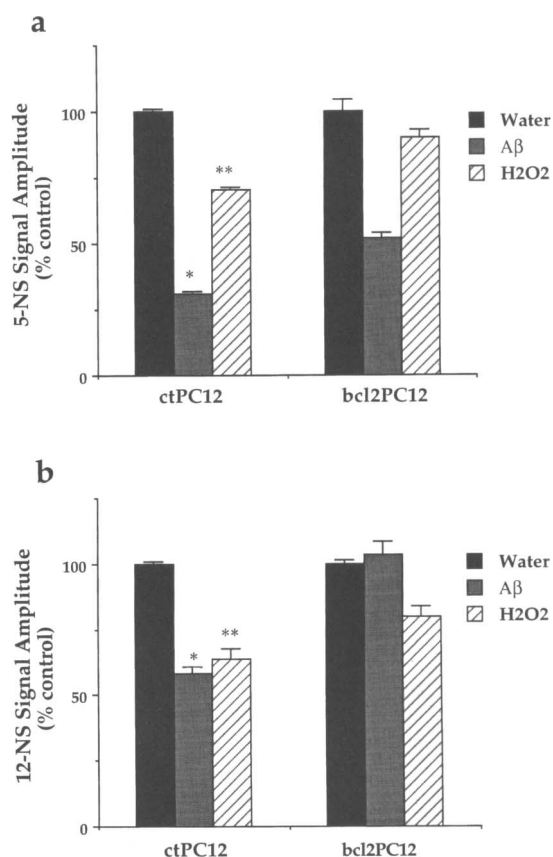


FIG. 5. Effects of A β and H₂O₂ on 5-NS and 12-NS signal amplitudes in isolated mitochondrial membranes from ct-PC12 and Bcl-2-PC12 cells. 5-NS (**a**) and 12-NS (**b**) were incorporated into mitochondrial membrane fractions, which were then exposed to water (vehicle), 400 μ M A β , or 3 mM H₂O₂ for 15 min. EPR signals were recorded and peak amplitudes quantified. There was no difference in basal rates of incorporation between ct-PC12 and Bcl-2-PC12 cells, and values are the means \pm SEM of four to six separate experiments. * p < 0.01, compared with water value in ct-PC12 cells and A β value in Bcl-2-PC12 cells; ** p < 0.01, compared with water value in ct-PC12 cells and H₂O₂ value in Bcl-2-PC12 cells.

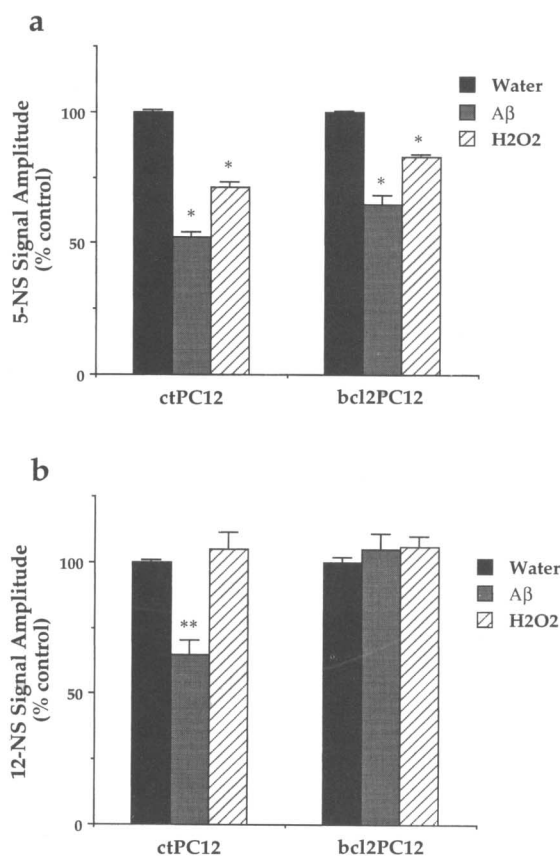


FIG. 6. Effects of $A\beta$ and H_2O_2 on 5-NS and 12-NS signal amplitudes in isolated plasma membranes from ct-PC12 and Bcl-2-PC12 cells. 5-NS (a) and 12-NS (b) were incorporated into plasma membranes, which were then exposed to water (vehicle), $400 \mu M$ $A\beta$, or $3 mM$ H_2O_2 for 15 min. EPR signals were recorded and peak amplitudes quantified. There was no difference in basal rates of incorporation between ct-PC12 and Bcl-2-PC12 cells, and values are the means \pm SEM of four to six separate experiments. * $p < 0.01$, compared with water value in ct-PC12 cells and $A\beta$ value in Bcl-2-PC12 cells; ** $p < 0.01$, compared with water value in ct-PC12 cells and H_2O_2 value in Bcl-2-PC12 cells.

chondrial membranes, it was conceivable that Bcl-2 might alter levels of membrane-associated cellular antioxidants. Therefore, we quantified levels of the major antioxidant enzymes and GSH in cytosolic, mitochondrial membrane, and plasma membrane fractions from ct-PC12 cells and Bcl-2-PC12 cells (Table 1). Levels of catalase activity were much lower in the membrane fractions than in the cytosolic fraction, and there were no significant differences in catalase activity between ct-PC12 and Bcl-2-PC12 cells in any of the three fractions. Levels of Cu/Zn-SOD activity were five- to 10-fold higher in cytosol than in the membrane fractions; cells overexpressing Bcl-2 had a significantly higher level of Cu/Zn-SOD activity in the cytosol, but levels of Cu/Zn-SOD activity in mitochondrial and plasma membrane fractions were not different in ct-PC12 and Bcl-2-PC12 cells (Table 1). Mn-SOD activity was

higher in the mitochondrial membrane fraction than in the other fractions in both ct-PC12 and Bcl-2-PC12 cells; Bcl-2 overexpression had no effect on Mn-SOD activity in any of the three fractions. Glutathione peroxidase activity was greater in the cytosolic fraction than in the mitochondrial fraction; activity of this enzyme was undetectable in the plasma membrane. Cells overexpressing Bcl-2 had a significantly elevated level of glutathione peroxidase in the cytosolic fraction compared with ct-PC12 cells, whereas levels of this enzyme were not significantly different in mitochondrial membranes from ct-PC12 and Bcl-2-PC12 cells (Table 1). GSH was undetectable in mitochondrial and plasma membrane fractions from ct-PC12 and Bcl-2-PC12 cells. In the cytosolic fraction, GSH levels were significantly greater (30% increase) in cells overexpressing Bcl-2.

DISCUSSION

The present findings demonstrate that Bcl-2 is present in plasma and mitochondrial membrane fractions, wherein it acts locally to suppress lipid peroxidation. In PC12 cells overexpressing Bcl-2, Bcl-2 was found not only in nuclear and mitochondrial membranes but also in plasma membrane fractions. This novel finding, when taken together with prior studies showing localization of Bcl-2 to membranes of mitochondria, endoplasmic reticulum, and nucleus (Hockenbery et al., 1993; Krafewski et al., 1993), indicates that Bcl-2 can associate with each of the major membrane systems in cells. The purity of isolated fractions was confirmed using specific markers for either plasma membrane (Na^+ , K^+ -ATPase and GLUT3) or mitochondrial (F_1/F_0 -ATPase) elements. These data suggest that the an-

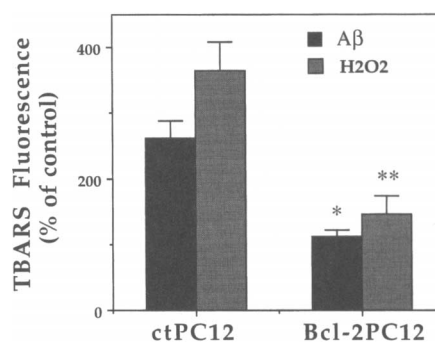


FIG. 7. Effects of $A\beta$ and H_2O_2 on TBARS in mitochondrial membranes from ct-PC12 and Bcl-2-PC12 cells. Lipid peroxidation, as indicated by thiobarbituric acid reactivity, was determined in purified mitochondrial membranes exposed to water (control), $400 \mu M$ $A\beta$, or $3 mM$ H_2O_2 for 15 min. Fluorescence was measured in arbitrary units, as described in Materials and Methods, and control values for ct-PC12 and Bcl-2-PC12 cells were $3,450 \pm 270$ and $3,604 \pm 396$, respectively. Values are the means \pm SEM of two separate experiments. * $p < 0.05$, compared with $A\beta$ -treated ct-PC12 cells; ** $p < 0.01$, compared with H_2O_2 -treated ct-PC12 cells.

TABLE 1. Antioxidant enzyme and GSH content of cytosolic, mitochondrial, and plasma membrane fractions from control PC12 cells and PC12 cells overexpressing Bcl-2

	ct-PC12			Bcl-2-PC12		
	Cytosol	Mito	PM	Cytosol	Mito	PM
Catalase	11.4 ± 0.8	4.2 ± 0.1	5.8 ± 0.3	15.5 ± 0.9	4.4 ± 0.2	5.1 ± 0.1
Cu/Zn-SOD	12.6 ± 0.2	2.7 ± 0.2	1.4 ± 0.3	22.7 ± 2.3 ^a	3.2 ± 0.3	2.2 ± 0.4
Mn-SOD	0.3 ± 0.2	1.6 ± 0.4	0.3 ± 0.1	0.3 ± 0.1	1.7 ± 0.1	0.3 ± 0.1
GSH perox.	54.7 ± 2.3	26.1 ± 4.6	ND	117.5 ± 20 ^a	34.5 ± 3.5	ND
GSH	74 ± 2.7	ND	ND	104 ± 2.0 ^a	ND	ND

Antioxidant enzyme activities (catalase, Cu/Zn-SOD, Mn-SOD, glutathione peroxidase) and GSH levels were quantified in cytosolic (Cytosol), mitochondrial (Mito), and plasma membrane (PM) fractions, as described in Materials and Methods. Values for enzyme activities are expressed as units of activity/mg of protein, and values for GSH levels as pmol/ μ g of protein. Values are the means \pm SEM of determinations made in three to four experiments. ND, not detectable.

^a $p < 0.01$ compared with corresponding value in ct-PC12 cells.

choring mechanisms needed to localize Bcl-2 to the plasma membrane are present in cells and hence raise the possibility that under pathophysiological conditions, Bcl-2 may associate with plasma membranes to facilitate defense against external oxidants. This scenario has not been established experimentally, but isolation of the putative intracellular signals that mediate Bcl-2 insertion into the plasma membrane warrants further research. However, because we used a cell line that overexpresses Bcl-2, we cannot rule out the possibility that such overexpression facilitates association of Bcl-2 with the plasma membrane. The possibility that overexpression of any membrane-associated protein may prevent oxidative injury by acting as a sink for free radicals is precluded by studies showing that overexpression of wild-type presenilin 1 has no effect on reactive oxygen species levels induced by several apoptotic insults, whereas overexpression of mutant presenilin 1 enhances oxidative stress (Guo et al., 1997).

In either crude postnuclear fractions or pure mitochondrial or plasma membrane fractions, the paramagnetism of both 5-NS (located near the lipid-water interface) and 12-NS (located deep within the lipid bilayer) was significantly decreased by A β and/or H₂O₂. Studies in several different laboratories have shown that A β is a potent inducer of membrane lipid peroxidation (Behl et al., 1994; Butterfield et al., 1994; Mark et al., 1995, 1997*a,b*) by a mechanism that appears to involve direct interaction with the membrane lipid bilayer (Goodman et al., 1996; Hensley et al., 1994; Mason et al., 1996). We found that Bcl-2 provides significant protection against lipid peroxidation induced by either A β or H₂O₂ in intact PC12 cells and in isolated mitochondrial and plasma membranes. Bcl-2 was particularly effective in protecting against oxidative damage to the 12-NS probe, suggesting some specificity for deep layers of lipid membrane in the actions of Bcl-2. Although Bcl-2 has been repeatedly shown to block oxidative injury in intact cells, this is the first report to demonstrate direct antioxidant effects of Bcl-2 in isolated plasma and mitochondrial membranes,

indicating that Bcl-2's antioxidant effects are not dependent on interaction with cytosolic constituents. Although Bcl-2 has been reported to block neuronal death in an atmosphere with very low oxygen levels (Jacobson et al., 1993), the preponderance of data suggests that (direct or indirect) antioxidant actions of Bcl-2 contribute to its antiapoptotic action in many different cell death paradigms.

The concentrations of A β and H₂O₂ used in the present study to disrupt spin label incorporation, although higher than generally used to injure primary neurons (Whittemore et al., 1994; Kruman et al., 1997), are within the range of concentrations required to kill tumor cell lines such as PC12 cells (Forrest et al., 1994; Zhou et al., 1996). Moreover, in experiments using whole cells, the external oxidative stimulus is propagated and amplified intracellularly through various cascades, a scenario that is not possible using isolated membrane fractions, therefore requiring the use of higher concentrations of oxidative insults.

Although Bcl-2 overexpression has been shown to suppress lipid peroxidation (Kane et al., 1993; Myers et al., 1995) and intracellular accumulation of reactive oxygen species (Hockenbery et al., 1993; Kane et al., 1993), its mechanism of actions has not been resolved. Mitochondria-associated Bcl-2 has been estimated to represent ~30–50% of total cellular Bcl-2 protein (Newmeyer et al., 1994), and this localization has been hypothesized to reflect Bcl-2's antiapoptotic properties (Jacobson et al., 1993; Reed, 1994). Shifts in mitochondrial function, including decreases in transmembrane potential and in complex II activity, occur during early stages of the apoptotic process and are attenuated by Bcl-2 overexpression (Reed, 1994; Kruman et al., 1997). However, the importance of membrane insertion for Bcl-2's antiapoptotic effects is unclear because C-terminal truncation of Bcl-2, which prevents membrane insertion, still provides some protection against oxidative insults (Kane et al., 1993; Borner et al., 1994). This observation, however, does not rule out the possibility that Bcl-2 acts as a free radical scavenging protein, a possibility suggested by

our data demonstrating direct antiperoxidative actions of Bcl-2 in isolated membrane preparations. PC12 cells that overexpress Bcl-2 have been reported to have higher levels of intracellular GSH (Kane et al., 1993; Kruman et al., 1997), suggesting an indirect antioxidant mechanism of action of Bcl-2. However, GSH is a cytosolic protein, and we did not detect GSH in plasma membrane or mitochondrial fractions. Moreover, our analyses of antioxidant enzyme activity levels in the different fractions showed that the level of each of the major antioxidant enzymes (catalase, Cu/Zn-SOD, Mn-SOD, and glutathione peroxidase) was not different in plasma membrane or mitochondrial membrane fractions from control and Bcl-2-overexpressing cells. Our results did, however, confirm previous reports describing increased cytosolic Cu/Zn-SOD and glutathione peroxidase in Bcl-2-overexpressing PC12 cells (Ellerby et al., 1996). Levels of antioxidant enzymes were at or below the limit of detection in the plasma membrane fraction of both ct-PC12 cells and Bcl-2-PC12 cells. These findings indicate that it is very unlikely that differences in antioxidant enzyme levels contributed to the increased resistance of membranes from Bcl-2-overexpressing cells to peroxidative insults. Therefore, it appears that Bcl-2 can act locally in membranes without a requirement for cytosolic constituents. Ascribing to Bcl-2 direct antioxidant properties also helps to explain reports describing antiapoptotic effects even in cells lacking nuclei (Newmeyer et al., 1994) or functional mitochondria (Jacobson et al., 1993). Indeed, it has been demonstrated that Bcl-2 overexpression does not alter many aspects of mitochondrial function, including ATP levels, oxygen consumption, and respiratory chain activity (Petit et al., 1996). Bcl-2 has been shown, however, to act downstream of calcium regulation to decrease evoked free radical formation in response to oxidative insults (Kane et al., 1993), indicating that Bcl-2's antiapoptotic properties can be explained, at least in part, by its ability to scavenge free radicals. Nevertheless, our data do not preclude the possibility that Bcl-2 has protective properties in addition to its antioxidant ability.

Membrane lipid peroxidation is believed to contribute to neuronal cell injury and death in a variety of disorders including cerebral ischemia (Chan, 1994; Hall et al., 1994) and Alzheimer's disease (Behl et al., 1994; Butterfield et al., 1994; Mark et al., 1997a,b). Evidence for neuronal apoptosis in ischemic brain injury (Linnik et al., 1993; MacManus et al., 1993) and Alzheimer's disease (Loo et al., 1993; Su et al., 1994) is accumulating. The importance of lipid peroxidation to apoptosis is evidenced by recent findings demonstrating that apoptotic concentrations of A β and FeSO₄ induce the formation of the lipid peroxidation product 4-hydroxynonenal, an aldehydic product of membrane lipid peroxidation (Kruman et al., 1997). Direct application of 4-hydroxynonenal to cells initiates apoptosis, and Bcl-2 overexpression blocks apoptosis induced by 4-hydroxynonenal (Kruman et al., 1997). A better un-

derstanding of the antiperoxidative actions of Bcl-2 may allow development of novel therapeutic approaches to the many different neurodegenerative conditions that involve oxidative stress.

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