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**13. ABSTRACT** ***(Maximum 200 Words)***

Previous studies have demonstrated that exposure of estrogen-responsive breast cancer cells in culture to estrogen leads to overexpression of *bcl-2*. Contrary to the case for apoptosis induced by conventional anticancer drugs, *bcl-2* overexpression potentiates apoptosis induction by neocarzinostatin in neural crest tumor cells. We therefore examined the effects of neocarzinostatin on *bcl-2*-overexpressing breast cancer cells in culture. Unlike the case for neural crest tumor cells, overexpression of *bcl-2* in MCF-7 cells did not afford potentiation of apoptosis induction by the reduction-dependent enediyne neocarzinostatin. Instead, MCF-7 cells were protected from apoptosis induced by neocarzinostatin. Two critical mechanistic differences have been discovered to relate to this finding. While *bcl-2* overexpression in neural crest tumor cells leads to alteration of glutathione handling in the direction of greater reducing potential, *bcl-2* overexpression in MCF-7 cells does not alter cellular redox potential or glutathione handling. Furthermore, neocarzinostatin treatment of neural crest tumor cells results in conversion of Bcl-2 protein to its pro-apoptotic cleavage products. This enzymatic cleavage is induced by caspase 3, an enzyme that is not expressed in MCF-7 cells. Predictably, only apoptosis induced by neocarzinostatin in neural crest tumor cells is inhibited by an inhibitor of caspase 3 activity.

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## (5) INTRODUCTION

The proto-oncogene bcl-2 is expressed in 65-80% of human breast cancers (1,2). Approximately 50% of breast cancers are found to overexpress this gene (1,3). Overexpression of bcl-2, which results in overproduction of the 26 kD protein Bcl-2, has been shown to confer resistance to apoptotic cell death induced by a variety of stimuli, including chemotherapeutic-induced apoptosis in breast cancer (4,5). This is particularly important in the case of advanced, estrogen receptor-positive tumors, a circumstance in which Bcl-2 content correlates with clinical resistance to chemotherapy (5).

Recent studies in other cell lines have suggested that Bcl-2 exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (6,7). Among other biochemical effects, these studies specifically implicate an increase in cellular reduced glutathione (GSH) content in this alteration in redox potential. This makes particular sense because other studies link the apoptotic process to exposure to reactive oxygen species (8-10). One potential strategy for overcoming Bcl-2-mediated chemotherapeutic resistance is to take advantage of the increase in cellular GSH and, therefore, free sulfhydryl content by using chemotherapeutic agents that require reduction by sulfhydryl compounds for their activity. We have recently explored in vitro the efficacy of one such group of agents, the enediynes, in pheochromocytoma cells that were genetically engineered to overexpress bcl-2 (11).

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in some tumor cell lines in culture (12). Like other naturally-occurring enediynes, NCS is actually a prodrug that requires sulfhydryl activation for efficacy. As such, the cytotoxicity of NCS has been demónstrated to vary directly with the sulfhydryl content of the cell (13-15). This information led to our prediction that, contrary to the case for all other chemotherapeutic agents studied, overexpression of bcl-2 and the resulting shift in redox potential of the cell would potentiate the induction of apoptosis by NCS.

We have shown that, in PC12 rat pheochromocytoma cells that have been bcl-2- or control-transfected, bcl-2 overexpression does indeed potentiate the apoptosis-inducing activity of NCS by increasing cellular levels of GSH (11). The reduction-dependent enediyne prodrugs are therefore the only class of drug that has been demonstrated to work best in those tumor cells that have become resistant to other known chemotherapeutic agents.

Although the naturally-occurring enediynes have been difficult to implement clinically because of toxicity issues (16), recent advances in pharmacology and pharmaceutics have begun to get around these problems. Modifying the NCS chromophore structure to produce other more efficacious and/or less toxic enediynes (17), masking the protein component with inert polymers (18), using enediynes adjunctively with cell-selective activating agents (13), and coupling the newer enediynes to monoclonal antibodies directed at tumor-specific antigens (19) have all proved useful in producing enediyne candidates for human therapeutics. In fact, the last of these

approaches is now in Phase I/II clinical trials for ovarian carcinoma (L. Hinman, personal communication), and has shown promise in a breast cancer cell line in culture (19). The 4-fold gap in sensitivity to NCS that we observed between bcl-2- and control-transfected PC12 cells (11) has led us to speculate that the enediynes would be both more efficacious and safer in these patients than in those whose tumors do not overexpress bcl-2.

These interesting and promising preliminary results led us to predict that breast cancer cells that overexpress bcl-2 would have a higher GSH content and therefore be more susceptible to the effects of the enediynes than are breast cancer cells that do not overexpress this gene or normal cells. We further predicted that we could augment the difference in enediyne sensitivity between bcl-2-native normal and -overexpressing tumor cells by increasing the availability of cysteine, the rate-limiting substrate for GSH synthesis. Furthermore, we predicted that by increasing the Bcl-2 content of estrogen receptor-positive breast cancer cells with tamoxifen (20) or estrogen (4), we could increase the effectiveness of enediynes against these cells, raising the possibility of adjunctive treatment with enediynes and tamoxifen. This would translate clinically into an improved therapeutic index for treatment of breast cancer with the enediynes.

## (6) BODY

### (6)-1. Experimental Methods, Assumptions, and Procedures

All of the methods we proposed for use in these studies are currently established in our laboratory using cultured cell systems (11,12,21-29). We describe below the specific hypotheses that were tested and the methods that were used over the two years of Department of Army funding.

(6)-1.1: General Methods Applicable to All Tasks and Technical Objectives

MCF-7 (estrogen receptor-positive) cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained as stock cultures in α-MEM (GIBCO-BRL) containing phenol red and supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum, and 2 μg/ml gentamicin sulfate (37°C, 5% $CO_2$). Where indicated, studies were conducted under four sets of conditions known to alter bcl-2 expression in this cell line in predictable ways: maintenance of the conditions of the stock cultures (E+; bcl-2-positive at "resting" levels; 4,5); addition to the stock culture conditions of 1 nM 17ß-estradiol and maintenance in the estradiol-enriched medium for 48 hr prior to study (E++; 3-fold enhancement of bcl-2 content over resting levels; 5); maintenance for 7 days prior to study in phenol red-free DMEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (Sigma Chemical Corp., St. Louis, MO) for 45 min at 45°C (E-; 6-fold reduction of bcl-2 levels relative to resting state; 4). In addition, E+ cells were studied after a four-day exposure to tamoxifen ($10^{-6}$M; condition T+), a condition the in vivo analogue of which is associated with induction of bcl-2 (20). In all cases, the same lot of fetal bovine serum (GIBCO-BRL) was used for all conditions in each experiment, and to the extent possible, for all experiments.

In addition, clonal transfectants of MCF-7 human breast cancer cells were obtained from Drs. Charles Rudin and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in ref. 30), respectively.

(6)-1.2: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on bcl-2 content of MCF-7 cells]

For all studies, relative bcl-2 content of native MCF-7 cells maintained under each condition was assayed by Western blotting as we have previously described (11). In the case of MCF-7 transfectants, stably transfected clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

(6)-1.3: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

To determine the GSH and total glutathione (GSH+GSSG) contents of MCF-7 cells containing different amounts of bcl-2, E+, E++, E-, and T+ cells ($10^7$ of each) were washed free of medium, and suspended in 1 ml of phosphate-buffered saline (PBS). The suspension was homogenized and assayed for GSSG and GSH+GSSG by the method of Tietze (31) or for GSH by the Thio-Glo method (32). For the Tietze method, the rate of change in the $OD_{412}$ was measured spectrophotometrically over a period of 3 min. This rate was converted to the total glutathione concentration by plotting on a simultaneously run standard curve for $\Delta OD_{412}$ versus total glutathione concentration (constructed using GSH standard solutions). The concentration of GSSG alone was determined by an identical procedure performed on samples that were treated with N-ethylmaleimide prior to assay, to eliminate the reaction of GSH with DTNB (31). The cellular contents of GSH and GSH+GSSG were then calculated for each condition. Each determination was performed in triplicate. Values so obtained for each experiment were compared between E+ and E++, E-, or T+ cells using Student's t test, as we have done in our published work (24,25).

(6)-1.4: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

An NCS concentration-response study was conducted for E+, E++, E-, and T+ MCF-7 cells and MCF-7 transfectants, as we have previously described for pheochromocytoma cells (11). Cells plated in 6-well tissue culture plates were treated with a range of concentrations of NCS (0-0.5 μg/ml) for 1 hr at 37°C. Cultures were then washed free of NCS, and adherent cell number was determined daily in control- and NCS-treated E+, E++, E-, and T+, and MCF-7 transfectant cultures as we have previously described for neuroblastoma and pheochromocytoma cells (11,21,33). The statistical significance of differences between E+ and E++, E-, and T+ cells, in turn, was assessed for each concentration of NCS using Student's t test, with p maximally 0.05 being considered significant.

(6)-1.5: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript co-authored by Drs. Schor, Kagan and his laboratory colleagues, and Thompson and his laboratory colleagues has been published in Oncogene (34). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-1.6: Tasks 5, 6 (Determination of glutathione metabolic enzyme activities and efflux rate in MCF-7 cells with varying expression of bcl-2)

Clonal transfectants of MCF-7 human breast cancer cells, two of which expressed bcl-2 in approximately 100-fold excess over native levels, and two of which were mock-transfectants with native bcl-2 expression levels, were obtained from Drs. Charles Rudin

and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in ref. 30), respectively. Homogenates of these cells were assayed at before and at various timepoints after incubation with N-acetylcysteine (10 mM) for the activities of γ-glutamylcysteine synthetase (GCS) and glutathione reductase (GR) by the methods of Seelig and Meister (35) and Carlburg and Mannervik (36), respectively. Statistical comparisons (n = 3 per condition) were made using Student's t test with a $p < 0.05$ considered to be statistically significant.

(6)-1.7: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells with cis-parinaric acid)

The membranes of E-, E+, and E++ MCF-7 cells were loaded with cis-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (32), as an in situ probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (37).

(6)-1.8: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to GSH oxidation membrane lipid peroxidation)

Our previous studies have demonstrated that the diazo radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (32). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (38,39). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.

In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17β-estradiol (E2; 0 - $5 \times 10^{-7}$ M;

Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.

PnA served as an in situ probe for phospholipid oxidation, as we have previously described (32,37). GSH was measured as detailed above [section (6)-1.3].

(6)-1.9: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been published in Biochemical Biophysical Research Communications (40). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-1.10: Task 10 (Determination of the effects of bcl-2-overexpression in MCF-7 cells on the increase in glutathione afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 0.5 - 4 hr. Cells were washed free of extracellular N-acetylcysteine and assayed at various timepoints for total glutathione content by the spectrophotometric method of Tietze (31).

(6)-1.11: Task 11 (Determination of the effects of bcl-2-overexpression in MCF-7 cells on the change in sensitivity to NCS afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 4 hr. Cells were washed free of extracellular N-acetylcysteine (to ensure that NCS did not get activated by this compound extracellularly), and an adherent cell number concentration-response curve to NCS was constructed for each transfectant as we have done in our previously published studies (11). The $ED_{50}$ for NCS was determined for each transfectant as we have previously done for apoptosis induction in SH-SY5Y cells (12,22). The $ED_{50}$s obtained with and without N-acetylcysteine were compared for each transfectant using Student's t test, as we have previously described (22).

(6)-1.12: Task 12 (Preparation of a manuscript describing the findings relative to Tasks 10-11)

The results of these studies form an integral part of the abovementioned manuscript published in Oncogene (34).

## (6)-2. Results and Discussion

(6)-2.1: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on bcl-2 content of MCF-7 cells]

We have measured the effects of addition to and subtraction from the medium of estradiol to the Bcl-2 content of MCF-7 cells in culture. Our first experiments involved using complete medium as the control, and adding various amounts of estradiol to the medium, as originally described in the proposed Methods. These studies, all using the same batch of serum for constitution of the medium, demonstrated no change in the Bcl-2 content of the cells. Our assumption was that the serum-replete medium contained suffient estradiol that neither our addition of small amounts of this compound nor our partial depletion of estradiol with charcoal treatment made a significant change in the Bcl-2 content. We surmounted this problem in two ways. First, we obtained estrogen-free medium commercially stripped (by column chromatography) of estrogen, and performed our studies by adding back various amounts of estradiol. This study conclusively demonstrated the estrogen concentration-dependent increase in concentration of Bcl-2 in the cells (See Figure 1). In addition, we have obtained from the laboratory of Dr. Craig Thompson (University of Chicago, Chicago, IL) two clones of MCF-7 cells that have been transfected with the bcl-2 gene, and their mock-transfected counterparts. We have confirmed overproduction of Bcl-2 in the bcl-2-transfected cells, and have maintained all of these lines in our laboratory.

(6)-2.2: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

In the first year of funding, we measured the total glutathione content of the transfectants obtained from Dr. Thompson's laboratory. Accompanying the increase in Bcl-2 content afforded by the bcl-2 transfection, there was a small but significant increase in glutathione content (see Figure 2).

Recently, we have been able to maintain MCF-7 cells in estrogen-depleted (E-) medium, medium with a physiological estrogen concentration (E+), and media containing each of two estrogen-excess concentrations (E++). With our co-investigators in the laboratory of Dr. Valerian Kagan, we have demonstrated that long-term E2 incubation leads to an increase in total glutathione content of the cells (see Figure 3).

(6)-2.3: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by bcl-2 transfection (11). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. To determine the generalizability of these findings to the overexpression of bcl-2 in different biological systems, similarly transfected MCF-7 human breast cancer cells were examined for the effects of bcl-2 overexpression on sensitivity to NCS. Unlike PC12

cells, bcl-2 overexpression protected MCF-7 cells from death induced by NCS (Figure 4). At each concentration, the survival of the bcl-2 transfectants exceeds that of the mock transfectants.

Attempts to perform the same set of studies in cells exposed to E++, E+, E-, and T+ media, or E- medium and subsequent transfer to graded E concentrations were thwarted by the tendency of estrogen-deprived cells to lift off of the culture surface over the final 5-10 days of the experimental exposure. Although biochemical studies (e.g., glutathione measurements) can be made on these detached cells, the serial assessment of cell culture growth is technically not feasible using our current techniques.

(6)-2.4: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript describing these findings has been published in Oncogene (34). This manuscript is co-authored by NF Schor, C Rudin, A-R Hartmann, CB Thompson, Y Tyurina, and VE Kagan. The support of the Department of the Army Breast Cancer Program has been acknowledged in this manuscript, and it is appended to this report.

(6)-2.5: Tasks 5, 6 (Determination of glutathione metabolic enzyme activities and efflux rate in MCF-7 cells with varying expression of bcl-2)

Examination of the GCS and GR content of MCF-7 transfectants indicates that there is no difference between bcl-2- and mock-transfectants either before or at timepoints up to 6 hours exposure to N-acetylcysteine (10 mM). Glutathione handling appears to be unaltered in MCF-7 cells by overproduction of Bcl-2.

(6)-2.6: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells with cis-parinaric acid)

Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown in Table 1. PnA served as an in situ probe for phospholipid oxidation, as we have previously described (32,37).

(6)-2.7: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to GSH oxidation membrane lipid peroxidation)

Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in statistically significant peroxidation of all four phospholipid species examined (Table 2). The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine (PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without sufficient change in the overall phospholipid composition of the membranes to induce necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to non-E2-exposed cells that did not reach statistical significance; Figure 5A). This wholesale protection of all phospholipids studied from peroxidation suggests a direct antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant protection of PS and, at the highest E2 concentration only, PE (Figure 5B). This pattern is suggestive of Bcl-2-mediated protection from enactment of apoptosis (38,39), rather than direct protection from membrane phospholipid peroxidation per se. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (32). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated ($5x10^{-7}$ M) cells similarly exposed ($p < 0.01$, Student's t test).

In light of the previous reports of glutathione-dependent (41) and independent (42-44) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Figure 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to bcl-2 overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (6).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (41), but may be enhanced by induction of estrogen metabolism (45). In contrast, bcl-2 induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

(6)-2.8: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been published in Biochemical Biophysical Research Communications (40). The co-authors of this manuscript are Drs. Schor, Kagan, Tyurin, and Tyurina. The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-2.9: Task 10 (Determination of the effects of bcl-2-overexpression in MCF-7 cells on the increase in glutathione afforded by incubation with N-acetylcysteine)

Treatment of mock- and bcl-2-transfected MCF-7 cells with N-acetylcysteine resulted in GSH content kinetics that resembled those seen with mock-transfected PC12 cells (Figure 2). There was no significant difference between the MCF-7 bcl-2- and mock-transfectants in this regard.

(6)-2.10: Task 11 (Determination of the effects of bcl-2-overexpression in MCF-7 cells on the change in sensitivity to NCS afforded by incubation with N-acetylcysteine)

In contrast to the case for bcl-2-transfected PC12 cells, previously shown by our group to increase in sensitivity to NCS after incubation with N-acetylcysteine, the concentration-response curve of bcl-2-transfected MCF-7 cells to NCS is unaffected by N-acetylcysteine treatment (Figure 6). The sensitivity to NCS of mock-transfected cells is similarly unaffected by N-acetylcysteine exposure (data not shown). These results are in accord with our finding that bcl-2-transfected MCF-7 cells do not demonstrate altered handling of glutathione.

(6)-2.11: Task 12 (Preparation of a manuscript describing the findings relative to Tasks 10-11)

The results of these studies form an integral part of the abovementioned manuscript published in Oncogene (34).

(6)-2.12: Additional tasks accomplished during the one-year extension of DoD funding

Accomplishment of the proposed tasks allowed us to determine that Bcl-2 overproduction in MCF-7 cells does not result in potentiation of NCS-induced apoptosis, unlike the case for neural crest tumor cells. Our studies also indicated that one reason for this is the difference between these two cell types in the effects of Bcl-2 production on glutathione handling. However, if overexpression of bcl-2 in neural crest tumor cells only increased the activation of NCS by glutathione, its protein product, Bcl-2, would be expected nonetheless to block apoptosis downstream of the action of this drug, presumably at the level of inhibition of cytochrome c release from mitochondria (46,47). That this is not the case is demonstrated by our finding of potentiation by Bcl-2 of reduction-dependent chemotherapeutic agent-induced oxidation and externalization of membrane phosphatidylserine (48), a late event in the apoptosis final common pathway. Clearly, the distal anti-apoptotic effects of Bcl-2 have been thwarted in this system;

indeed, a pro-apoptotic effect appears likely. Because of reports that cleavage products of Bcl-2 are pro-apoptotic (49), we used the Western blotting technique described for Task 1 to examine the effects of NCS treatment on Bcl-2 itself in MCF-7 and neural crest tumor cells. Figure 7 demonstrates the time-dependent cleavage of Bcl-2 to its pro-apoptotic counterpart in Bcl-2-overproducing PC12 neural crest tumor cells treated with NCS. As is shown in Figure 8 however, MCF-7 cells treated similarly do not demonstrate Bcl-2 cleavage. Furthermore, as Bcl-2 cleavage is thought to be the result of the activity of caspase 3, we examined caspase 3 expression in PC12 cells and in our MCF-7 transfectants. As has been reported for native MCF-7 cells, our MCF-7 transfectants do not express caspase 3. We are currently planning to determine the predictive value of caspase 3 expression for Bcl-2-mediated potentiation of NCS-induced apoptosis. A manuscript describing these studies has been submitted for publication in Cancer Research (50). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

## (7) KEY RESEARCH ACCOMPLISHMENTS

- Determination of the effects of bcl-2 overexpression on the GSH content of MCF-7 breast cancer cells.
- Determination of the effects of bcl-2 overexpression on the susceptibility of MCF-7 breast cancer cells to NCS-induced apoptosis.
- Determination of the mechanistic differences between MCF-7 and PC12 cells that determine the differential handling of GSH consequent to bcl-2 overexpression.
- Determination of the molecular differences between MCF-7 and PC12 cells that determine the differential mechanism of apoptosis signaling and susceptibility to NCS-induced apoptosis.

## (8) REPORTABLE OUTCOMES

Manuscripts

Schor, N. F., Rudin, C. M., Hartman, A.-R., Thompson, C. B., Tyurina, Y. Y., and Kagan, V. E. Cell line dependence of Bcl-2-induced alteration of glutathione handling. Oncogene 19: 472-476, 2000.

Schor, N. F., Tyurina, Y. Y., Fabisiak, J. P., Tyurin, V. A., Lazo, J. S., and Kagan, V. E. Selective oxidation and externalization of membrane phosphatidylserine: Bcl-2-induced potentiation of the final common pathway for apoptosis. Brain Research 831: 125-130, 1999.

Schor, N. F., Tyurina, Y. Y., Tyurin, V. A., and Kagan, V. E. Differential membrane antioxidant effects of immediate and long-term estradiol treatment of MCF-7 breast cancer cells. Biochem. Biophys. Res. Commun. 260: 410-415, 1999.

Tyurina, Y. Y., Shvedova, A. A., Kawai, K., Tyurin, V. A., Kommineni, C., Quinn, P. J., Schor, N. F., Fabisiak, J. P., Kagan, V. E.. Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. Toxicology, in press, 2000.

Kagan, V. E., Fabisiak, J. P., Shvedova, A. A., Tyurina, Y. Y., Tyurin, V. A., Schor, N. F., Kawai, K. Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. FEBS Lett., in press, 2000.

Liang, Y., Nylander, K. D., and Schor, N. F. Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis By Bcl-2. Submitted to Cancer Research, 2000.

Abstracts

Schor, N. F., Liang, Y., Kagan, V. E., Tyurina, Y. Y., Tyurin, V., and Nylander, K. D. Exploitation of the overexpression of bcl-2 in the therapy of breast cancer. Proc Era of Hope DoD BCRP Meeting, p. 747, 2000.

## (9) CONCLUSIONS

Our results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (41), but may be enhanced by induction of estrogen metabolism (45). In contrast, bcl-2 induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). Our studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

Some studies of the effects of bcl-2 overexpression on ROS handling in neural cells have suggsted a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (6,7,32). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (51). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (52), and that GSH depletion does not abolish the protective effects of Bcl-2 (45). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is millieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well documented (53).

Our studies of NCS treatment of PC12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (4,5,54-58), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (11). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in bcl-2-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (59). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to

compensate for consumption of reducing equivalents is aberrant in some bcl-2-overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (60). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (61). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (39), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells make NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.

That incubation with N-acetylcysteine accentuates the difference in GSH handling between bcl-2- and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in bcl-2-overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an in vitro assay of GSH accumulation after N-acetylcysteine incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.

Our previous studies have demonstrated that bcl-2 overexpression potentiates enediyne-induced apoptosis in some cell lines and not in others. We now show that potentiation of enediyne-induced apoptosis in bcl-2-transfected PC12 cells is associated with cleavage of Bcl-2 protein. Although Bcl-2 itself is an anti-apoptotic protein, it has recently been reported that the cleavage product of this protein is pro-apoptotic (49,62). Bcl-2 cleavage has been reported to occur after Asp-34, and to result in production of a pro-apoptotic cleavage product lacking the N-terminal 34 amino acids of Bcl-2 (49). This is consistent with the 23 kD cleavage product that was detected in response to NCS treatment. Cleavage of Bcl-2 was not detected in cisplatin-treated, bcl-2-transfected PC12 cells or NCS-treated, bcl-2-transfected human MCF-7 cells.

That this Bcl-2 cleavage requires the activity of caspase 3 is shown by two lines of evidence. NCS treatment of MCF-7 cells, cells previously described and demonstrated herein not to express caspase 3, does not result in cleavage of Bcl-2. [The absence of caspase 3 expression in MCF-7 cells is the result of a 47-base pair deletion within exon of 3 of the caspase 3 gene (63).] Furthermore, the caspase 3-specific inhibitor Ac-DEVD-CHO blocks both the cleavage of Bcl-2 resulting from NCS treatment and the potentiation of NCS-induced apoptosis seen with bcl-2 overexpression. This is consistent with previous reports of the abrogation of cleavage of Bcl-2 when caspase 3 was immunodepleted from extracts of 293 cells. Conversely, immunodepletion of caspase 7 did not affect Bcl-2 cleavage in this system (62). It is thus clear that caspase 3 plays a critical role in the cleavage of Bcl-2 in bcl-2-transfected PC12 cells. While this role is likely to be a direct one, it is also possible that caspase 3 activates a downstream protease that, in turn, directly cleaves Bcl-2.

It is of interest that, despite their lack of caspase 3 expresison, MCF-7 cells still undergo apoptosis induced by a host of exogenous stimuli, including Vitamin D (64), staurosporine (63), the diazo radical initiator, AMVN (40), and NCS (34). Caspase 3 is clearly not involved in apoptosis in these systems. However, when the gene for caspase 3 was tranfected into MCF-7 cells, the gene was expressed, and caspase 3 protein was activated, resulting in cleavage of Bcl-2 (62).

The mechanism of apoptosis induction by NCS in MCF-7 cells may involve pro-apoptotic changes other than the cleavage of Bcl-2. Our preliminary studies indicate that treatment of MCF-7 cells with NCS results in downregulated expression of Bcl-2 and upregulated expression of its pro-apoptotic analogue, Bax. The resulting decrease in the Bcl-2/Bax ratio has been shown in other systems to trigger the release of cytochrome c from the mitochondria (65), and to thereby induce apoptosis (66,67). Modulation of the Bcl-2/Bax ratio represents an alternative mechanism of potentiation of apoptosis in cells lacking caspase 3 and therefore unable to cleave Bcl-2 to a pro-apoptotic species.

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## (9) APPENDICES

Figure Legends

Figure 1. Western blot for human Bcl-2 performed on estradiol (E2)-deprived (10 d) MCF-7 cells after 14 d replacement with E2 (0 - 5 x $10^{-7}$ M). A whole cell homogenate of $10^6$ cells was applied to each lane. (A) Photograph of representative lanes of the Western blot depicting bcl-2-transfected PC12 cells as a positive control and MCF-7 cells initially estrogen-deprived and subsequently treated with 5 x $10^{-7}$ M E2. The numbers to the left of the gel photograph indicate the running position on the gel of various molecular weight standards. (B) Densitometric vertical scans of each lane of the Western blot stained for Bcl-2.

Figure 2. Effects of bcl-2 transfection on accumulation of GSH in MCF-7 cells continuously incubated with N-acetylcysteine (NAcCys; 10 mM). The results at t = 0 represent the native GSH content of each of these transfectants. The results of single determinations from each of two mock- and two bcl-2-transfected clones of MCF-7 cells are shown. A second independent experiment gave comparable results.

Figure 3. Effect of immediate and long-term exposure to E2 and treatment with AMVN on glutathione content of MCF-7 breast cancer cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 μM) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo™ assay. Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with $p < 0.0001$ (Student's t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E2-exposed cells.

Figure 4. Effects of bcl-2 transfection on sensitivity of cultured tumor cells to NCS. In all cases, cells were treated for 1 h with NCS on day 0 and counted daily. Points represent the mean of counts from three separate high power fields expressed as a percent of the count on day 0. Error bars signify the SEM. (A) PC12 pheochromocytoma cells; (B) MCF-7 breast cancer cells.

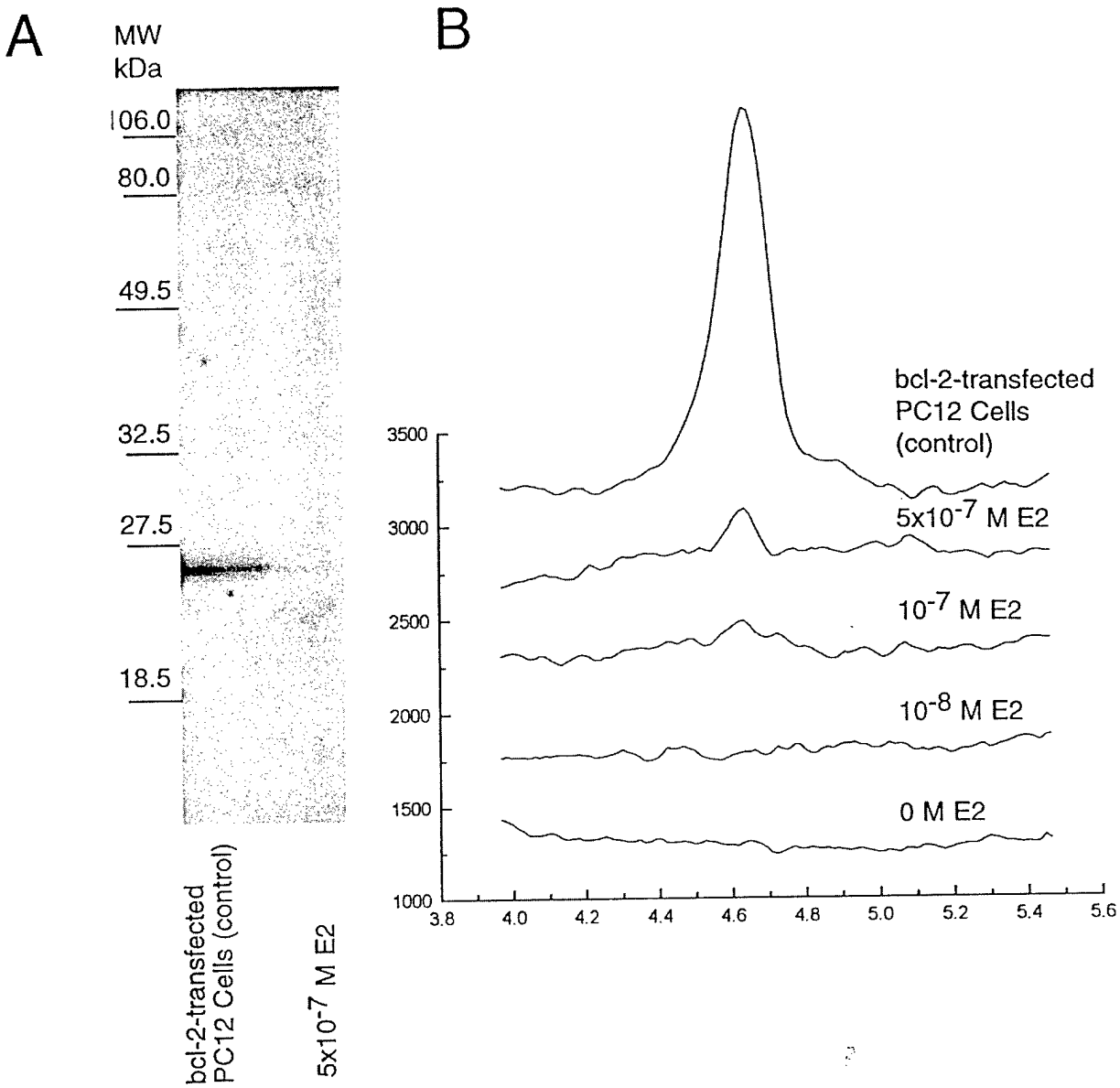
Figure 5. Effect of treatment with estradiol (E2) on oxidation of cis-parinaric acid-labeled phospholipids induced by AMVN in MCF-7 breast cancer cells. MCF-7 cells were treated with E2 (0 - 5 x $10^{-7}$ M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 μM; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA. Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS ($p < 0.02$ relative to AMVN alone at all E2 concentrations; Student's t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment afforded statistically significant protection against oxidation of PS alone ($p < 0.03$ relative to AMVN alone at $10^{-8}$ M and 5 x $10^{-7}$ M E2; Student's t test).

Figure 6. Effect of N-acetylcysteine (10 mM; 4 h) pretreatment on NCS sensitivity of PC12 pheochromocytoma cells (A) and MCF-7 cells (B). NCS treatment (1 H) was performed on sister cultures immediately after washout of N-acetylcysteine (NAcCys) or vehicle (no NACys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. SEMs of the triplicate determinations are plotted but in some cases are too small to be resolved on the plot. Adherent cell counts are shown during mid-log phase growth (day 5 for PC12 cells and day 3 for MCF-7 cells).

Figure 7. NCS induces Bcl-2 cleavage in bcl-2-transfected PC12 cells. Mock- and bcl-2-transfected PC12 cells were treated with NCS (20 nM; 1h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western Blot analysis. Results of staining with an antibody for Bcl-2 (Santa Cruz Biotechnology) are shown. Mock-transfected PC12 cells: left-most five lanes; bcl-2-transfected PC12 cells: right-most five lanes. Note that mock-transfected PC12 cells do not contain detectable Bcl-2.

Figure 8. NCS did not induce Bcl-2 cleavage in bcl-2-transfected MCF-7 cells. Mock- and bcl-2 transfected MCF-7 cells were treated with NCS (20 nM; 1h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western Blot analysis. Results of staining with an antibody for Bcl-2 (Santa Cruz Biotechnology) are shown. (A) neo.1 mock-transfected cells: left-most five lanes; bcl-2.1 bcl-2-transfected cells: right-most five lanes. (B) neo.2 mock-transfected cells: left-most five lanes; bcl-2.3 bcl-2-transfected cells: right-most five lanes.

# FIGURE 1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5.0
5.2
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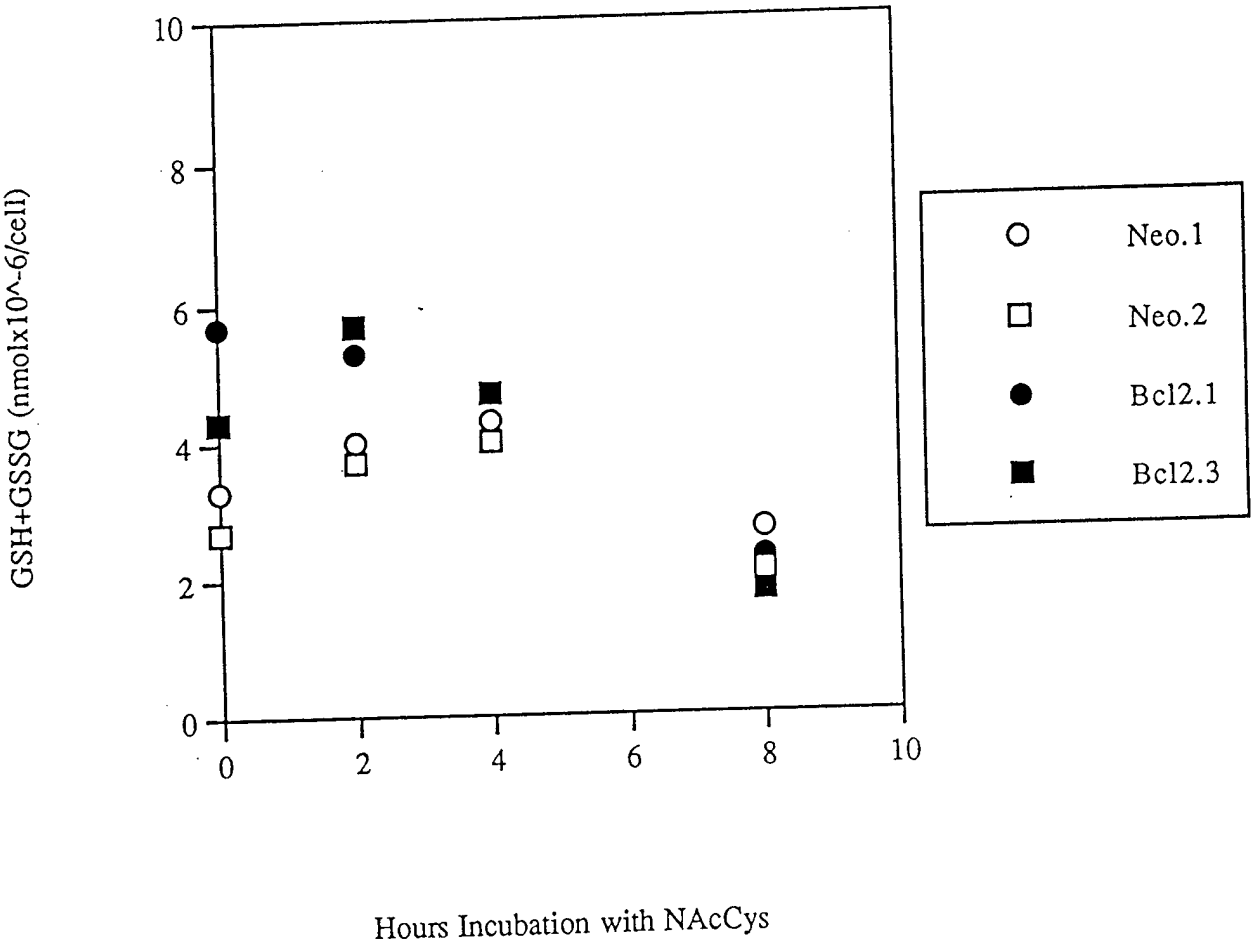
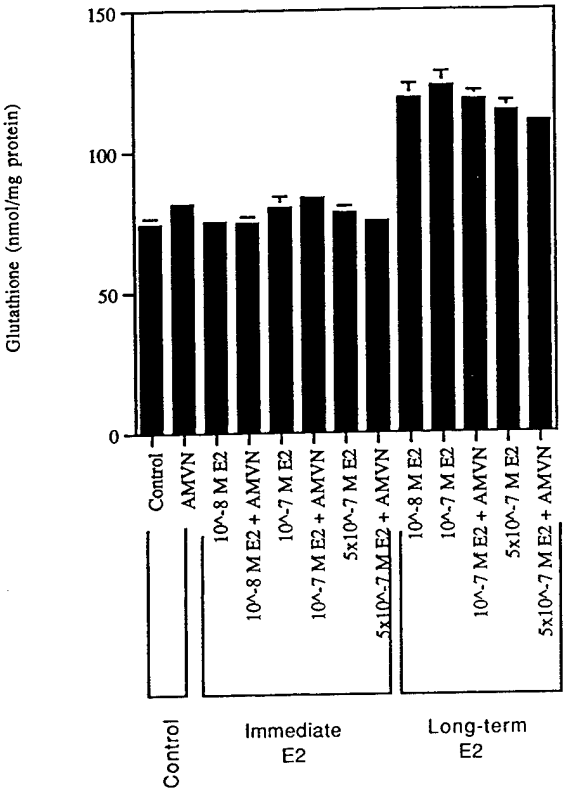
bcl-2-transfected
PC12 Cells
(control)
5x10-7 M E2
10-7 M E2
10-8 M E2
0 M E2

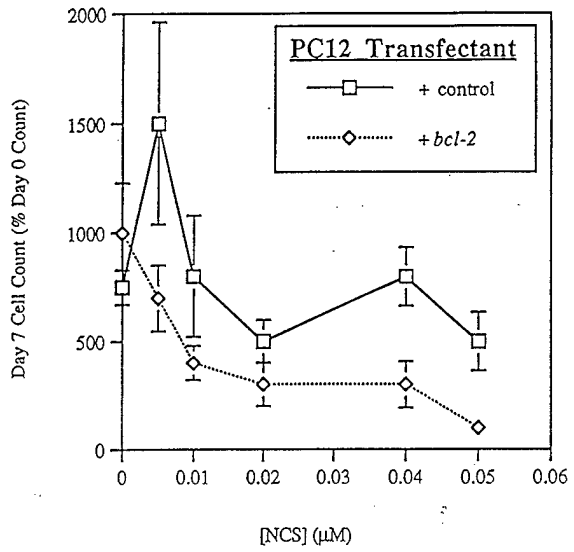
# FIGURE 2


GSH+GSSG (nmolx10^-6/cell)
10
8
6
4
2
0
0
2
4
6
8
10
Neo.1
Neo.2
Bcl2.1
Bcl2.3
Hours Incubation with NAcCys

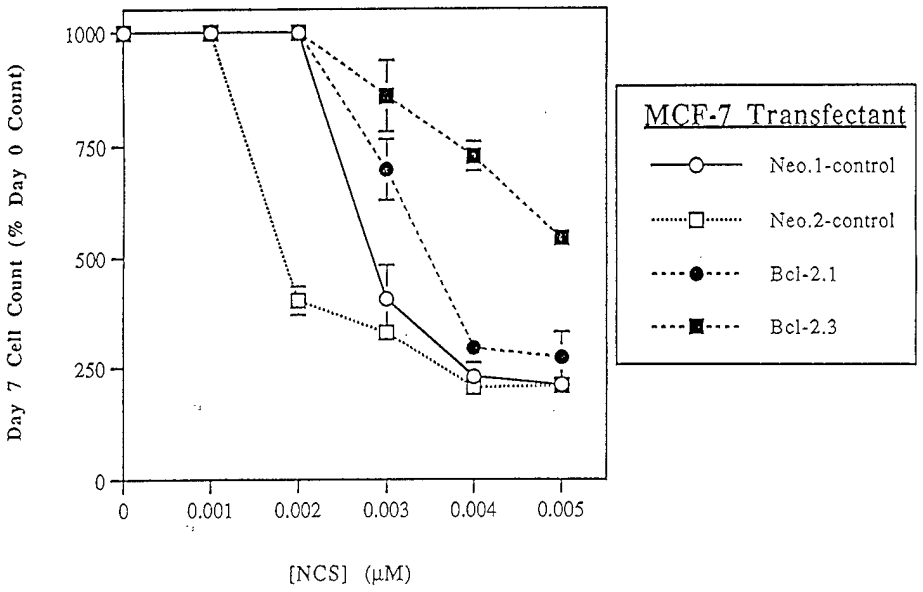
# FIGURE 3


Glutathione (nmol/mg protein)
150
100
50
0
Control
AMVN
10^-8 M E2
10^-8 M E2 + AMVN
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
10^-8 M E2
10^-7 M E2
10^-7 M E2 + AMVN
5x10^-7 M E2
5x10^-7 M E2 + AMVN
Control
Immediate E2
Long-term E2

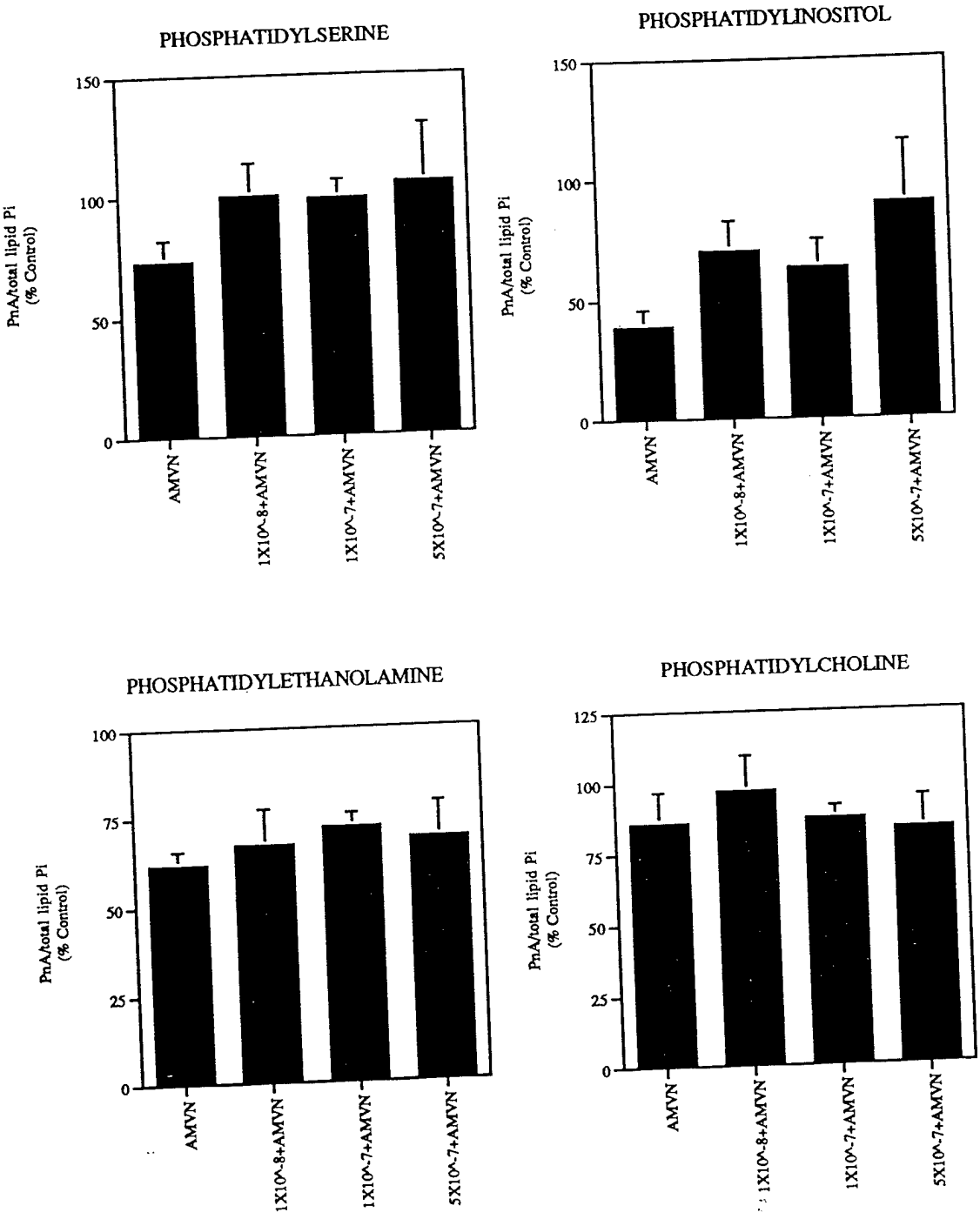
# FIGURE 4 A


PC12 Transfectant
+ control
+ bcl-2
Day 7 Cell Count (% Day 0 Count)
2000
1500
1000
500
0
0
0.01
0.02
0.03
0.04
0.05
0.06
[NCS] (µM)

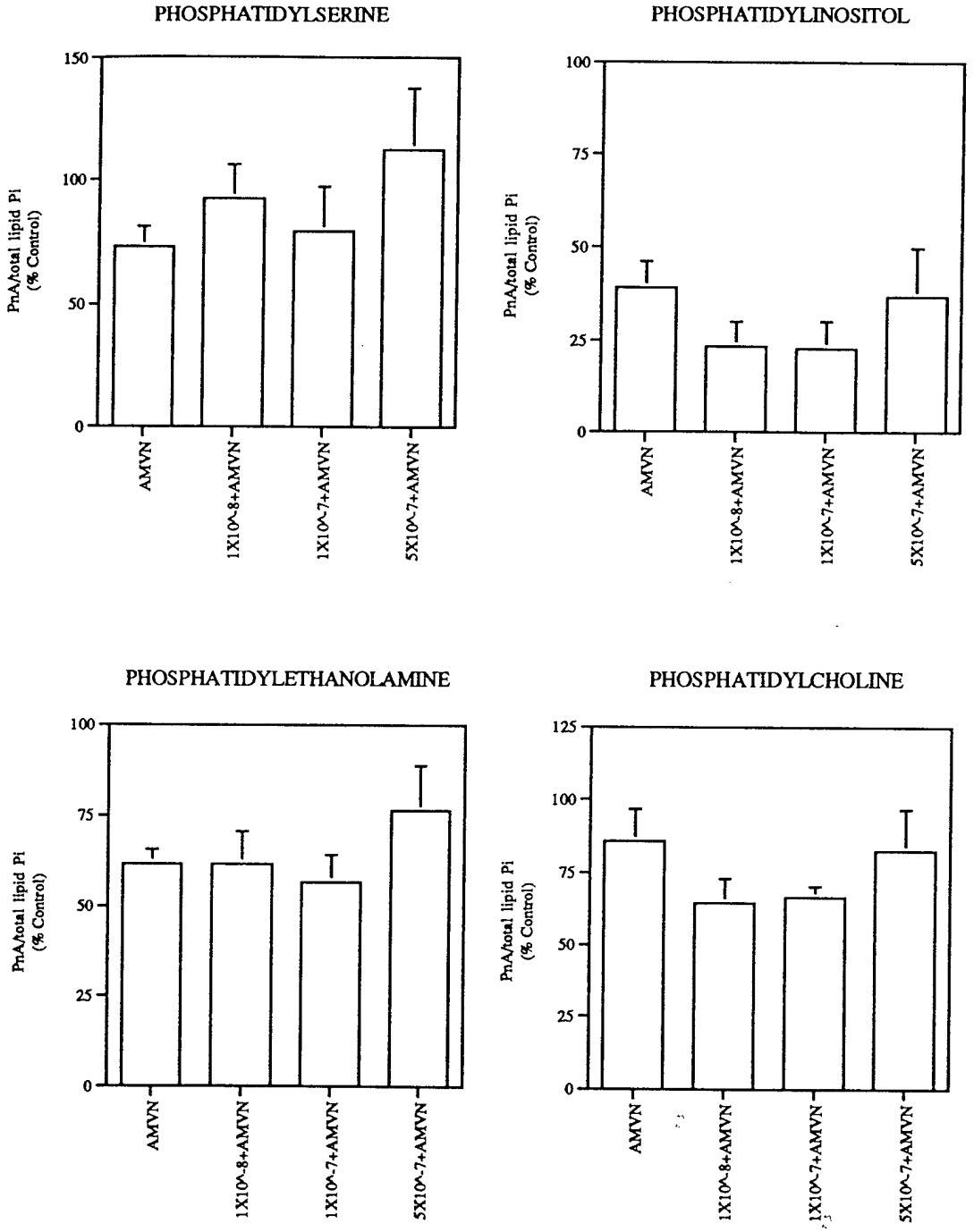
# FIGURE 4 B


Day 7 Cell Count (% Day 0 Count)
1000
750
500
250
0
0
0.001
0.002
0.003
0.004
0.005
[NCS] (μM)
MCF-7 Transfectant
Neo.1-control
Neo.2-control
Bcl-2.1
Bcl-2.3

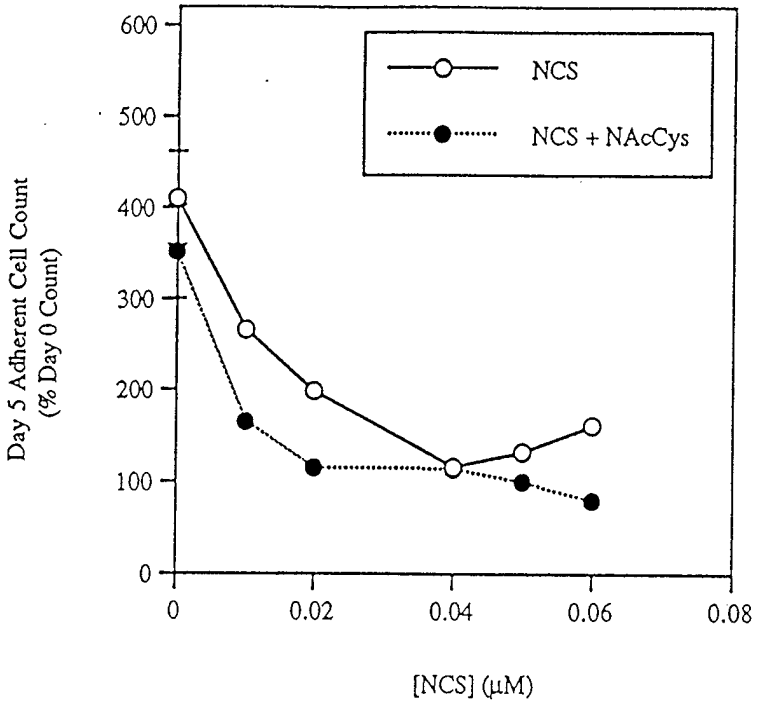
# FIGURE 5 A


PHOSPHATIDYLSERINE
PnA/total lipid Pi
(% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLINOSITOL
PnA/total lipid Pi
(% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLETHANOLAMINE
PnA/total lipid Pi
(% Control)
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLCHOLINE
PnA/total lipid Pi
(% Control)
125
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

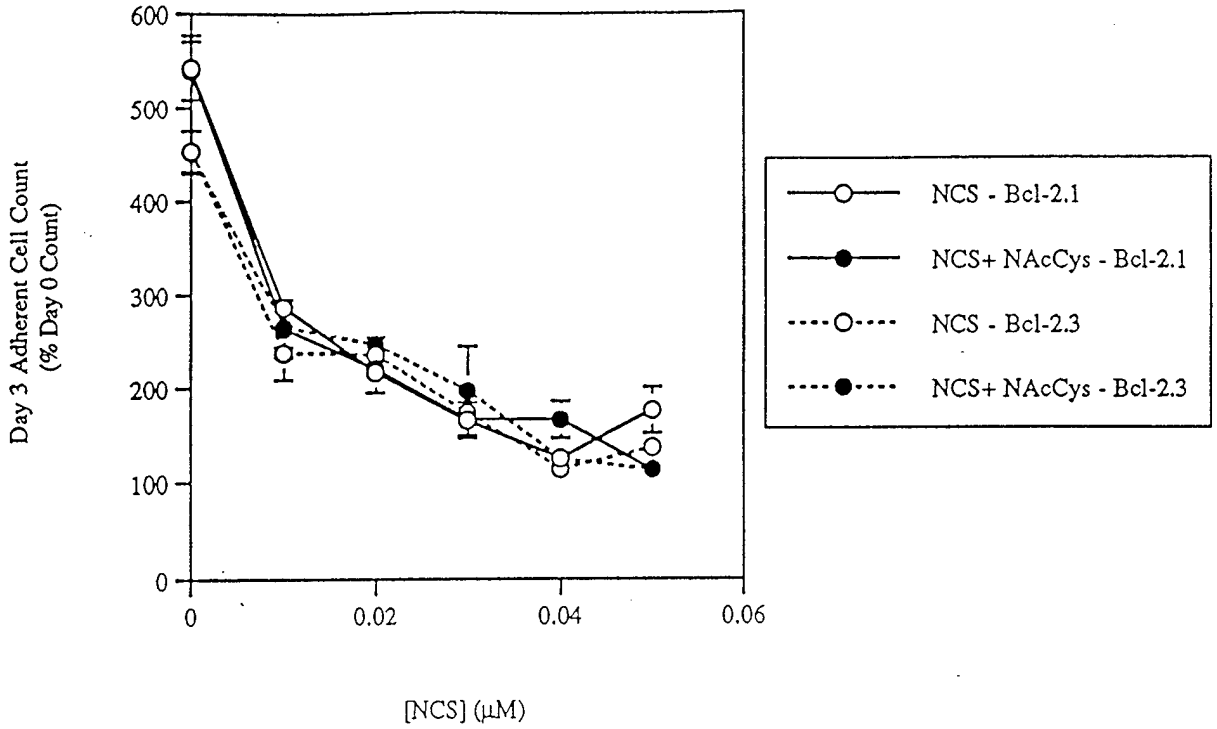
# FIGURE 5 B


PHOSPHATIDYLSERINE
PnA/total lipid Pi (% Control)
150
100
50
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLINOSITOL
PnA/total lipid Pi (% Control)
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLETHANOLAMINE
PnA/total lipid Pi (% Control)
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN
PHOSPHATIDYLCHOLINE
PnA/total lipid Pi (% Control)
125
100
75
50
25
0
AMVN
1X10^-8+AMVN
1X10^-7+AMVN
5X10^-7+AMVN

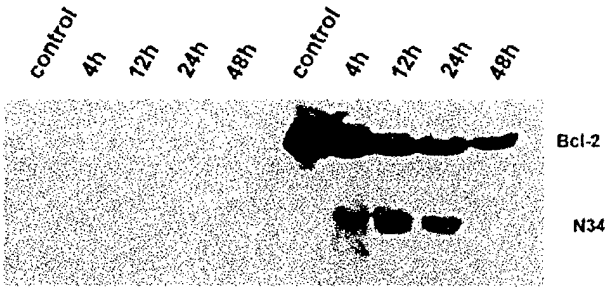
# FIGURE 6 A


600
500
400
300
200
100
0
0
0.02
0.04
0.06
0.08
NCS
NCS + NAcCys
Day 5 Adherent Cell Count
(% Day 0 Count)
[NCS] (μM)

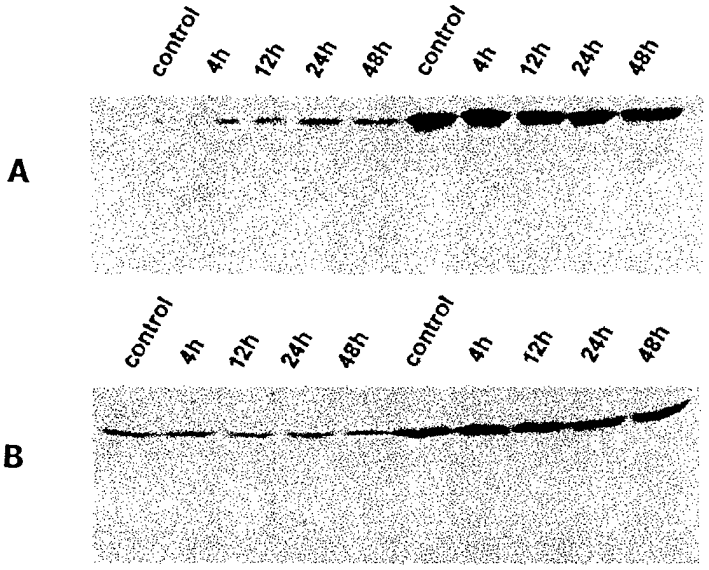
# FIGURE 6 B


Day 3 Adherent Cell Count
(% Day 0 Count)
600
500
400
300
200
100
0
0
0.02
0.04
0.06
[NCS] (μM)
NCS - Bcl-2.1
NCS+ NAcCys - Bcl-2.1
NCS - Bcl-2.3
NCS+ NAcCys - Bcl-2.3

# FIGURE 7



# FIGURE 8


control
4h
12h
24h
48h
control
4h
12h
24h
48h
A
control
4h
12h
24h
48h
control
4h
12h
24h
48h
B

TABLES

**TABLE 1: Specific Incorporation of *cis*-Parinaric Acid (PnA) Into Membrane Phospholipids of MCF-7 Breast Cancer Cells.** MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined.

| Phospholipids | Specific Incorporation (mol PnA : mol phospholipid) |
|---|---|
| Phosphatidylcholine | 1 : 4 |
| Phosphatidylethanolamine | 1 : 10 |
| Phosphatidylserine | 1 : 13 |
| Phosphatidylinositol | 1 : 31 |

**TABLE 2: Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-detected Levels of *cis*-Parinaric Acid (PnA).** MCF-7 cells were membrane-loaded with PnA and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 $\mu$M). Peroxidation of PnA in the various membrane phospholipids was determined.

| | ngPnA/mg Pi | | | |
|---|---|---|---|---|
| | **PI** | **PE** | **PS** | **PC** |
| **Control (n=13)** | 35.5±14.9 | 242.3±25.6 | 37.7±6.5 | 1105.4±117.6 |
| **AMVN (n-5)** | 13.9±2.5 | 149.4±9.5 | 27.6±3.1 | 948.6±120.5 |
| "p" vs. Control | 0.006 | < 0.001 | 0.005 | 0.023 |

**TABLE 3: Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells.** MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 $\mu$M; 2 h; 37°C). Phospholipid composition was determined by HPTLC. All data are expressed as mean ± SEM, and n = 6 for each value. *p < 0.05 vs. control cells (Student's t test).

| Phospholipid | Percent of Total Phospholipids | |
|---|---|---|
| | Control | AMVN |
| Diphosphatidylglycerol | 2.33 ± 0.48 | 3.07 ± 0.41 |
| Phosphatidylethanolamine | 27.85 ± 0.28 | 25.61 ± 0.92 |
| Phosphatidylcholine | 47.62 ± 0.24 | 48.94 ± 0.88 |
| Phosphatidylserine | 5.63 ± 0.15 | 5.76 ± 0.33 |
| Phosphatidylinositol | 7.89 ± 0.27 | 6.90 ± 0.37 |
| Sphingomyelin | 8.33 ± 0.03 | 8.14 ± 0.30 |
| Lysophosphatidylcholine | 0.48 ± 0.11 | 1.14 ± 0.25* |

## PERSONNEL RECEIVING SALARY SUPPORT FROM THIS RESEARCH EFFORT

1. Nina Felice Schor, M.D., Ph.D.
2. Valerian E. Kagan, Ph.D.
3. Yulia Y. Tyurina, Ph.D.
4. Karen D. Nylander, R. N.

# PHOSPHOLIPID SIGNALING IN APOPTOSIS:

# PEROXIDATION AND EXTERNALIZATION OF PHOSPHATIDYLSERINE

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Running title: phosphatidylserine peroxidation in apoptosis

Keywords: phosphatidylserine, apoptosis, oxidative stress, antioxidants

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## Abstract

The role of phospholipids in apoptosis signaling and the relationship between oxidation of phosphatidylserine and its redistribution in the plasma membrane were studied. A novel method for detection of site-specific phospholipid peroxidation based on the use of cis-parinaric acid as a reporter molecule metabolically integrated into membrane phospholipids in living cells was employed. When several tissue culture cell lines and different exogenous oxidants were used, the relationship between oxidation of phosphatidylserine and apoptosis has been revealed. Plasma membrane was the preferred site of phosphatidylserine oxidation in cells. It was shown that selective oxidation of phosphatidylserine precedes its translocation from the inside to the outside surface of the plasma membrane during apoptosis. A model is proposed in which cytochrome c released from mitochondria by oxidative stress binds to phosphatidylserine located at the cytoplasmic surface of the plasma membrane and induces its oxidation. Interaction of peroxidized phosphatidylserine with aminophospholipid translocase causes inhibition of the enzyme relevant to phosphatidylserine externalization.

## Introduction

Phospholipids are the dominant lipid constituents of the membranes of animal cells where they are arranged in a bilayer configuration and act as the matrix for the support and organization of the different membrane proteins. In addition to this structural role many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli. Such signals invariably involve redistribution and/or metabolism (hydrolysis, oxidation) of the target phospholipid, the products of which either modulate protein functions directly or are further converted to products that serve this function (Wilton, 1998; Ohanian et al., 1998; Malisan et al., 1999). Phosphoinositol-dependent pathways and eicosanoid cascades are two prominent examples of the important role(s) that phospholipids play in cell physiology (Martin, 1998; de Jonge et al., 1996; Versteeg et al., 1999).

More recently, lipid metabolism and turnover have been shown to be associated with initiation and/or progress of cell death. Metabolism of ceramides, for example, is known to be a factor culminating in apoptosis (Perry and Hannun, 1998; Mathias et al., 1998). Oxidation of the unsaturated fatty acyl residues of phospholipids, such as formation of lipid peroxides, can lead either to apoptosis or necrotic cell death depending on the particular stimulus and the extent of phospholipid involvement (Smith, 1987; Pacifici et al., 1994a; Glaesser et al., 1996; Kaneko and Baba, 1999). While there is strong evidence that oxidative stress, in general, and lipid peroxidation, in particular, are involved in both initiation and mediation of apoptosis, the specific mechanism(s) that

translate peroxidation of one or more classes of phospholipids into the recognizable biochemical and morphological stages of apoptosis remains unclear.

Recent studies have provided evidence that membrane phospholipid asymmetry is disturbed in one of the early stages of apoptosis (Reno et al., 1998). Specifically, a translocation of phosphatidylserine (PS) from the internal surface to external surface of the plasma membrane appears to be a fundamental mechanism by which apoptotic cells are recognized and eliminated by phagocytic macrophages (Fadok et al., 1992, 1998; Hampton et al., 1996). PS externalization could arise either through inactivation of aminophospholipid translocase, whose normal function maintains the asymmetric distribution of PS in cells (Verhoven et al., 1995; Bruckheimer and Schroit, 1996) or by accelerated reversal of movement of the phospholipid. Aminophospholipid translocase is a membrane-bound ATP-dependent enzyme whose normal role is to transport aminophospholipids, PE and PS, from the external to the internal leaflet of plasma membrane. Inhibition of this enzyme results in disruption of membrane phospholipid asymmetry and exposure of PS to the outside of the cell (Martin and Pagano, 1987). Another enzyme, phospholipid scramblase, transports multiple phospholipids, including PS, bidirectionally. Activation of this enzyme is required to initiate the loss of membrane asymmetry in apoptosis (Bratton et al., 1997).

Another promising line of investigation appears to be to link a disturbance of mitochondrial electron transport in early stages of apoptosis with the generation of free radicals the action of which results in peroxidation of membrane phospholipids, increased mitochondrial permeability and release of cytohrome c (Mignotte and

Vayssiere, 1998). Apoptotic events are known to include cytochrome c release from mitochondria and its accumulation in cytosol. This is associated with activation of caspase-9, which then leads to processing and activation of other caspases (Green and Reed, 1998).

Over the past few years our research group has been examining the hypothesis that susceptibility of PS to oxidation is augmented by binding of basic proteins, in particular cytochrome c, which are released from mitochondria and bind to the acidic phospholipid. Oxidation of PS, in turn, is an early step in the process of apoptosis.

## Measurements of Phospholipid Peroxidation in Live Cells

Experimental approaches to demonstrate involvement of phospholipid oxidation in cell homeostasis and signaling processes have proved to be relatively difficult because of inherent problems of quantitating phospholipid peroxidation in living cells. This is largely due to a remarkably efficient remodelling/repair of oxidized phospholipids. For example, phospholipid hydroperoxides have long been known to be efficient substrates for endogenous phospholipases and particularly phospholipase $A_2$ (Kagan, 1988). More recent studies have shown that accumulation of phospholipid hydroperoxides in membranes not only stimulates phospholipase $A_2$-catalyzed hydrolysis of hydroperoxy-containing molecular species but accelerates hydrolysis of non-oxidized phospholipids in membranes (Salgo et al., 1992). Subsequent reacylation of monoacylglycerophospholipids is also stimulated by phospholipid peroxidation thereby augmenting phospholipid turnover. Thus, acylcarnitine-dependent reacylation of

lysophospholipids is elevated many-fold as a consequence of oxidative stress (Pacifici et al., 1994b). Such phospholipid remodeling masks potential role(s) that oxidatively modified phospholipids may play in apoptotic pathways.

To address these problems we have developed a novel method of detecting phospholipid oxidation based on the use of cis-parinaric acid (PnA) as a reporter molecule for lipid peroxidation in living mammalian cells (Ritov et al., 1996). PnA is a naturally-occurring, 18-carbon fatty acid containing four conjugated double bonds; it has a confirmation similar to that of other polyunsaturated fatty acid residues normally associated with membrane phospholipids (Welti, 1982). Oxidative destruction of any part of the conjugated double bond system of PnA results in the disappearance of its characteristic fluorescence at 420 nm (Kuypers et al., 1987) so that measurements of fluorescence emission intensity can be used to monitor lipid oxidation. PnA can be metabolically incorporated into different classes of membrane phospholipids of cells cultured under physiological conditions (Rintoul and Simoni, 1977) and used as a sensitive and specific reporter system to measure oxidative stress in membranes in living cells. This method has proved to be successful in demonstrating the involvement of oxidation of different phospholipid classes in living cells subjected to oxidative stress using a variety of oxidants (Ritov et al., 1996; Kagan et al., 1998). It is therefore possible to detect oxidation of specific phospholipids with a level of precision sufficient to identify potential involvement of phospholipid metabolism in cell physiological events.

## Relationship between Phospholipid Peroxidation and Apoptosis

The response of cells in tissue culture to oxidative stress has been assessed by exposing cells containing metabolically labeled parinaroyl-phospholipids to exogenous oxidants and measuring susceptibility of different phospholipids to oxidation. A lipid-soluble azo-initiator, 2,2'-azobis(2,4-dimethylvaleronitrile), AMVN, is a preferred source of free radicals for such experiments because of its unique ability to generate peroxyl radicals exclusively within the hydrophobic environment of membranes (Niki, 1990; Krainev and Bigelow, 1996). When HL60 cells pre-labeled with PnA were exposed to AMVN, lipid peroxidation could be easily detected from fluorescence of individual phospholipid classesresolved by HPLC. It was found that exposure to AMVN resulted in oxidation of all PnA-labeled phospholipids in HL60 cells, but in an amount that was beyond the level that could be detected as a change in phospholipid composition of the cells. Exposure to AMVN also induces apoptosis in these cells as evidenced by (i) DNA fragmentation, (ii) appearance of apoptotic nuclei, (iii) externalization of PS (Fabisiak et al., 1998a). Thus, the nuclear phenotype in cells exposed to AMVN is characterized by chromatin condensation, fragmentation and internucleosomal DNA cleavage. This is associated with significant changes in the distribution of PS in the plasma membrane such that asymmetry tends to be lost and the phospholipid appears on the surface of the cell. There is also a strong correlation between the appearance of annexin V/propidium iodide positive HL60 cells and the oxidative stress induced by exposure to AMVN (Fabisiak et al., 1998a).

It follows that if oxidative stress is related to initiation or mediation of apoptosis then protection against such stress should arrest the process. The major lipid-soluble antioxidant within cell membranes is vitamin E (Burton and Ingold, 1986). The ability of antioxidants and antioxidant enzymes to protect cells from apoptosis has been suggested previously (Hockenbery et al., 1993; Kane et al., 1993). To investigate the role of antioxidants as possible protectors against oxidative stress, a homologue of vitamin E, such as 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), was used to determine the extent of protection provided against AMVN-induced peroxidative modification of specific classes of phospholipids in the membranes of HL60 cells. It is well known that PMC is an efficient lipid antioxidant that randomly protects lipids from oxidation (Kagan et al., 1990). In HL60 cells exposed to AMVN, PMC greatly attenuated the loss of PnA fluorescence in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), but oxidation of PS was not prevented by the antioxidant. In model experiments in which liposomes prepared from PnA-labeled phospholipids of HL60 cells were exposed to AMVN, substantial oxidation of all phospholipids, similar to that seen in intact cells, was demonstrated. However, in this cell-free system PMC was fully able to protect all phospholipids, including PS.

The significance of this failure of PMC to protect PS from oxidation in HL60 cells oxidatively stressed by exposure to AMVN was apparent from the progress of apoptosis in cells treated with PMC. The presence of PMC during exposure of the HL60 cells to AMVN failed to block the formation of DNA ladders and other AMVN-induced apoptotic changes in nuclear morphology. Moreover, externalization of PS following treatment with the azo initiator of peroxyl radicals was also unaffected by the presence of PMC

consistent with flow cytometry measurements of annexin V binding to externalized PS (Fabisiak et al., 1998a).

## Control of Phospholipid Peroxidation at the Level of Gene Expression

Bcl-2 is one of a family of proteins that can inhibit apoptosis. These proteins have also been shown to protect membrane lipids from peroxidation during exposure to oxidative stress (Kane et al., 1993; Hockenbery et al., 1993). In PC12 pheochromocytoma cells exposed to AMVN a uniform pattern of peroxidation of PnA-labeled membrane phospholipids is observed and this is associated with a concentration dependent induction of apoptosis (Tyurina et al., 1997). The specific rate of oxidation was found to be greater for PS than for any of the other phospholipid classes (Fig. 1). Products of the bcl-2 gene protect PC12 cells against apoptosis induced by oxidative stress and prevent oxidation of all the phospholipids, including PS, during exposure of cells to relatively low concentrations of AMVN, it is noteworthy that protection of PS oxidation by bcl-2 gene products was significantly less than that of the other phospholipid classes and also that full protection against apoptosis was not observed (Tyurina et al., 1997). Thus Bcl-2 only partly protects PS against oxidation and does not completely prevent apoptosis induced by AMVN in PC12 cells.

## Responses to Different Oxidative Stresses

The relationship between oxidation of PS and redistribution of the phospholipid in the plasma membrane is central to our understanding of the role of the phospholipid in

apoptosis. The use of a variety of different exogenous oxidants to explore the relationship between oxidative stress, PS and apoptosis has been particularly revealing. The use of different oxidants has suggested that the form and type of radicals involved may influence the type of response observed in a particular cell line.

Experiments using paraquat as the source of oxidative stress in 32D cells have, for example, clearly shown that selective oxidation of PS precedes its translocation from inside to the outside surface of the plasma membrane and apoptosis (Fabisiak et al., 1997). Apoptosis in response to paraquat is characterized by chromatin condensation and fragmentation, internucleosomal DNA cleavage, and loss of cell viability within 24 h of toxin exposure. Paraquat induced early and selective oxidation of PnA, primarily in PS, which was subsequently followed by apoptosis. PS peroxidation preceded its appearance on the cell surface by several hours. Overexpression of Bcl-2 afforded significant protection against paraquat-induced apoptosis as well as peroxidation of PnA metabolically integrated into PS. Quinacrine, similarly to Bcl-2, attenuated the selective oxidation of PS and blocked paraquat-induced apoptosis in 32D cells (Fabisiak et al., 1998b).

The protection afforded by Bcl-2 against apoptosis also differs depending on the type of oxidative stress. As with the effect of oxidative stress induced by AMVN treatment, exposure of mock-transfected PC12 cells to the antitumor drug, neocarzinostatin, results in peroxidation of all membrane phospholipid classes. The proportion of apoptotic nuclei in cells following exposure to neocarzinostatin was, however, greater in bcl-2-transfected compared with mock-transfected cells (Cortazzo and Schor, 1996). It is evident that in

these cells Bcl-2 does not provide protection of cells against apoptosis induced by this antitumor drug and, in contrast to AMVN, overexpression of Bcl-2 does not protect the cell from PS oxidation or the appearance of PS on the cell surface induced by neocarzinostatin (Schor et al., 1999).

Glutamate-induced cytotoxicity is mediated primarily through necrosis. Neither PC12 nor PC12/bcl-2 cells underwent apoptosis in response to cytotoxic doses of glutamate. Significant oxidation of PnA-labeled PE and PC and their protection by products of the bcl-2 gene were observed in PC12 cells treated with glutamate (15 mM), but even with such high concentrations of glutamate there was no significant oxiation of parinaroyl-PS in these cells (Tyurin et al., 1998).

Phenol alone caused substantial oxidation of PnA-labeled phospholipids in normal human epidermal keratinocytes. Two major phospholipids – PE and PC were primary targets for peroxidation in keratinocytes (Fig. 2). PS was found to be resistant to phenol-induced oxidation and the morphology of treated keratinocytes does not show differences from that of control cells (Shvedova et a., 1999). In contrast, a significantly increased number of apoptotic cells and oxidation of all phospholipids, including PS, were detected after treatment of normal human epidermal keratinocytes with AMVN (Shvedova et al., 1999). Moreover, exposure of human keratinocytes to cumene hydroperoxide caused both enhanced peroxidation of PS and its externalization documenting execution of apoptotic program in the cells (Shvedova et al., in preparation).

Another organic hydroperoxide, tert-BuOOH, produced site-specific oxidative stress in cardiomyocyte membrane phospholipids. The oxidant caused loss of parinaroyl-PS while other phospholipids remained unchanged (Gorbunov et al., 1998). Interestingly, tert-BuOOH selectively oxidized PS the oxidation and externalization on the cell surface of which may be associated with apoptosis in cardiomyocytes (Maulik et al., 1998). It is noteworthy that in the presence of the NO-donor, PAPANONOate, the oxidative effect of tert-BuOOH was no longer observed. Hence, the protective effect of NO may be interpreted as the ability of NO to prevent tert-BuOOH-induced apoptosis in cardiomyocytes (Gorbunov et al., 1998). This, however, is not always the case. In HL60 cells treatment with the NO donor, PAPANONOate, completely protected all phospholipids, including PS, from oxidation induced by AMVN, but it did not inhibit externalization of PS and had no effect on other markers of apoptosis following treatment with AMVN (Fabisiak et al., 1999).

In summary, our results clearly demonstrate that oxidative modification of PS may be a common pathway in apoptotic signaling in cells challenged with oxidants. Two important questions are i) does peroxidation of PS occur specifically in plasma membranes, and if so ii) what are the mechanism(s) through which oxidants induce site-specific oxidation of PS? In our preliminary experiments, we isolated different subcellular organelles from PnA-labeled HL60 cells to determine whether PS is indeed the preferred peroxidation substrate in plasma membranes. We found that in HL60 cells challenged with tert-BuOOH oxidation of PS was maximal in plasma membranes (Fig. 3) and endoplasmic reticulum membranes. In these organelles, PS peroxidation exceeded the oxidation of other classes of phospholipids by more than 2-fold. Importantly, tert-BuOOH caused

apoptosis in HL60 cells as evidenced by PS externalization and appearance of condensed apoptotic nuclear morphology. If one assumes that cytochrome c released from mitochondria into cytosol is involved in catalysis of PS oxidation then it seems likely that enhanced PS oxidation would be confined to the cytosolic surfaces of plasma membrane and endoplasmic reticulum membranes. This model emphasizes the central role of early release of cytochrome c from mitochondria not only as one of apoptosis-initiating factors but also as a catalyst of PS peroxidation related to inhibition of aminophospholipid translocase and subsequent PS externalization. An overall scheme linking release of cytochrome c from mitochondria with PS transformation in the plasma membrane is presented in Fig. 4. Future studies are in progress to examine this model.

## Acknowledgements

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## Figure Legends:

**Figure 1.** Protective effect of *bcl-2* transfection on oxidation of parinaroyl-phosphatidylserine and apoptosis induced by AMVN in PC12 pheochromocytoma cells.

**A** – AMVN induced oxidation of parinaroyl-phosphatidylserine in mock- and *bcl-2*-transfected PC12 pheochromocytoma cells. Cells pre-labeled with PnA were incubated with different concentrations of AMVN for 2h at 37°C then lipids were extracted and resolved by HPLC.

**B** – AMVN induced apoptosis in mock- and *bcl2*-transfected PC12 pheochromocytoma cells. Cells were incubated for up to 100 min in the presence of 0.75 mM of AMVN. Percentage of cell demonstrating apoptosis was manually determined for each of three high-power fields using fluorescence microscopy of acridine orange-ethidium bromide stained cells.

All data are means ± SEM. *$P<0.05$.

**Figure 2.** Effect of phenol and AMVN on oxidation of phospholipids and induction of apoptosis in normal human epidermal keratinocytes.

**A** – Oxidation of phospholipids in normal human epidermal keratonocytes induced by phenol. PnA pre-labeled keratinocytes were incubated in the presence of phenol (50 μM) for 2 h at 37°C, then lipids were extracted and resolved by HPLC.

**B** – Oxidation of phospholipids in normal human epidermal keratinocytes induced by AMVN. PnA pre-labeled keratinocytes were incubated in the presence of AMVN (500 μM) for 2 h at 37°C, then lipids were extracted and resolved by HPLC.

**C** – Effect of phenol and AMVN on appearance of hypodiploid keratinocytes. Keratinocytes were incubated with phenol (50 μM) or AMVN (500 μM) for 2 h at 37°C then apoptosis (number of hypodiploid cells) was determined by flow cytometry.

All data are means ± SEM

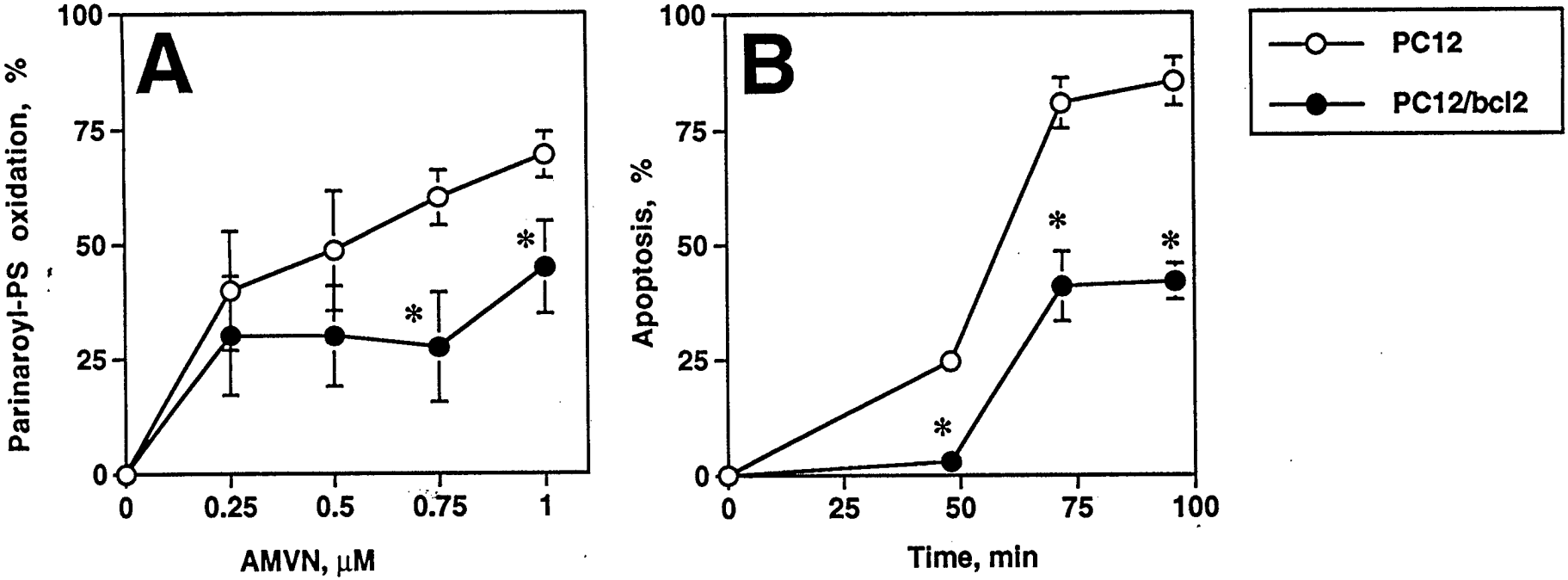
**Figure 3.** Normal-phase HPLC chromatograms of total lipids extracted from plasma membrane of (**A**) control HL60 cells and (**B**) HL60 cells treated with *tert*-BuOOH.

Fluorescence emission intensity, (excitation at 324 nm, emission at 420 nm) was measured in the column effluent. Cells were incubated in the presence and absence of *tert*-BuOOH (150 μM) for 20 min then BHT (10 μM) was added and subcellular organelles were isolated. Lipids from plasma membrane were extracted and resolved by HPLC. PnA- *cis*-parinaric acid; DPG – diphosphatidylglycerol; PI – phosphatidylinositol;

PE – phosphatidylethanolamine; PS – phosphatidylserine; PC – phosphatidylcholine, SPH – sphingomyelin.

**Figure 4.** A model scheme of the role of early cytochrome *c* release from mitochondria in oxidation and externalization of phosphatidylserine in plasma membrane

Figure 1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Parinaroyl-PS oxidation, %
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75
50
25
0
0
0.25
0.5
0.75
1
AMVN, μM
*
*
B
Apoptosis, %
100
75
50
25
0
0
25
50
75
100
Time, min
*
*
*
PC12
PC12/bcl2

Figure 2

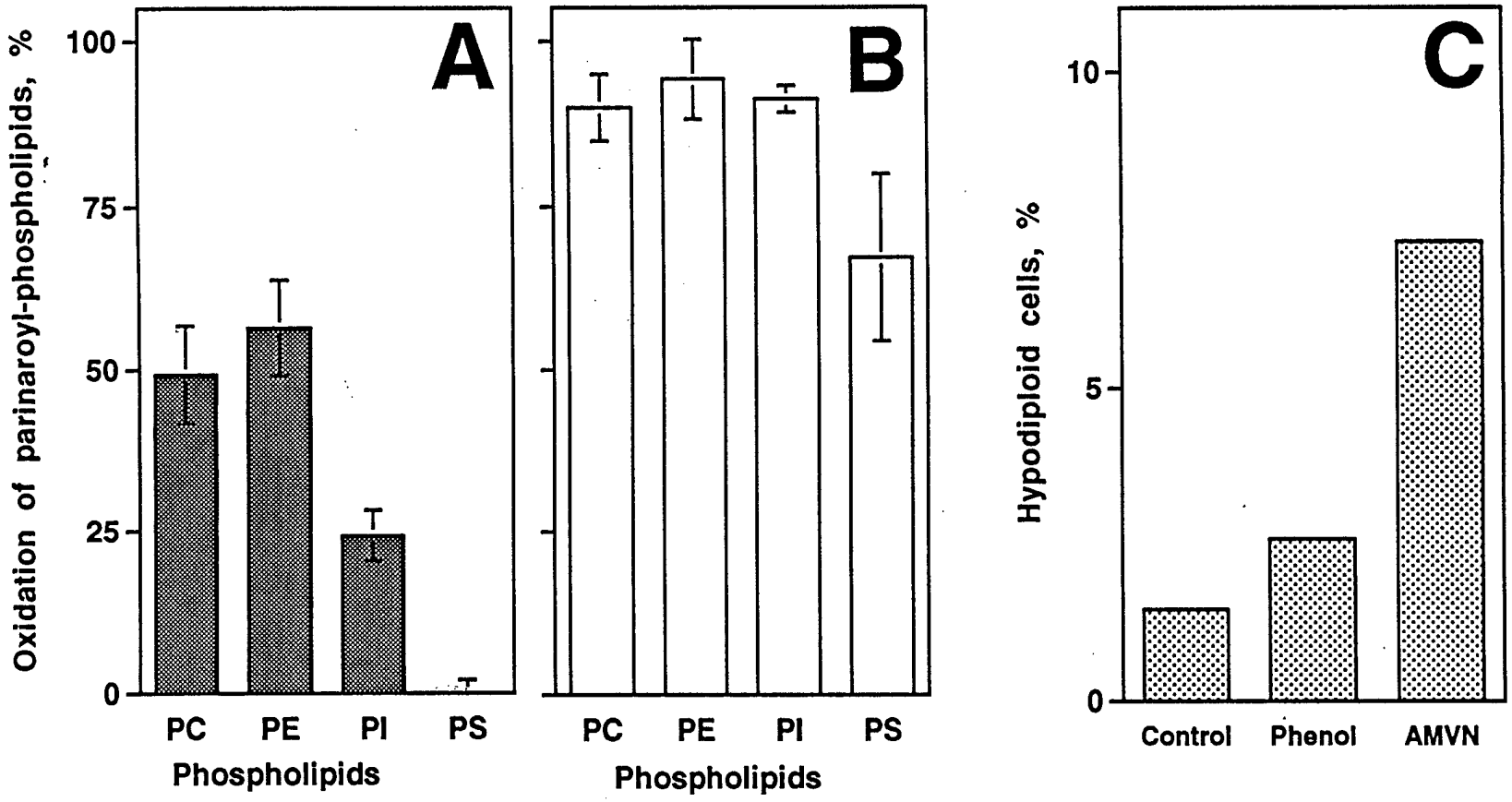
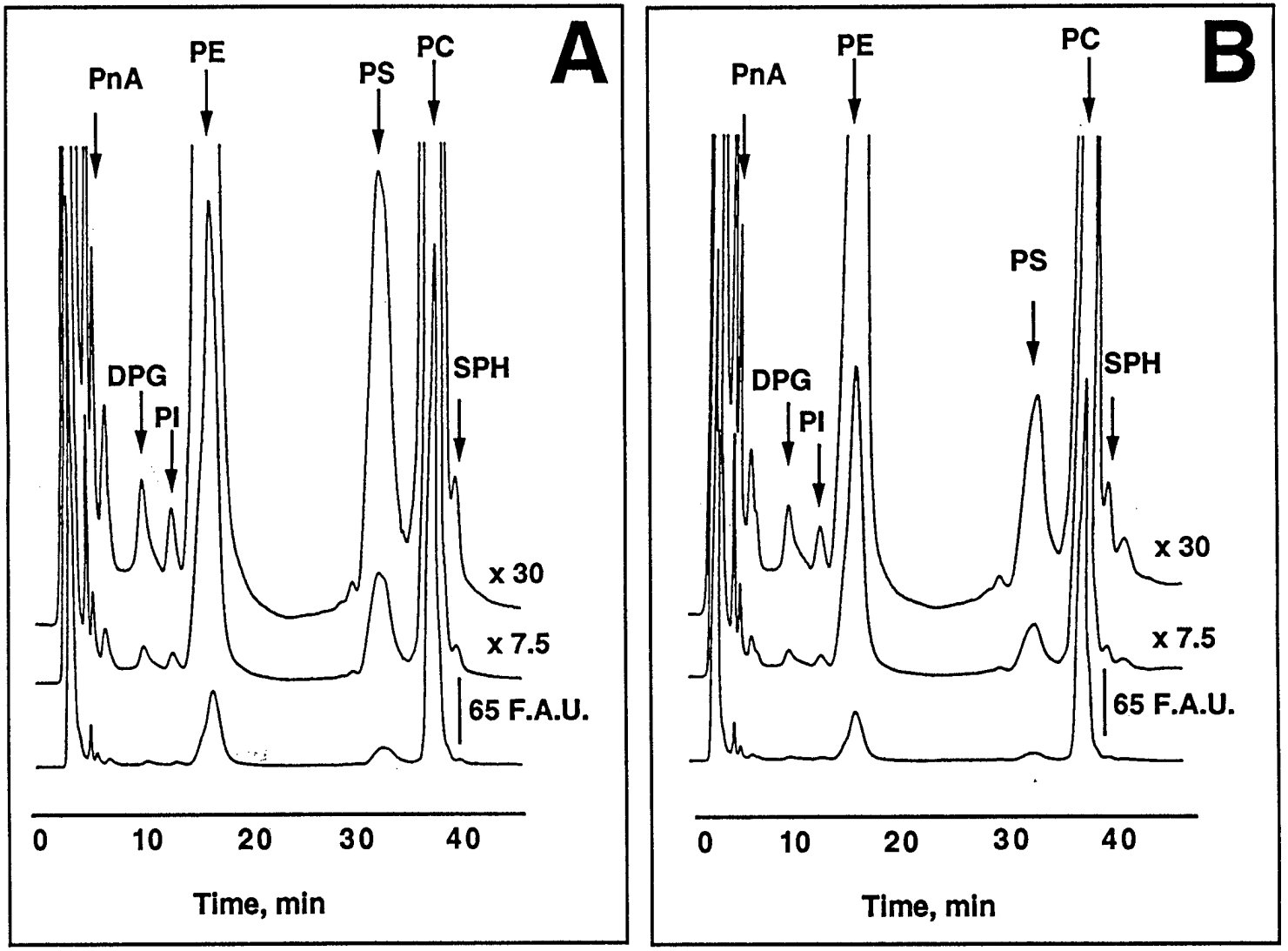

A
B
C
Oxidation of parinaroyl-phospholipids, %
100
75
50
25
0
PC
PE
PI
PS
Phospholipids
Hypodiploid cells, %
10
5
0
Control
Phenol
AMVN

Figure 3


A
PnA
PE
PS
PC
DPG
PI
SPH
x 30
x 7.5
65 F.A.U.
0
10
20
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40
Time, min
B
PnA
PE
PC
PS
DPG
PI
SPH
x 30
x 7.5
65 F.A.U.
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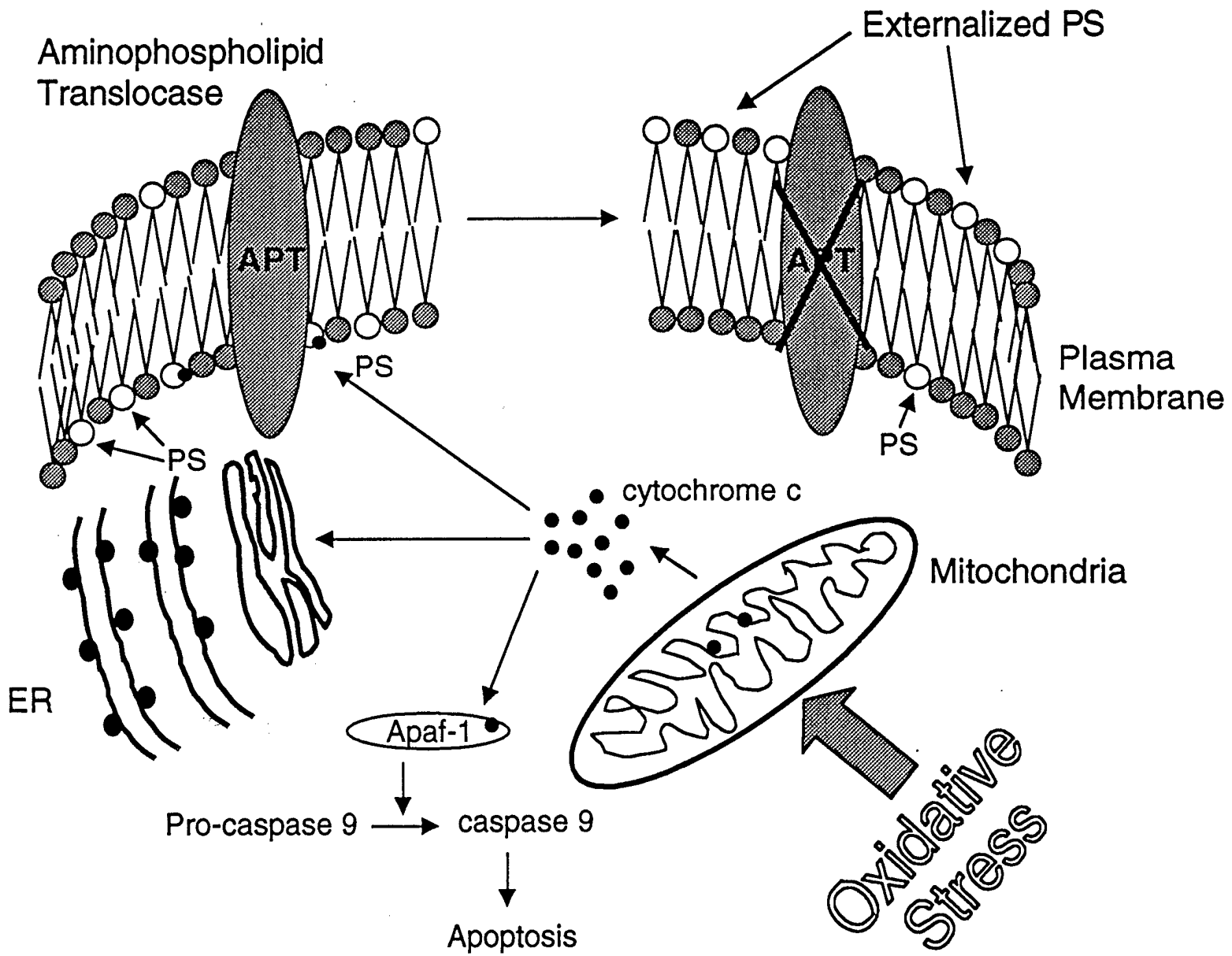
10
20
30
40
Time, min

**Figure 4**


Aminophospholipid
Translocase
APT
PS
PS
Externalized PS
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Membrane
cytochrome c
Mitochondria
ER
Apaf-1
Pro-caspase 9
caspase 9
Apoptosis
Oxidative
Stress

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# Differential Membrane Antioxidant Effects of Immediate and Long-Term Estradiol Treatment of MCF-7 Breast Cancer Cells

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**Previous studies have documented the direct antioxidant effects of estradiol, and it is tempting to ascribe the antiapoptosis effects of estradiol to its scavenging of reactive oxygen species. However, recent reports have also demonstrated that long-term exposure of MCF-7 human breast cancer cells to estradiol results in estrogen receptor- and estradiol dose-dependent overexpression of the antiapoptosis gene, *bcl-2*. We have used the pattern of protection of membrane phospholipids from oxidation as a probe to separate these direct and indirect effects of estradiol from one another. Immediate exposure to estradiol non-specifically protects all membrane phospholipids from oxidation by the diazo radical initiator, AMVN. This implies the direct antioxidant activity of estradiol in this system. In contrast, long-term exposure, with associated increased expression of *bcl-2*, protects only phosphatidylserine, the oxidation of which is a critical component of the final common pathway for apoptosis. This *bcl-2*-mediated indirect effect of estradiol is accompanied by prevention of apoptosis in MCF-7 cells.** © 1999 Academic Press

Direct, estrogen receptor-independent antioxidant effects have been identified for estrogen in a number of systems (1–3). Synergy with glutathione has been demonstrated in some, leading to the proposal of combined estrogen-antioxidant therapy for neurodegenerative disorders thought to have their origins in the generation of reactive oxygen species (4). This direct effect of estrogen is not exposure time-dependent; that is, it is observed immediately upon addition of estrogen to the system.

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Similarly, the antiapoptosis protein, Bcl-2, has been hypothesized to play direct (5) and indirect (6) roles in altering the redox state of the cell. Studies demonstrating an estradiol concentration- and estrogen receptor-dependent induction of the *bcl-2* gene in MCF-7 human breast cancer cells (7) have led us to predict that estradiol would also have an indirect antioxidant and antiapoptotic effect on these cells. Unlike its direct effects, the induction of *bcl-2* by estradiol requires long-term (i.e., days to weeks) estradiol exposure.

Our previous studies have demonstrated that the diazo radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (8). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (9, 10). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.

## MATERIALS AND METHODS

MCF-7 cells (ATCC, Rockville, MD) were maintained as adherent monolayers in 75-mm$^2$ tissue culture flasks (Life Technologies,

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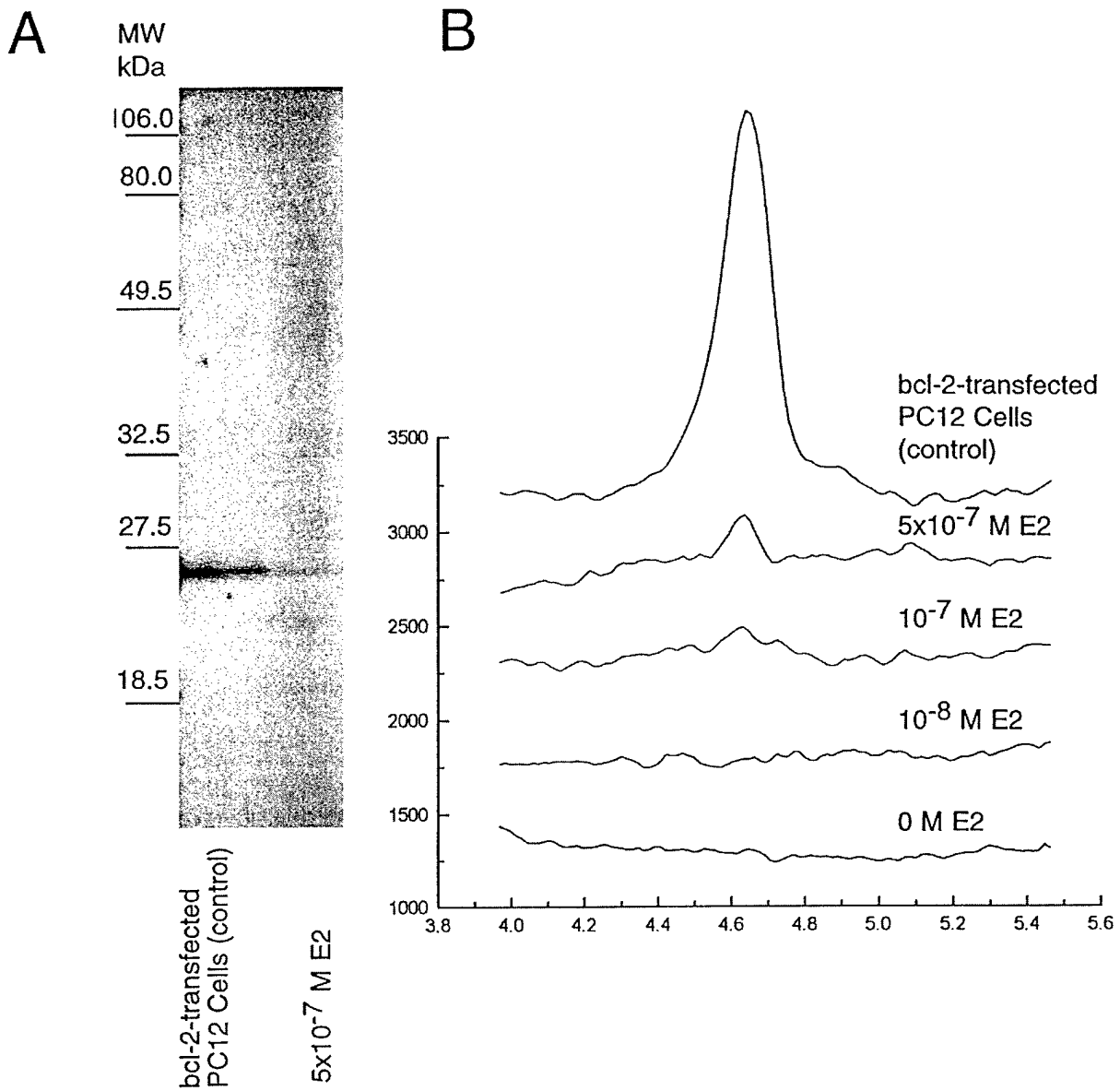


**FIG. 1.** Western blot for human Bcl-2 performed on estradiol (E2)-deprived (10 d) MCF-7 cells after 14 d replacement with E2 ($0–5 \times 10^{-7}$ M). Western blotting was performed as we have previously described (8). A whole cell homogenate of $10^6$ cells was applied to each lane. (A) Photograph of representative lanes of the Western blot depicting *bcl-2*-transfected PC12 cells (8) as a positive control and MCF-7 cells initially estrogen-deprived and subsequently treated with $5 \times 10^{-7}$ M E2 as described in Materials and Methods. The numbers to the left of the gel photograph indicate the running position on the gel of various molecular weight standards. (B) Densitometric vertical scans of each lane of the Western blot stained for Bcl-2.

Grand Island, NY) fed twice weekly with α-MEM (Mediatech, Herndon, VA) supplemented with 5% FBS (Atlanta Biologicals, Norcross, GA), 0.3% glucose, 2 mM L-glutamine (Life Technologies, Grand Island, NY), and 2 μg/ml gentamicin sulfate (Biofluids, Rockville, MD). In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17β-estradiol (E2; $0–5 \times 10^{-7}$ M; Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.

The membranes of the cells were loaded with *cis*-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (8), as an *in situ* probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (11).

TABLE 1

Specific Incorporation of *cis*-Parinaric Acid (PnA) into Membrane Phospholipids of MCF-7 Breast Cancer Cells

| Phospholipids | Specific incorporation (mol PnA:mol phospholipid) |
|---|---|
| Phosphatidylcholine | 1:4 |
| Phosphatidylethanolamine | 1:10 |
| Phosphatidylserine | 1:13 |
| Phosphatidylinositol | 1:31 |

*Note.* MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined as we have previously described (11).

TABLE 3

Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells

| Phospholipid | Percent of total phospholipids | |
|---|---|---|
| | Control | AMVN |
| Diphosphatidylglycerol | 2.33 ± 0.48 | ± 0.41 |
| Phosphatidylethanolamine | 27.85 ± 0.28 | 25.61 ± 0.92 |
| Phosphatidylcholine | 47.62 ± 0.24 | 48.94 ± 0.88 |
| Phosphatidylserine | 5.63 ± 0.15 | 5.76 ± 0.33 |
| Phosphatidylinositol | 7.89 ± 0.27 | 6.90 ± 0.37 |
| Sphingomyelin | 8.33 ± 0.03 | 8.14 ± 0.30 |
| Lysophosphatidylcholine | 0.48 ± 0.11 | 1.14 ± 0.25* |

*Note.* MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 μM; 2 h; 37°C). Phospholipid composition was determined by HPTLC as we have previously described (8). All data are expressed as mean ± SEM, and n = 6 for each value. *$p < 0.05$ vs. control cells (Student's t test).

## RESULTS AND DISCUSSION

As is shown in Fig. 1, long-term E2 supplementation resulted in a concentration-dependent increase in the Bcl-2 content of the cells. Immediate E2 supplementation did not affect cellular Bcl-2 content (data not shown). Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown in Table 1. PnA served as an *in situ* probe for phospholipid oxidation, as we have previously described (8, 11). Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in statistically significant peroxidation of all four phospholipid species examined (Table 2). The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine (PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without sufficient change in the overall phospholipid composition of the membranes to induce necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to non-E2-exposed cells that did not reach statistical significance; Fig. 2A). This wholesale protection of all phospholipids studied from peroxidation suggests a direct antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant protection of PS and, at the highest E2 concentration only, PE (Fig. 2B). This pattern is suggestive of Bcl-2-mediated protection from enactment of apoptosis (9, 10), rather than direct protection from membrane phospholipid peroxidation *per se*. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (8). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated ($5 \times 10^{-7}$ M) cells similarly exposed ($p < 0.01$, Student's t test).

In light of the previous reports of glutathione-dependent (4) and independent (12–14) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione

TABLE 2

Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-Detected Levels of *cis*-Parinaric Acid (PnA)

| | ngPnA/mg Pi | | | |
|---|---|---|---|---|
| | PI | PE | PS | PC |
| Control (n = 13) | 35.5 ± 14.9 | 242.3 ± 25.6 | 37.7 ± 6.5 | 1105.4 ± 117.6 |
| AMVN (n = 5) | 13.9 ± 2.5 | 149.4 ± 9.5 | 27.6 ± 3.1 | 948.6 ± 120.5 |
| "p" vs. Control | 0.006 | <0.001 | 0.005 | 0.023 |

*Note.* MCF-7 cells were membrane-loaded with PnA (11) and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 μM). Peroxidation of PnA in the various membrane phospholipids was determined as we have previously described (11).

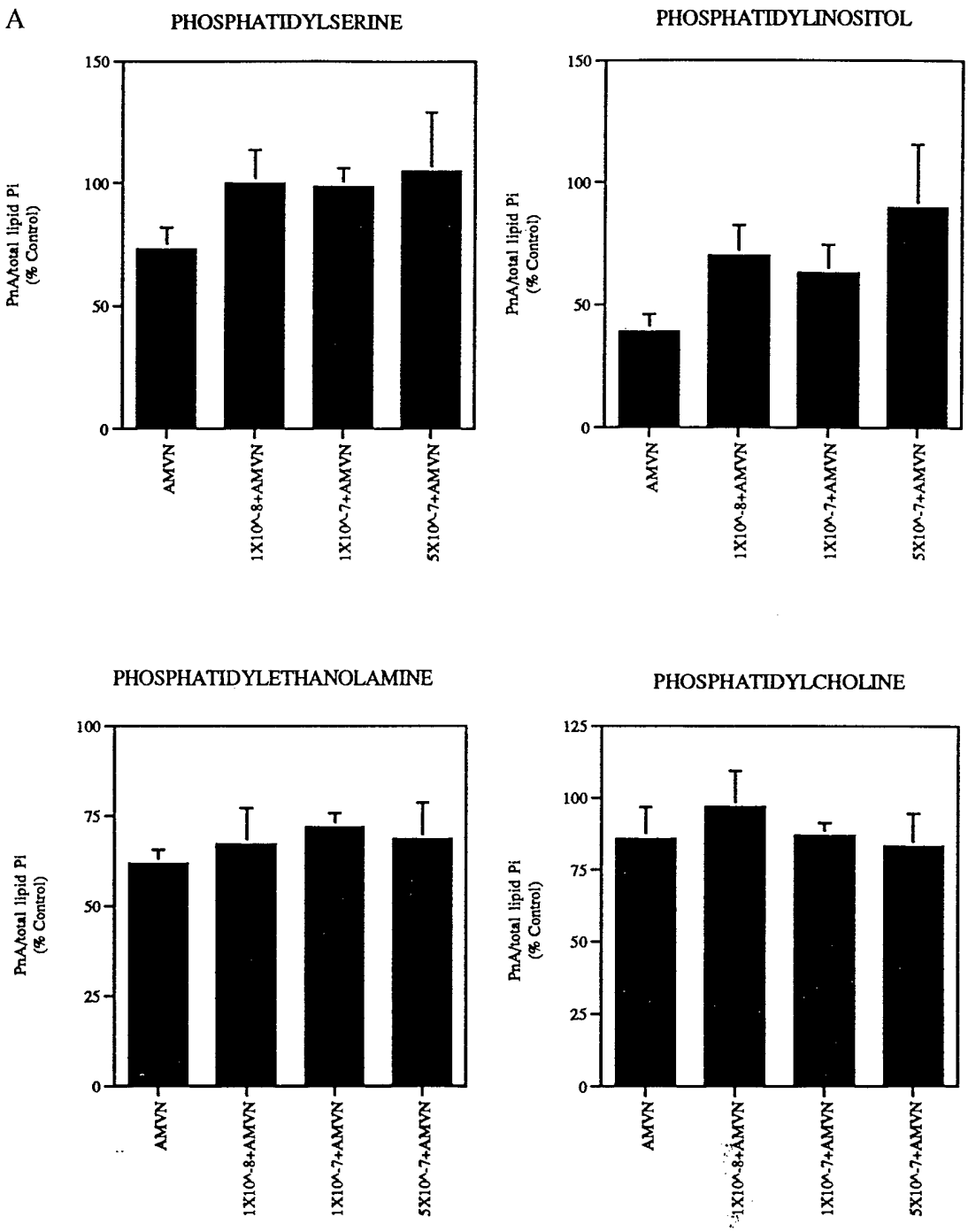


**FIG. 2.** Effect of treatment with estradiol (E2) on oxidation of *cis*-parinaric acid (PnA)-labeled phospholipids induced by AMVN in MCF-7 breast cancer cells. MCF-7 cells were treated with E2 ($0–5 \times 10^{-7}$ M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 $\mu$M; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA as we have previously described (8, 11). Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS ($p < 0.02$ relative to AMVN alone at all E2 concentrations; Student's t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment afforded statistically significant protection against oxidation of PS alone ($p < 0.03$ relative to AMVN alone at $10^{-8}$ and $5 \times 10^{-7}$ M E2; Student's t test).

content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Fig. 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the

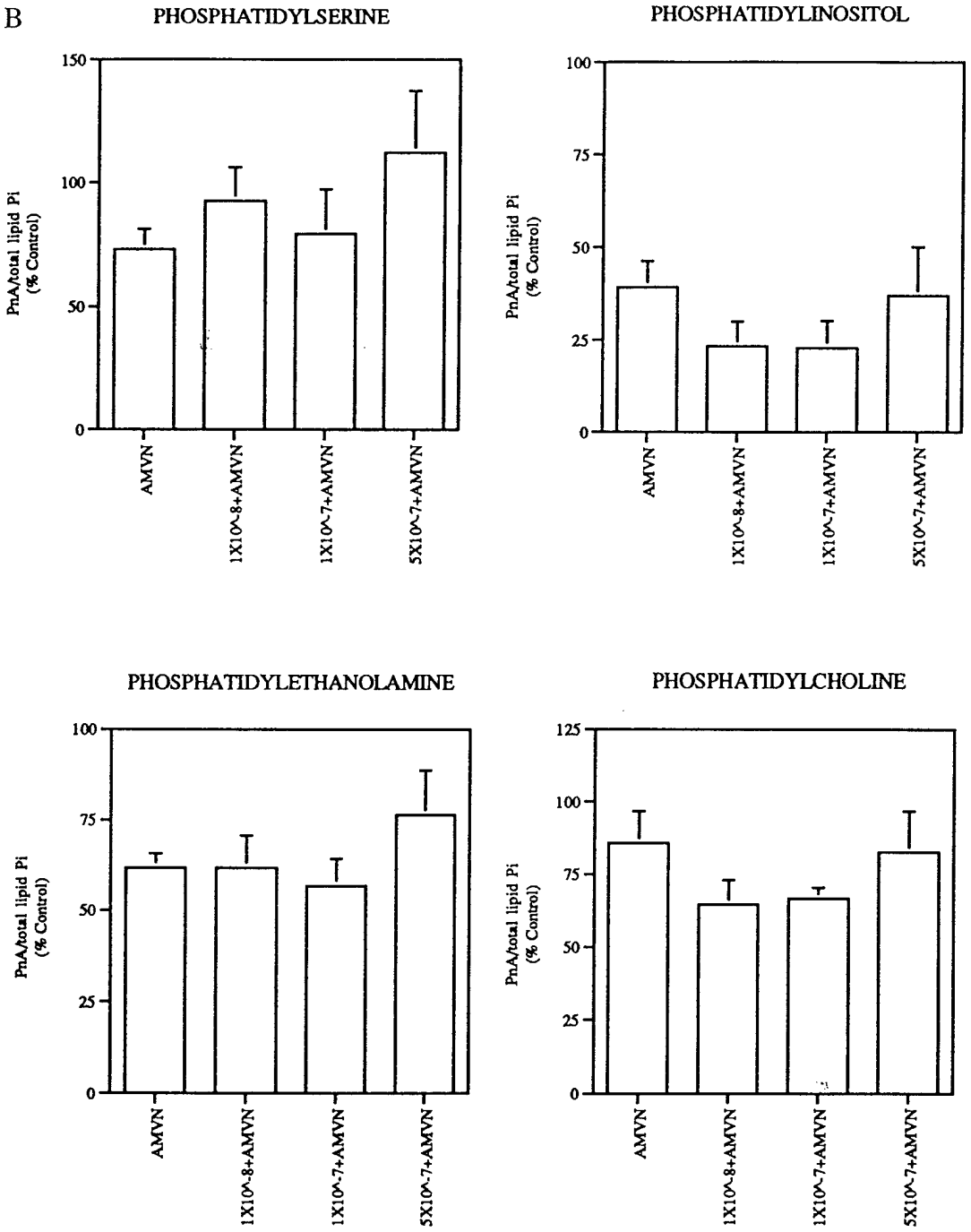


**FIG. 2**—*Continued*

concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to *bcl-2* overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (15).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (4), but may be enhanced by induction of estrogen metabolism (1). In contrast, *bcl-2* induction associated with long-

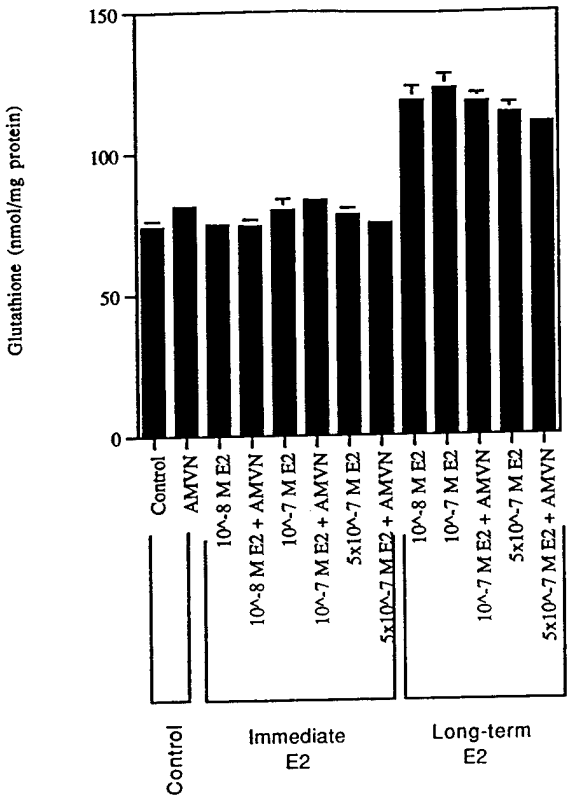


**FIG. 3.** Effect of immediate and long-term exposure to E2 and treatment with AMVN on glutathione content of MCF-7 breast cancer cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 $\mu$M) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo assay (8). Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with $p < 0.0001$ (Student's t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E2-exposed cells.

term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (7). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechnisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

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Research report

# Selective oxidation and externalization of membrane phosphatidylserine: Bcl-2-induced potentiation of the final common pathway for apoptosis

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cally block the apoptotic pathway distal to that inciting event (i.e., at a point in the final common apoptosis pathway). We have therefore attempted to resolve this seeming paradox by examining the effects of NCS on changes associated specifically with the common apoptosis pathway in mock- and *bcl-2*-transfected PC12 cells. Previous studies in our laboratory have demonstrated the role of peroxidation and externalization of membrane-bound phosphatidylserine (PS) early in the enactment phase of the apoptosis pathway [6]. They have further shown the activity of Bcl-2 in preventing peroxidation of PS and other membrane phospholipids by paraquat or the azo-initiator of lipid peroxyl radicals, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN; [6,15]). We now report the effects of Bcl-2 on membrane phospholipid peroxidation and PS externalization in PC12 cells treated with NCS.

## 2. Materials and methods

### 2.1. Cell lines and transfectants

Mock- and *bcl-2*-transfected PC12 cells, both selected as polyclonal populations [5], were the kind gift of Dr. Dale E. Bredesen (Burnham Institute, La Jolla, CA). These cells were maintained as adherent monolayers as we have previously described [2].

### 2.2. Incorporation of parinaric acid (PnA) into membrane phospholipids

Membrane phospholipids of both transfectants were labeled with PnA as we have described previously [15].

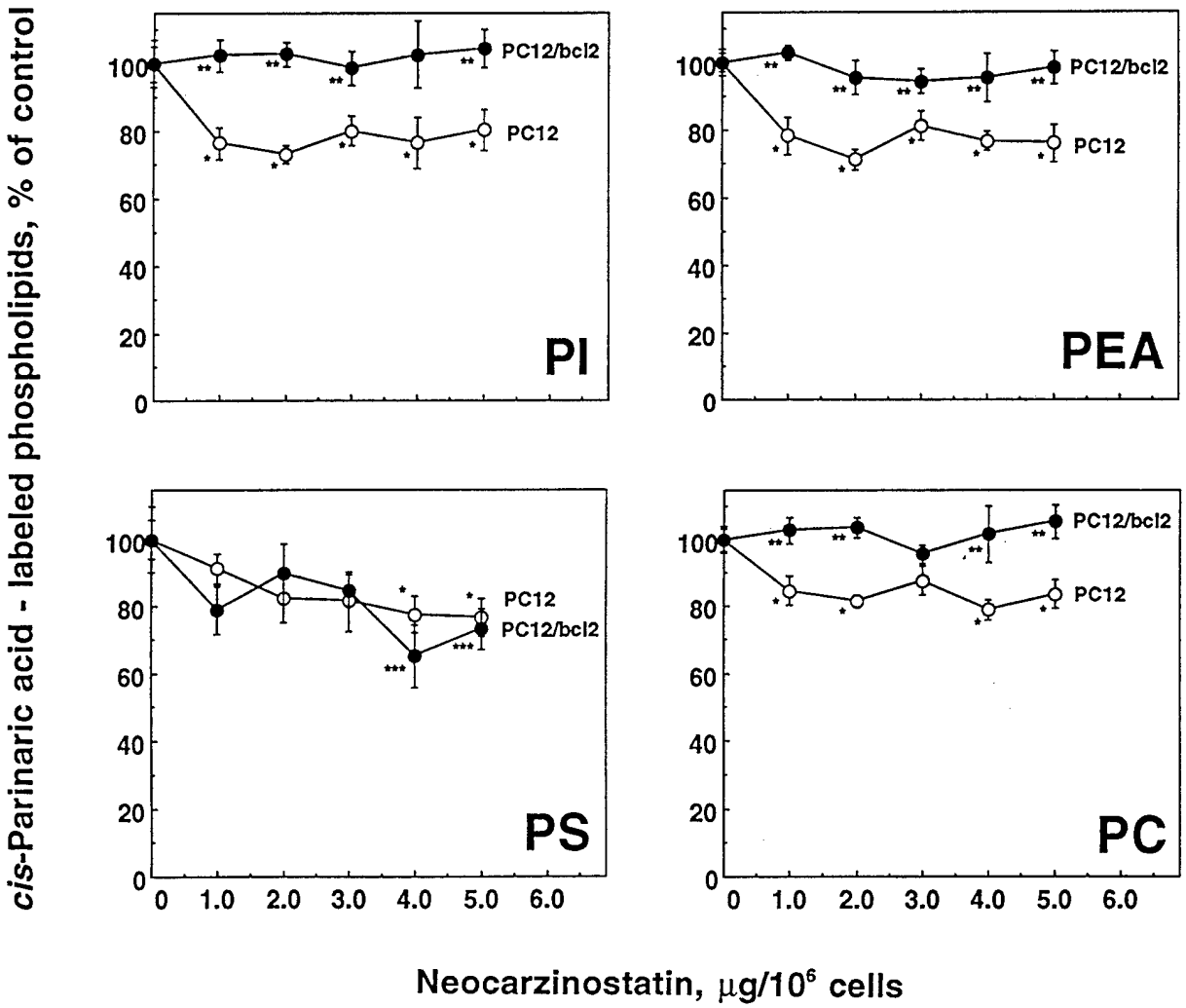


Fig. 1. Neocarzinostatin-induced oxidation of *cis*-parinaric acid-labeled [12,15] phospholipids in mock- and *bcl-2*-transfected PC12 cells. Each point represents the mean ± S.E.M. of five determinations made in two independent experiments. Abbreviations: PnA, *cis*-parinaric acid; NCS, neocarzinostatin; PC12, mock-transfected PC12 cells; PC12/bcl-2, *bcl-2*-transfected PC12 cells; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Student's *t*-test analysis: $^{*}p < 0.05$ vs. vehicle-treated PC12 cells; $^{**}p < 0.05$ vs. NCS-treated PC12 cells; $^{***}p < 0.05$ vs. vehicle-treated PC12/bcl-2 cells.

Briefly, mock- and *bcl-2*-transfected PC12 cells were incubated with a PnA-human serum albumin complex for 2 h at 37°C in L1210 medium. After incubation, lipids were extracted and resolved by HPLC.

### 2.3. Fluorescent labeling and analysis of external membrane phospholipids

Derivatization of intact cells with fluorescamine was performed as we have previously described [12]. Two-dimensional HPTLC was then performed on a total lipid extract of $10^6$ logarithmically-growing cells using chloroform:methanol:28% ammonium hydroxide::65:25:5 in the first dimension and chloroform:acetone:methanol:glacial acetic acid:water::50:20:10:10:5 in the second dimension.

### 2.4. Visualization of externalized PS

Because of the specific propensity of annexin V to bind to PS only on the extracellular face of the cell membrane, this compound has been used to 'stain' cells for the presence of this translocated PS [6,10,11,14,16,17]. Dual staining of mock- and *bcl-2*-transfected cells with propidium iodide and fluorescein-labeled annexin V [6] was performed at 24 h after completion of a 1 h exposure to NCS.

### 2.5. Dose–response studies of enediynes

Dose–response studies of the enediynes NCS and C1027 in apoptosis induction in mock- and *bcl-2*-transfected PC12 cells were performed as we have described previously [2]. In each case and for each drug, the $ED_{50}$ for apoptosis induction was defined and determined as detailed by Hartsell et al. [8].

## 3. Results

### 3.1. Membrane lipid peroxidation by NCS in mock- and bcl-2-transfected PC12 cells

The membranes of mock- and *bcl-2*-transfected PC12 cells were saturated with PnA as a probe for membrane phospholipid oxidation [12,15]. The time course and pattern of incorporation of PnA into membrane phospholipids did not differ significantly between mock- and *bcl-2*-transfected PC12 cells (data not shown). Similar to the case for AMVN treatment, a 1 h exposure of mock-transfected PC12 cells to NCS (37°C; 1–5 $\mu g/10^6$ cells) resulted in statistically significant (20–30%) peroxidation of phosphatidylinositol (PI), phosphatidylethanolamine (PEA), phosphatidylcholine (PC), and PS (Fig. 1). However, unlike the case for AMVN treatment, overproduction of Bcl-2 did not protect PC12 cells from PS oxidation in-

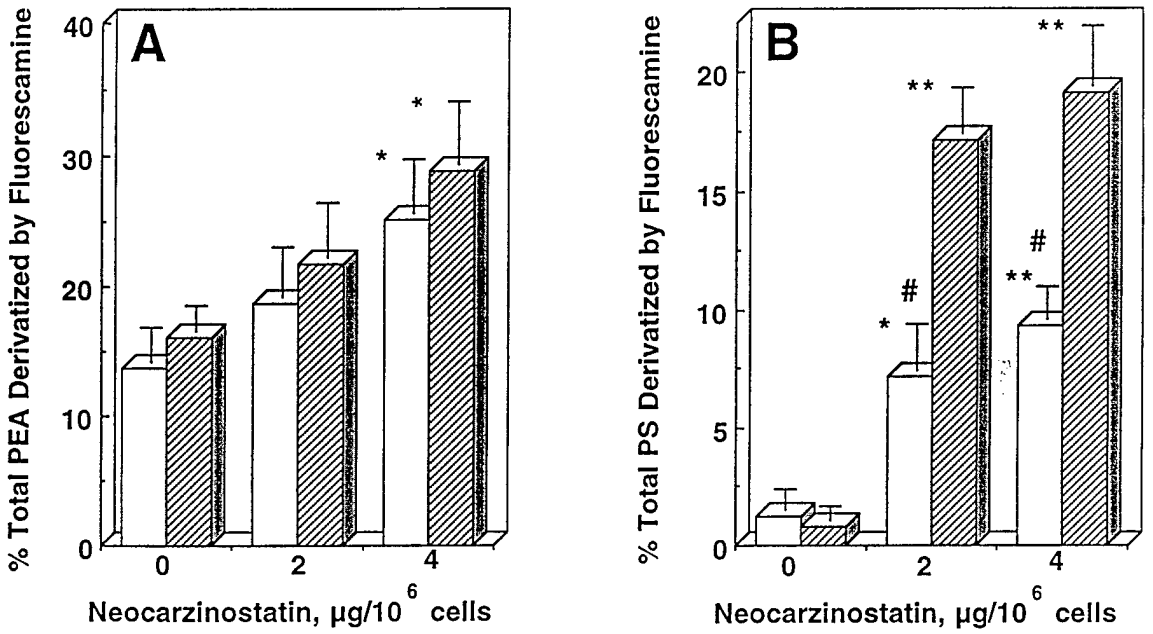


Fig. 2. Neocarzinostatin- (NCS-) induced externalization of phosphatidylethanolamine (PEA; panel A) and phosphatidylserine (PS; panel B). Mock- and *bcl-2*-transfected PC12 cells were incubated with NCS (0–4 $\mu g/10^6$ cells) for 1 h at 37°C in the dark in L1210 medium. The cells were then rinsed twice and external phospholipids were derivatized with fluorescamine. The total cell lipids were then extracted and analyzed by two-dimensional HPTLC [12]. Open bars, mock-transfected cells; hatched bars, *bcl-2*-transfected cells. Data shown represent the mean ± S.E.M. of five determinations in three independent experiments. $^*p < 0.05$ vs. vehicle-treated cells; $^{**}p < 0.001$ vs. vehicle-treated cells; $^{\#}p < 0.01$ vs. *bcl-2*-transfected cells treated with the same concentration of NCS.

duced by exposure to NCS. The selectivity of this phenomenon is demonstrated by the statistically significant protection by Bcl-2 of all other membrane phospholipids studied from NCS-induced peroxidation.

### 3.2. Externalization of PS in mock- and bcl-2-transfected PC12 cells treated with NCS

Immediately after a 1 h exposure to NCS, both PEA and PS are externalized in an NCS concentration-dependent fashion, as determined by direct fluorescamine staining of external membrane phospholipids (Fig. 2). Externalization of PS is approximately 2-fold greater in *bcl-2*-transfected than in mock-transfected PC12 cells ($p < 0.01$). In contrast, externalization of PEA did not differ significantly between the transfectants at any NCS concentration.

Neither transfectant exposed to control conditions demonstrated staining with annexin V (Fig. 3a,d), indicating that membrane PS normally faces intracellularly in these cells. Treatment with NCS resulted in *bcl-2*-transfected cells that stained with annexin V, even at 0.5 μg/ml, the lowest NCS concentration examined (Fig. 2e,f). In contrast, no mock-transfected cells stained with annexin V at 0.5 μg/ml NCS (Fig. 3b), and exposure to 2.5 μg/ml NCS resulted in scant staining of an occasional cell (Fig. 3c). These studies suggest that, unlike the case for treatment with AMVN or paraquat, Bcl-2 does not abrogate, but rather potentiates, PS oxidation and externalization in PC12 cells.

### 3.3. Role of reductive activation in potentiation of NCS-induced apoptosis by Bcl-2

From a mechanistic standpoint, potentiation by Bcl-2 of NCS-induced apoptosis ($ED_{50}$: PC12, 0.02 μM; PC12/Bcl-2, 0.005 μM) is dependent on the requirement for reductive activation of this prodrug. Mock- and *bcl-2*-transfected PC12 cells treated with the reduction-independent, autoactivating enediyne, C1027, demonstrated *bcl-2*-induced protection from, rather than potentiation of apoptosis ($ED_{50}$: PC12, $1 \times 10^{-5}$ μM; PC12/Bcl-2, $2 \times 10^{-5}$ μM).

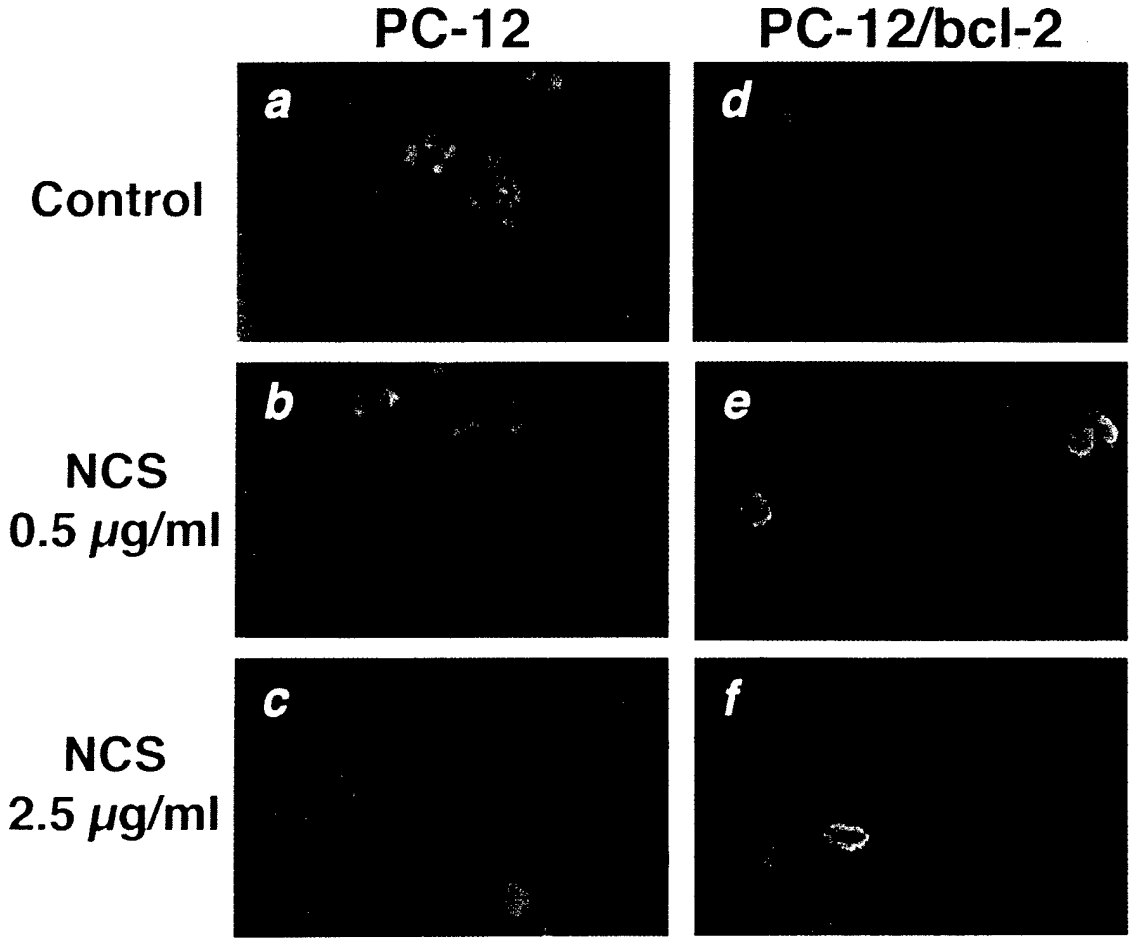


Fig. 3. Representative fluorescence micrographs of control and NCS-treated mock- and *bcl-2*-transfected PC12 cells dual-stained with propidium iodide and fluorescein conjugated annexin V [6]. PC12 cells transfected with *bcl-2* (d–f) and mock-transfected PC12 cells (a–c) following 24 h exposure to 0.5 μg/ml (0.05 μM; b,e) or 2.5 μg/ml (0.25 μM; c,f) NCS. Vehicle-treated cells are shown in panels a and d. Each of the panels shown is representative of five different high-power fields (400 ×).

## 4. Discussion

Recent studies have demonstrated the protective effect of Bcl-2 against apoptosis produced by direct triggering of the final common apoptosis pathway [13,20]. Our previous observation that Bcl-2 overproduction potentiates the induction of apoptosis in PC12 cells by NCS would appear to contradict this notion. If indeed Bcl-2 acts only at a distal common point in the induction of apoptosis, then NCS-induced apoptosis should be abrogated by Bcl-2, even if Bcl-2 directly or indirectly enhances the activation of NCS. The present studies demonstrate potentiation by Bcl-2 of events in the final common apoptosis pathway.

In T lymphocytes, initiation of apoptosis is accompanied by down-regulation of the ATP-dependent aminophospholipid translocase and activation of a nonspecific phospholipid scramblase [6,10]. Both of these changes result in transfer of PS from the inner to the outer surface of the cell membrane. The effects of Bcl-2 on PS externalization induced by NCS was studied both by direct fluorescamine staining of external membrane phospholipids and annexin V staining of external PS in intact cells. The results of these studies indicate that potentiation by Bcl-2 of NCS-induced apoptosis is accompanied by selective maintenance of vulnerability to oxidation of PS and potentiation of early events in the common pathway for apoptosis.

The potentiation of NCS-induced apoptosis has been hypothesized to be related to increased activation of this enediyne in the highly reducing intracellular milieu of *bcl-2*-transfected cells [2,5]. The present studies comparing autoactivating and reduction-requiring enediynes definitively demonstrate the role of reductive activation in this model. While it is possible that NCS-induced apoptosis is not susceptible to the regulatory influence of Bcl-2, it is perhaps more likely that increased activation of NCS results in consumption of species critical to the anti-apoptosis effects of Bcl-2, or in the quantitative overriding of the protective activity of Bcl-2. In its most specific application, the potentiation, by overexpression of *bcl-2* in PC12 cells, of NCS activity and PS oxidation and externalization suggests that in vitro screening for such potentiation could be used to identify chemoresistant tumors that might be responsive to treatment with enediynes. In a more general sense, this potentiation raises the caveat that, in neurodegenerative disorders for which gene therapy with *bcl-2* or its relatives has been suggested, such therapy must be carefully assessed in light of other aspects of the pharmacological and physiological environment in which the cells in question sit.

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Oncogene (2000) 19, 472–476

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SHORT REPORT

# Cell line dependence of Bcl-2-induced alteration of glutathione handling

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**Bcl-2 has been associated with both oxidative and antioxidative effects *in vivo*. Moreover, despite evidence that Bcl-2 is antiapoptotic by virtue of its effect on reactive oxygen species and their scavengers, Bcl-2 exerts its antiapoptotic effects even under anaerobic conditions. The reasons for the variable relationship between Bcl-2 and reactive oxygen species are not clear. The present studies demonstrate that the impact of Bcl-2 on glutathione (GSH) metabolism is cell line-dependent. Bcl-2 overproduction in PC12 cells is associated with increased functional thiol reserves, increased reductive activation of chemotherapeutic prodrugs, and GSH accumulation after treatment with N-acetylcysteine. In contrast, Bcl-2-overproducing MCF-7 breast cancer cells demonstrate neither altered GSH handling nor potentiation of chemotherapeutic prodrug reduction. These findings indicate that the effects of Bcl-2 on GSH handling are millieu-dependent. This could account for the variable effects of Bcl-2 in *in vivo* systems. Furthermore, since our previous studies have demonstrated that reduction-dependent prodrugs may be useful chemotherapeutic agents against tumors that demonstrate altered GSH handling, screening *in vitro* for alteration of GSH handling may predict responsiveness of such tumors to these reduction-dependent agents.** *Oncogene* (2000) **19**, 472–476.

**Keywords:** oxidative stress; apoptosis; glutathione

The induction and enactment of apoptosis have been associated with the generation of reactive oxygen species. In keeping with this association, overproduction of the antiapoptosis protein, Bcl-2, is associated in some systems with increased tolerance to oxidant stress (Kane *et al.*, 1993; Albrecht *et al.*, 1994; Tyurina *et al.*, 1997), an increase in the cellular content of reducing species (Kane *et al.*, 1993), and prevention of the release of cytochrome c from the mitochondrion (Yang *et al.*, 1997; Kluck *et al.*, 1997). However, in contradistinction to these studies, others have reported antiapoptosis activity of Bcl-2 in anaerobic systems (Jacobson and Raff, 1995) and have even ascribed a pro-oxidant function to Bcl-2 (Steinman, 1995).

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Glutathione (GSH) is one of several antioxidant species the metabolism of which is thought to be altered by Bcl-2 overproduction. We have previously discussed the implications of this alteration for apoptosis induction during cancer chemotherapy with reduction-dependent agents (Cortazzo and Schor, 1996) and in the course of neurodegenerative disease (Schor *et al.*, 1999). We examine herein the metabolism of GSH in native and bcl-2-transfected cell lines.

Mock- and bcl-2-transfected PC12 pheochromocytoma cells were prepared and isolated as a polyclonal population as previously published (Kane *et al.*, 1993). Mock-and bcl-2-transfected MCF-7 human breast cancer cells were generated by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in Boise *et al.*, 1993), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

Incubation of bcl-2-transfected PC12 cells with the GSH precursor N-acetylcysteine (NAcCys; 10 mM) for up to 8 h results in a GSH level 5-times baseline ($P<0.002$) by 4 h of treatment (Figure 1a). In contrast, similar incubation of mock-transfected PC12 cells results in only a 1.6-fold increase in intracellular GSH ($P<0.002$, Student's *t*-test) that is apparent by 1 h and plateaus until at least 4 h of incubation. Without additional NAcCys supplementation, the GSH content of bcl-2-transfected cells declines and the difference in GSH content between the two transfectants loses statistical significance ($P>0.05$) by 12 h after addition of NAcCys to the medium (data not shown).

Analogous treatment of mock- and bcl-2-transfected MCF-7 cells with NAcCys results in GSH content kinetics that resemble those seen with mock-transfected PC12 cells (Figure 1b). There was no significant difference between the MCF-7 bcl-2- and mock-transfectants in this regard.

NAcCys-induced GSH accumulation could result from augmented GSH cycling in response to oxidative consumption. As such, we have examined the effects of bcl-2 transfection on the ability of PC12 cells to exchange and recycle GSH reducing equivalents (so-called 'functional thiol reserves'; Hubel *et al.*, 1997) in response to treatment with NCS, a known 'consumer' of GSH. We have done this by measuring the generation of glutathionyl radical (Stoyanovsky *et al.*, 1996) and the decline in GSH content (Langmuir *et al.*, 1996) after a 1 h incubation with NCS. Formation of

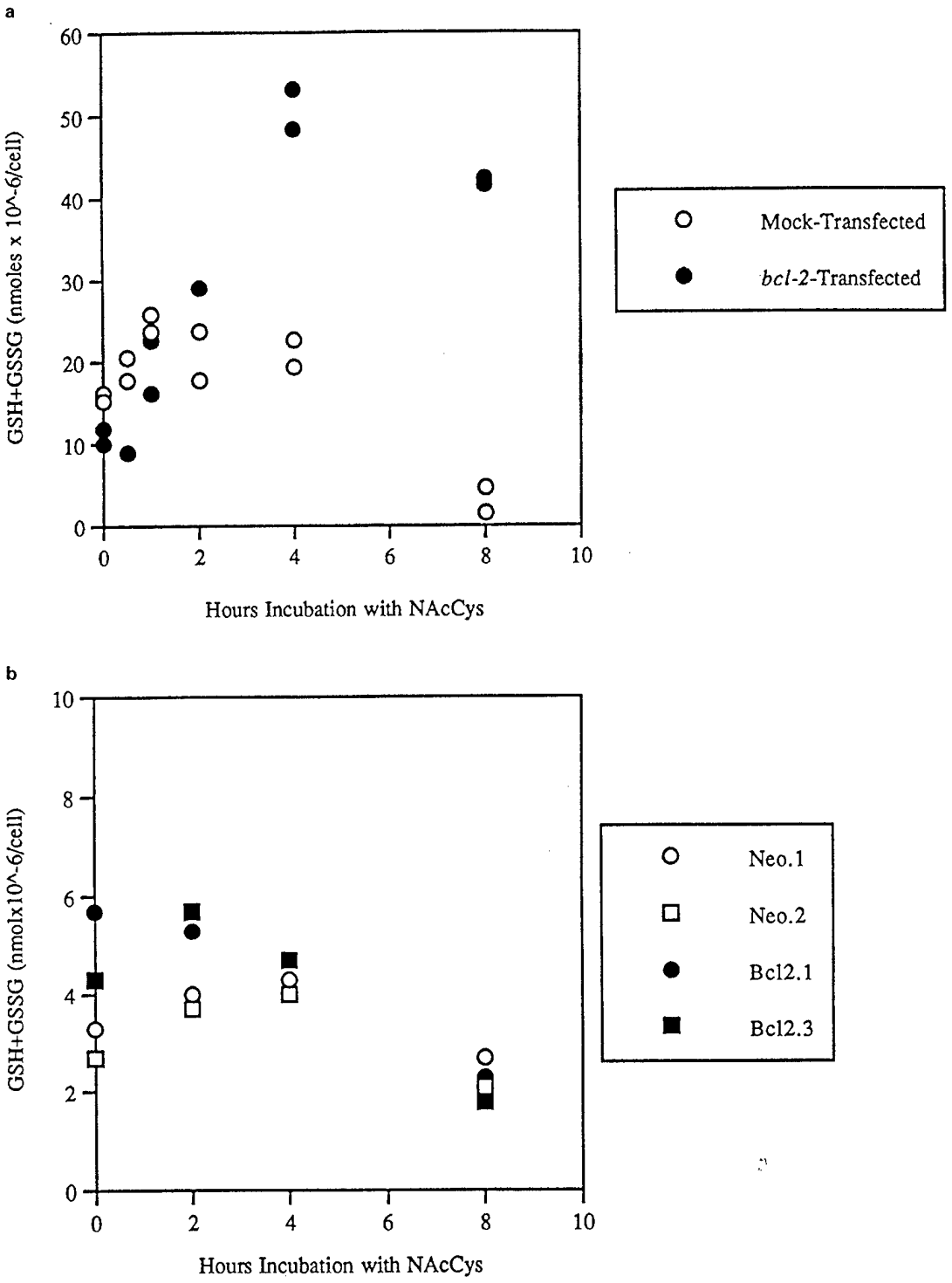


**Figure 1** Effects of bcl-2 transfection on accumulation of GSH in tumor cells continuously incubated with N-acetylcysteine (NAcCys; 10 mM). GSH was measured for the results depicted using the method of Tietze (1969). Independently obtained samples assayed using the Thio-Glo™ method (Langmuir *et al.*, 1996) gave analogous results. (**a**) PC12 pheochromocytoma cells. Mock- and bcl-2-transfected PC12 pheochromocytoma cells were prepared and isolated as a polyclonal population as previously published (Kane *et al.*, 1993). Four independent experiments were performed and gave comparable results. The results of duplicate determinations from one of these experiments are shown. (**b**) MCF-7 breast cancer cells. Mock- and bcl-2-transfected MCF-7 human breast cancer cells were generated by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in Boise *et al.*, 1993), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology). The results of single determinations from each of two mock- (Neo.1-mock and Neo.2-mock) and two bcl-2-transfected (Bcl-2.1 and Bcl-2.2) clones of MCF-7 cells are shown. A second independent experiment gave comparable results

the glutathionyl radical mirrors oxidative GSH consumption (Stoyanovsky *et al.*, 1996).

Treatment of bcl-2-transfected PC12 cells with NCS (0.4 nmol/$10^6$ cells) results in the trapping of approximately 4 nmol/mg protein ($P<0.005$, Student's *t*-test) of glutathionyl radical and a decline in GSH content of 1.4 nmol/mg protein ($P<0.0005$) over 1 h (Table 1). In sharp contrast, treatment of mock-transfected PC12

**Table 1** Glutathione handling in mock- and bcl-2-transfected PC12 cells

| | *Mock-transfectant* | | *bcl-2 transfectant* | |
|---|---|---|---|---|
| | *Pre-NAcCys* | *Post-NAcCys* | *Pre-NAcCys* | *Post-NAcCys* |
| GS• Produced during NCS treatment (nmol/mg protein) | 0.3±0.04 | NA | 4.1±0.8 ($P<0.005$) | NA |
| GSH consumed during NCS treatment (nmol/mg protein) | 0.1±0.01 | NA | 1.4±0.41 ($P<0.0005$) | NA |
| GCS (relative activity per cell) | 3.3±0.4 | 2.1±0.2 | 3.2±0.4 | 2.3±0.2 |
| GR (relative activity per cell) | 5.8±0.2 | 3.3±0.2 | 4.2±0.2 | 3.3±0.2 |
| $t_{efflux}$ for GSH (h) | 6.0 ($r^2=0.98$) | ND | 5.5 ($r^2=0.91$) | ND |

Formation of glutathionyl radical (GS•) and GSH consumption were measured during a 1 h treatment with NCS (0.4 nmol/$10^6$ cells). GS• was measured as its 5,5 dimethyl-1-pyrroline N-oxide- (DMPO-) trapped adduct (Stoyanovsky *et al.*, 1996). GSH was measured by the Thio-Glo™ method (Langmuir *et al.*, 1996). The activities of glutathione reductase (GR) and γ-glutamylcysteine synthetase (GCS) were measured by the methods of Carlburg and Mannervik (1985) and Seeling and Meister (1985), respectively, before and after incubation of the cells with N-acetylcystine (NAcCys; 10 mM, 4 h). The time constant for GSH efflux ($t_{efflux}$ for GSH) was measured by determination of the GSH content of the cells (Tietze, 1969) at various time points (0.5–24 h) during inhibition of new GSH synthesis (BSO, 1 mM). A straight line was fitted to a semi-log plot of the data for each of the two transfectants, and $r^2$ for each line is given in the Table. In all cases, statistical significance of the differences in the appropriate direction between the mock and bcl-2 transfectants was determined by one-tailed Student's *t*-test. For values attaining significance (defined as $P<0.05$, bcl-2 transfectant vs mock transfectant), the *P* values are provided in the Table. NA, not applicable; ND, not determined

cells with NCS results in no signficant change in the cumulative generation of glutathionyl radical or the cellular content of GSH. Parallel studies of the effect of NCS treatment on cellular protein thiol content revealed no significant change in either PC12 transfectant (data not shown).

GSH accumulation in bcl-2-transfected PC12 cells is not simply the result of increased GSH synthetic capacity. We have measured the activities of the GSH synthetic enzymes glutathione reductase (GR; Carlburg and Mannervik, 1985) and γ-glutamylcysteine synthetase (GCS; Seelig and Meister, 1985) in mock- and bcl-2-transfected PC12 cells both before and after 4 h treatment with 10 mM NAcCys. There was no difference between the transfectants with respect to GCS activity either in the native state or after incubation with NAcCys (Table 1). Furthermore, the observed lower native GR activity in bcl-2-transfected PC12 cells relative to mock-transfected cells is counter to the predicted increase in GR activity hypothesized to result in increased intracellular GSH levels after bcl-2 transfection. There was no observed difference in GR activity after NAcCys treatment between the transfectants.

The observed GSH accumulation is also not the result of decreased GSH efflux from the cell. The rate of efflux of GSH from native mock- and bcl-2-transfected PC12 cells was approximated by treatment of the cells with buthionine sulfoximine (BSO; 1 mM) to block new GSH synthesis, followed by timed measurement of total GSH per cell (Tietze, 1969). There was no significant difference in the rate of decrease in the total cellular GSH content of the two transfectants after BSO treatment (Table 1).

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by bcl-2 transfection [Cortazzo and Schor, 1996; concentration inducing apoptosis in 50% of cells ($EC_{50}$) decreases from >0.06 to 0.01 μm]. This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. The difference in effect of bcl-2 transfection on GSH handling in PC12 and MCF-7 cells led us to predict that bcl-2 transfection would not potentiate and might even prevent apoptosis in MCF-7 cells treated with NCS.

As predicted, bcl-2 overexpression in MCF-7 cells did not potentiate death induced by NCS ($EC_{50}$ increases from 0.0025±0.0005 to 0.0045±0.0009 μm). The small protective effect of bcl-2 overexpression in this system did not quite reach statistical significance (*P* between 0.05 and 0.1, Student's *t*-test). However, at every concentration tested, the survival of the MCF-7 bcl-2 transfectants exceeds that of the mock transfectants (data not shown). Note also that mock-transfected MCF-7 cells are tenfold more sensitive than mock-transfected PC12 cells to the effects of NCS.

In light of the accumulation of GSH seen only in bcl-2-transfected PC12 cells after incubation with NAcCys, and the concentration-dependent role of GSH in activation of NCS (DeGraff and Mitchell, 1985; DeGraff *et al.*, 1985; Beerman *et al.*, 1977; Schor, 1992), we hypothesized that pretreatment of bcl-2-transfected PC12 cells with NAcCys would result in further potentiation of NCS-induced apoptosis, while the response of mock-transfected PC12 cells and mock- and bcl-2-transfected MCF-7 cells to NCS would remain unaltered by NAcCys. NAcCys treatment (4 h; 10 mM) of bcl-2-transfected PC12 cells prior to treatment with NCS results in a twofold shift in the concentration-response curve of such cells in the direction of increased potency of NCS (Figure 2; $P<0.002$, Student's *t*-test). In contrast, the concentration-response curve of bcl-2-transfected MCF-7 cells to NCS is unaffected by NAcCys treatment ($EC_{50}$s of 0.0017±0.0002 without NAcCys and 0.0018± 0.0002 μm with NAcCys; $P>0.05$, Student's *t*-test). The sensitivity to NCS of mock-transfected cells of both types is unaffected by NAcCys exposure (data not shown).

The induction of apoptosis in neural cells has been hypothesized to play a role in the morphological and physiological changes associated with a variety of developmental and pathological states (Narayanan, 1997; Gelbard *et al.*, 1997). For many of these, reactive oxygen species (ROS) have been implicated in the disease pathogenesis itself and/or as final common mediators of apoptosis (Wood and Youle, 1994, 1995). Similarly, species known to influence the induction and enactment of apoptosis have been proposed as modulators of development and disease and cellular handling of ROS.

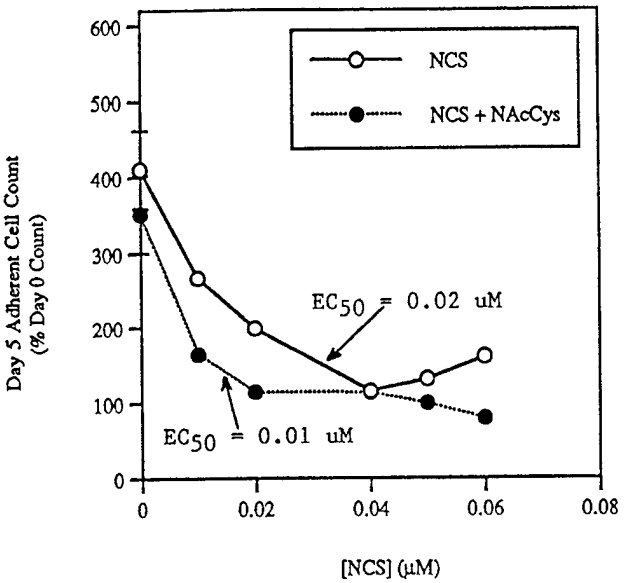


**Figure 2** Effect of NAcCys (10 mM; 37°C; 4 h) pretreatment on NCS sensitivity of bcl-2-transfected PC12 pheochromocytoma cells. NCS treatment (1 h; 37°C) was performed on sister cultures immediately after washout of NAcCys or vehicle (no NAcCys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. We have previously demonstrated the apoptotic nature of cell death induced by NCS in many cell lines (Hartsell *et al.*, 1995; Hartsell *et al.*, 1996; Cortazzo and Schor, 1996). We have also shown the equivalence of loss of adherence to the cell culture plate and apoptotic nuclear morphology in this model (Cortazzo and Schor, 1996). The s.e.m.s of the triplicate determinations are plotted, but are smaller than the width of the symbol at each point. At all NCS concentrations except 0.04 μm, the cell counts for NAcCys incubation differ from those for vehicle incubation at the $P<0.002$ level (Student's *t*-test)

Some studies of the effects of bcl-2 overexpression on ROS handling in neural cells have suggsted a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (Kane *et al.*, 1993; Albrecht *et al.*, 1994; Tyurina *et al.*, 1997). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (Steinman, 1995). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (Jacobson and Raff, 1995), and the GSH depletion does not abolish the protective effects of Bcl-2 (Kane *et al.*, 1993). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is millieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well-documented (Halliwell and Gutteridge, 1985).

Our studies of NCS treatment of PC12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (Dole *et al.*, 1994, 1995; Teixeira *et al.*, 1995; Bonetti *et al.*, 1996; Beham and MacDonnell, 1996; Campos *et al.*, 1993; Reber *et al.*, 1998), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (Cortazzo and Schor, 1996). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in bcl-2-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (Maeda, 1994). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to compensate for consumption of reducing equivalents is aberrant in some bcl-2-overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (Stoyanovsky *et al.*, 1996). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (Stoyanovsky *et al.*, 1996; Chin *et al.*, 1988). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (Schor *et al.*, 1999), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells makes NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.

That incubation with NAcCys accentuates the difference in GSH handling between bcl-2- and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in bcl-2-overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an *in vitro* assay of GSH accumulation after NAcCys incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.

These results have broader significance, as well. They indicate that the redox 'set-point' of a cell and the effects of Bcl-2 on it, may determine in dramatically different ways the implications for that cell of exposure to antimitotic, DNA cleaving, or potential apoptosis-inducing agents. As our previous studies implied, the effects of such agents are likely dictated by intrinsic properties of the cell, and not by the specific agent used to induce the effect (Smith *et al.*, 1998).

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# ROLE OF CASPASE 3-DEPENDENT Bcl-2 CLEAVAGE IN POTENTIATION OF APOPTOSIS BY Bcl-2

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## INTRODUCTION

In the case of most chemotherapeutic agents, overexpression of *bcl-2* leads to abrogation of apoptosis induction (1-4). However, in the case of reduction-dependent chemotherapeutic prodrugs, overexpression of *bcl-2* potentiates apoptosis induction (5-8). It was originally hypothesized that this potentiation results from a *bcl-2*-induced shift in the redox potential of the cell with increased functional thiol reserves and consequent potentiation of activation of these reduction-dependent prodrugs (5,8). However, if overexpression of *bcl-2* only increased the activation of the apoptosis-inciting drug, its protein product, Bcl-2, would be expected nonetheless to block apoptosis downstream of the action of this drug, presumably at the level of inhibition of cytochrome c release from mitochondria (9,10). That this is not the case is demonstrated by potentiation by Bcl-2 of reduction-dependent chemotherapeutic agent-induced oxidation and externalization of membrane phosphatidylserine (7), a late event in the apoptosis final common pathway. Clearly, the distal anti-apoptotic effects of Bcl-2 have been thwarted in this system; indeed, a pro-apoptotic effect appears likely.

Bcl-2 is one of a family of proteins, some of which, unlike Bcl-2, are pro-apoptotic (reviewed in references 11 and 12). Previous studies have demonstrated that Bcl-2 itself can be cleaved by caspase 3 to a pro-apoptotic Bcl-2 fragment (13). We have therefore examined the role of cleavage of Bcl-2 in Bcl-2-mediated potentiation of apoptosis.

## MATERIALS AND METHODS

**Cells and Cell Culture.** Mock-transfected (pBabe-puro) PC12 cells and PC12 cells transfected with the *bcl-2* gene ligated into the retrovial vector, pBabe, containing a puromycin resistance gene (*bcl-2*- pBabe-puro; 14) were provided by Dale E. Bredesen (La Jolla Cancer Institute, La Jolla, CA). Cells were maintained as adherent monolayers in DMEM made 10% in horse serum, 0.5% in fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1.1% in

penicillin/streptomycin (Life Technologies, Grand Island, NY). Cells were fed every 3-4 days, and biweekly, 1 μg/ml of puromycin was added to the medium. Cells were examined for Bcl-2 expression by Western blotting every 10 passages. Bcl-2 expression did not vary in either cell line over the course of these studies.

Mock- and *bcl-2* gene transfected MCF-7 human breast cancer cells were generated by electroporation (300mV, 960 mFD) with pSFFV-neo and pSFFV-*bcl-2* (plasmids described in reference 15), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Two mock- (Neo.1 and Neo. 2) and two *bcl-2*-transfected (Bcl-2.1 and Bcl-2.3) clones of MCF-7 cells were used for these studies (16). The MCF-7 cells were maintained as adherent monolayers in 75-$mm^2$ culture flasks (Life Technologies), and fed twice weekly with α-MEM (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum, 0.3% glucose, 2mM L-glutamine (Life Technologies), and 2μg/ml gentamicin sulfate (Biofluids, Rockville, MD).

**Neocarzinostatin and Cisplatin Treatment.** Mock- and *bcl-2*-transfected PC12 and MCF-7 cells were treated with either 0.02μM neocarzinostatin (NCS) or 10 μM cisplatin for 1 hour. Subsequently, the NCS or cisplatin was washed out and fresh medium was added. Cells were then incubated for varying lengths of time as indicated. For studies of the effects of the caspase 3 inhibitor Ac-DEVD-CHO on mock- and *bcl-2*-trancfected PC12 cells, 5 μM Ac-DEVD-CHO (PharMingen International, San Diego, CA) was added to the cells 1 hour prior to NCS or cisplatin treatment, and maintained in the medium thereafter.

**Western Blotting Analysis of Bcl-2 and Caspase 3 proteins.** At the indicated time points, PC12 and MCF-7 cells were lysed in RIPA buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS. 1 mM PMSF, 4 μg/ml aprotinin, 1 mM sodium orthovanadate). Subsequently the protein concentrations of the lysates were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a

standard. An aliquot of each lysate containing 500 μg of protein was loaded onto each lane and electrophoresed on a 15% SDS-polyacrylamide gel, followed by blotting on a nitrocellulose membrane (Bio-Rad Laboratories). After blotting, non-specific binding was blocked with 5% milk/PBS and the membrane was incubated with either anti-Bcl-2 (1:500, Santa Cruz Biotechnology) or anti-caspase 3 (1:1000, PharMingen International) antibodies diluted in 5% milk/PBS at 20°C for 2 hours, washed, and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Santa Cruz Biotechnology) for 1 hour. The membrane was finally washed and developed with Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) following the manufacturer's instructions.

**Determination of Adherent Cell Number.** Adherent cell number was determined daily in control and treated cultures, as we have decsribed previously for neuroblastoma cells (5,17-19). In case of Ac-DEVD-CHO caspase 3 inhibitor treament, 5 μM of the inhibitor was added 1 hour prior to NCS treatment. The statistical significance of differences between control and treated cultures was assessed in all cases using Student's *t* test, with $P \leq 0.05$ being considered significant.

***In Vivo* Studies of Effects of Cisplatin or NCS on Tumorigenesis and Tumor Growth from Mock- and *bcl-2*-transfected PC12 Cells.** Experiments involving tumorigenesis and tumor growth were performed on 5 – 7 week-old male NIH athymic mice injected subcutaneously with $10^6$ mock- or *bcl-2*-transfected PC12 cells into the left flank on day 0 of each study. Either cisplatin (0 – 25 mg/kg) or NCS (0 – 5 mg/kg) was administered intraperitoneally on day 1 (n = 4 for each dose). Mice were examined daily for grossly visible tumor, and, once tumors appeared, they were assessed by measurement of the largest and smallest diameter of each tumor on a daily basis. Animals were sacrificed by cervical dislocation on day 28 or 32; tumors were resected and weighed. Statistical significance of differences in growth rate and ultimate tumor weight was determined using Student's t test.

## RESULTS

*NCS induced Bcl-2 cleavage in* bcl-2 *transfected PC12 cells:* We have reported that *bcl-2*-transfected PC12 cells are more susceptible to NCS-induced apoptosis than mock-transfected cells (5). To begin to examine the possibility that cleavage of the Bcl-2 protein plays a role in the potentiation of NCS treatment-induced apoptosis, we first looked for cleavage of Bcl-2 in cells treated with NCS. For this study, *bcl-2*- and mock-transfected PC12 cells were subjected to NCS treatment and the cell lysate was harvested and analyzed by Western blotting. As shown in Fig. 1, 12 hours after NCS treatment, Bcl-2 protein was cleaved to an 21 kD protein in the *bcl-2*-transfected cells. The mock-transfected PC12 cells do not have detectable Bcl-2 or cleavage product.

*NCS did not induce Bcl-2 cleavage in* bcl-2 *-transfected MCF-7 cells:* We previously demonstrated that, unlike the case for PC12 cells, *bcl-2* transfection of breast cancer cells of the MCF-7 line does not lead to potentiation of NCS-induced apoptosis (16). In order to determine whether Bcl-2 cleavage after NCS treatment co-segregated with potentiation of apoptosis, we examined the effects of NCS treatment on Bcl-2 in MCF-7 cells. After NCS treatment, Bcl-2 was not cleaved in either *bcl-2*- or mock-transfected MCF-7 cells (Fig. 2).

*Cisplatin did not induce Bcl-2 cleavage in* bcl-2 *-transfected PC12 and MCF-7 cells:* Unlike the case for NCS, *bcl-2* transfection protects both PC12 and MCF-7 cells from cisplatin-induced apoptosis (5). We therefore examined whether cisplatin induced Bcl-2 cleavage in *bcl-2* transfected PC12 and MCF-7 cells. As shown in Fig. 3, there was no Bcl-2 cleavage in either cell line after cisplatin treatment.

*MCF-7 cells do not express caspase 3:* Caspase 3 has been shown to cleave Bcl-2 to pro-apoptotic peptides in other systems (13). In an effort to link the cleavage of Bcl-2 protein in

PC12 cells and the lack thereof in MCF-7 cells, we measured caspase 3 expression in each of our transfectants of PC12 and MCF-7 cells. As shown in Fig. 4, caspase 3 is expressed in *bcl-2*- and mock-transfected PC12 cells, but not in either *bcl-2*- or mock-transfected MCF-7 cells.

*The caspase 3 inhibitor DEVD-CHO abrogates NCS-induced apoptosis in* bcl-2 *transfected PC12 cells.* In order to further demonstrate the role of caspase 3 in the cleavage of Bcl-2 after NCS treatment, we pretreated *bcl-2*- and mock-transfected PC12 cells with the caspase 3 inhibitor DEVD-CHO (5 μM) for 1 hour before NCS treatment. As shown in Fig. 5, pretreatment with DEVD-CHO results in abrogation of apoptosis resulting from NCS treatment in *bcl-2*-transfected PC12 cells; in contrast, DEVD-CHO did not alter NCS-induced apoptosis in mock-transfected PC12 cells.

*Unlike the case for cisplatin treatment, xenografts of* bcl-2 *-transfected PC12 cells are more susceptible to NCS toxicity than are xenografts of mock-transfected PC12 cells:* Subcutaneous implants of *bcl-2*-transfected PC12 cells more often generate gross tumors than their mock-transfected counterparts. The tumors that form from them are palpable earlier and ultimately larger than those formed from implanted mock-transfected PC12 cells (Fig. 6A). Nonetheless, as is the case for *in vitro* treatment of *bcl-2*- and mock-transfected PC12 cells, implants from *bcl-2*-transfected cells are more susceptible to NCS-induced inhibition of their tumorigenic potential than are implants from mock-transfected cells (Fig. 6B).

An analogous study performed with intraperitoneal administration of cisplatin (10 mg/kg) demonstrated comparable depression of tumor growth rate with no effect on tumor incidence in murine xenografts of mock- and *bcl-2*-transfected PC12 cells (data not shown). At doses of cisplatin below 10 mg/kg, no depression of tumor growth rate was seen in either xenografted transfectant. In no case was Bcl-2-mediated potentiation of the anti-tumor effect seen. At doses of cisplatin above 10 mg/kg, lethal toxicity of the drug, evident before tumors are grossly

detectable in control animals, precluded evaluation of the effect of high-dose cisplatin on tumor growth.

## DISCUSSION

Our previous studies have demonstrated that *bcl-2* overexpression potentiates enediyne-induced apoptosis in some cell lines and not in others. We now show that potentiation of enediyne-induced apoptosis in *bcl-2*-transfected PC12 cells is associated with cleavage of Bcl-2 protein. Although Bcl-2 itself is an anti-apoptotic protein, it has recently been reported that the cleavage product of this protein is pro-apoptotic (13,20). Bcl-2 cleavage has been reported to occur after Asp-34, and to result in production of a pro-apoptotic cleavage product lacking the N-terminal 34 amino acids of Bcl-2 (13). This is consistent with the 21 kD cleavage product that was detected in response to NCS treatment. Cleavage of Bcl-2 was not detected in cisplatin-treated, *bcl-2*-transfected PC12 cells or NCS-treated, *bcl-2*-transfected human MCF-7 cells.

That this Bcl-2 cleavage requires the activity of caspase 3 is shown by two lines of evidence. NCS treatment of MCF-7 cells, cells previously described and demonstrated herein not to express caspase 3, does not result in cleavage of Bcl-2. [The absence of caspase 3 expression in MCF-7 cells is the result of a 47-base pair deletion within exon of 3 of the caspase 3 gene (21).] Furthermore, the caspase 3-specific inhibitor Ac-DEVD-CHO blocks both the cleavage of Bcl-2 resulting from NCS treatment and the potentiation of NCS-induced apoptosis seen with *bcl-2* overexpression. This is consistent with previous reports of the abrogation of cleavage of Bcl-2 when caspase 3 was immunodepleted from extracts of 293 cells. Conversely, immunodepletion of caspase 7 did not affect Bcl-2 cleavage in this system (20). It is thus clear that caspase 3 plays a critical role in the cleavage of Bcl-2 in *bcl-2*-transfected PC12 cells. While this role is likely to be a direct one, it is also possible that caspase 3 activates a downstream protease that, in turn, directly cleaves Bcl-2.

It is of interest that, despite their lack of caspase 3 expresison, MCF-7 cells still undergo apoptosis induced by a host of exogenous stimuli, including Vitamin D (22), staurosporine (21),

the diazo radical initiator, AMVN (7), and NCS (16). Caspase 3 is clearly not involved in apoptosis in these systems. However, when the gene for caspase 3 was tranfected into MCF-7 cells, the gene was expressed, and caspase 3 protein was activated, resulting in cleavage of Bcl-2 (20).

The mechanism of apoptosis induction by NCS in MCF-7 cells may involve pro-apoptotic changes other than the cleavage of Bcl-2. Our preliminary studies indicate that treatment of MCF-7 cells with NCS results in downregulated expression of Bcl-2 and upregulated expression of its pro-apoptotic analogue, Bax. The resulting decrease in the Bcl-2/Bax ratio has been shown in other systems to trigger the release of cytochrome c from the mitochondria (23), and to thereby induce apoptosis (24,25). Modulation of the Bcl-2/Bax ratio represents an alternative mechanism of potentiation of apoptosis in cells lacking caspase 3 and therefore unable to cleave Bcl-2 to a pro-apoptotic species.

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## FIGURE LEGENDS

Figure 1: NCS induces Bcl-2 cleavage in *bcl-2*-transfected PC12 cells. Mock- and *bcl-2*-transfected PC12 cells were treated with NCS (20 nM; 1h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western Blot analysis. Results of staining with an antibody for Bcl-2 (PharMingen, International) are shown. Mock-transfected PC12 cells: left-most five lanes; *bcl-2*-transfected PC12 cells: right-most five lanes. Note that mock-transfected PC12 cells do not contain detectable Bcl-2.

Figure 2: NCS did not induce Bcl-2 cleavage in *bcl-2*-transfected MCF-7 cells. Mock- and *bcl-2* transfected MCF-7 cells were treated with NCS (20 nM; 1h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western Blot analysis. Results of staining with an antibody for Bcl-2 (PharMingen, International) are shown. (A) neo.1 mock-transfected cells: left-most five lanes; bcl-2.1 *bcl-2*-transfected cells: right-most five lanes. (B) neo.2 mock-transfected cells: left-most five lanes; bcl-2.3 *bcl-2*-transfected cells: right-most five lanes.

Figure 3: Cisplatin did not induce Bcl-2 cleavage in *bcl-2*-transfected PC12 cells. Mock and *bcl-2*-transfected PC12 cells were treated with 10μM cisplatin for 1 h. At various time points, cells were harvested for Western blot analysis for Bcl-2. mock-transfected PC12 cells: left-most five lanes; *bcl-2*-transfected PC12 cells: right-most five lanes.

Figure 4: Caspase 3 expression in PC12 and MCF-7 cells. The lysate of the A431 cell line represents a known positive control. No caspase 3 were detected in any of the 4 different MCF-7 cell transfectants (neo.1; neo.2; bcl-2.1; bcl-2.3). In contrast, the two PC12 transfectants express caspase 3 in comparable amounts.

Figure 5 : The caspase 3 inhibitor, Ac-DEVD-CHO, increased the survival after NCS treatment of *bcl-2*-transfected, but not mock-transfected PC12 cells. Both mock- and *bcl-2*-transfected PC12 cells were incubated with 5μM of Ac-DEVD-CHO for 1 h before NCS treatment. The cells were then washed and incubated with fresh medium on day 0. Cells were counted daily as we have previously described (5,26). Plotted cell counts represent the mean day 7 counts for three high power fields, and are expressed as a percentage of the count on day 0± SEM. (A) mock-transfected PC12 cells; (B) *bcl-2*- transfected PC12 cells.

Figure 6 : NCS treatment is more effective against tumors derived from *bcl-2*-transfected cells than against those derived from mock-transfected cells. Nude mice were xenografted by subcutaneous injection with $10^6$ cells on day –1. NCS was administered as a single intraperitoneal dose on day 0. (A) Tumor footprint was computed daily as the product of the length (longest dimension) and width (perpendicular to length) of each tumor. Results shown are those obtained for day 30. (B) Gross tumor incidence (n = 4 for each cell line at each point) is plotted on day 30.

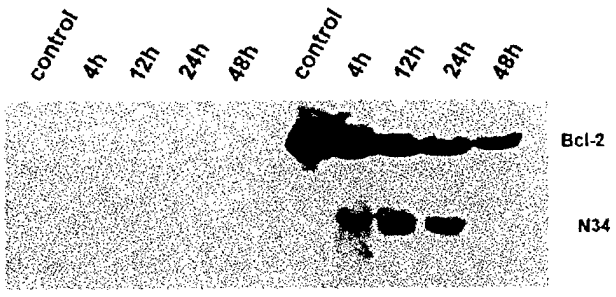



**Figure 1**

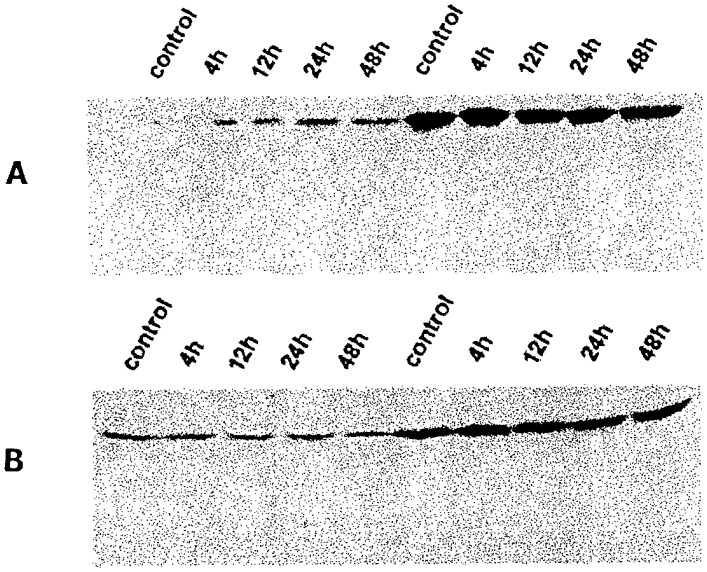

control
4h
12h
24h
48h
control
4h
12h
24h
48h
A
control
4h
12h
24h
48h
control
4h
12h
24h
48h
B

Figure 2

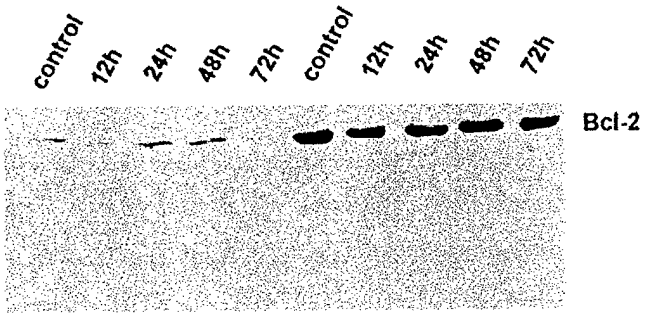



Figure 3

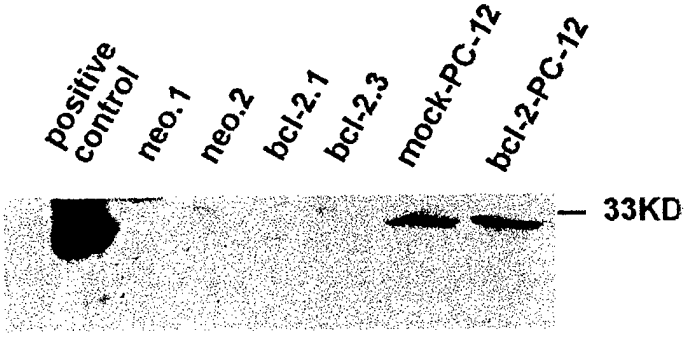
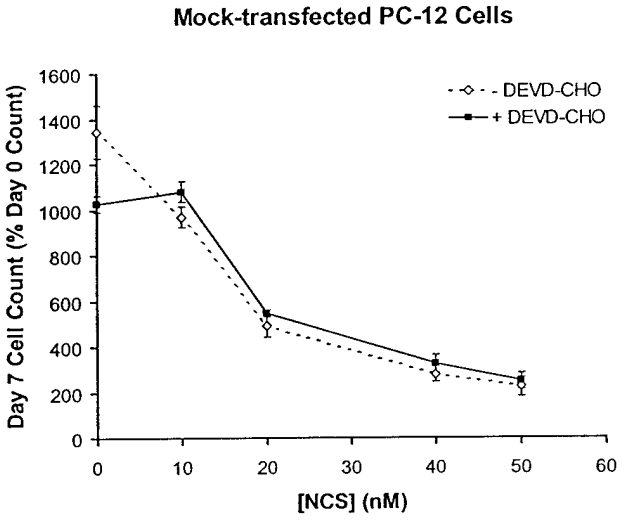

positive control
neo.1
neo.2
bcl-2.1
bcl-2.3
mock-PC-12
bcl-2-PC-12
33KD

Figure 4


Mock-transfected PC-12 Cells
- DEVD-CHO
+ DEVD-CHO
Day 7 Cell Count (% Day 0 Count)
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
[NCS] (nM)

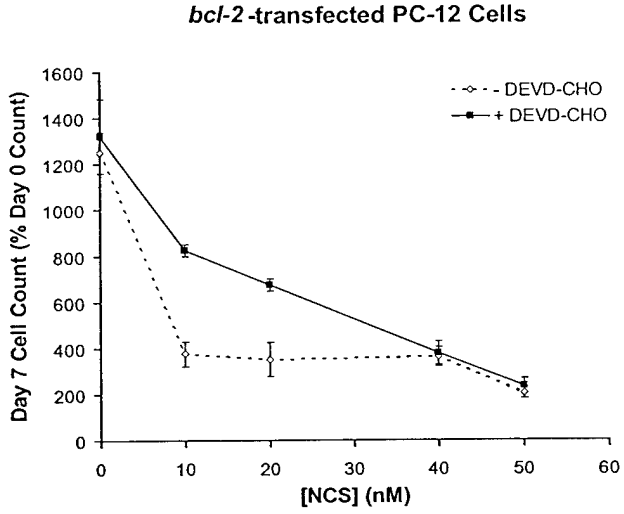
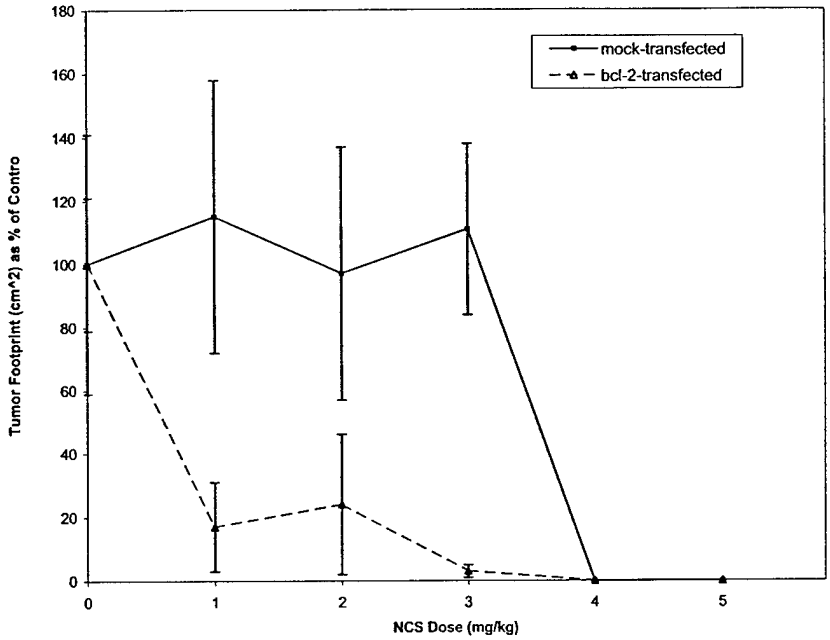
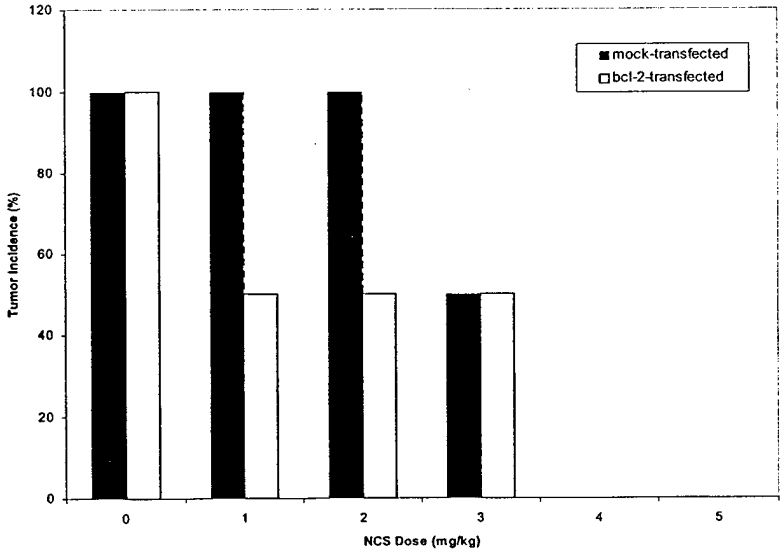

*bcl-2*-transfected PC-12 Cells
- DEVD-CHO
+ DEVD-CHO
Day 7 Cell Count (% Day 0 Count)
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
[NCS] (nM)

Fig 5

A


mock-transfected
bcl-2-transfected
Tumor Footprint (cm^2) as % of Contro
NCS Dose (mg/kg)
0
20
40
60
80
100
120
140
160
180
0
1
2
3
4
5

B


mock-transfected
bcl-2-transfected
Tumor Incidence (%)
NCS Dose (mg/kg)
0
20
40
60
80
100
120
0
1
2
3
4
5

# OXIDATIVE SIGNALING PATHWAY FOR EXTERNALIZATION OF PLASMA MEMBRANE PHOSPHATIDYLSERINE DURING APOPTOSIS

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*Abbreviations:* PS, phosphatidylserine; PE, phosphatidylethanolamine, PC, phosphatidylcholine, PI, phosphatidylinositol, DPG ,diphosphatidylgycerol; SPH ,sphingomyelin; PnA, *cis*-parinaric acid; APT, aminophospholipid translocase; PMC, 2,2,5,7,8-pentamethyl-6-hydroxy-chromane; AMVN, 2,2'-azobis(2,4-dimethylisovaleronitrile t-BuOOH, tert-butylhydroperoxide.

**Abstract**

Active maintenance of membrane phospholipid asymmetry is universal in normal cell membranes and its disruption with subsequent externalization of phosphatidylserine (PS) is a hallmark of apoptosis. Externalized phosphatidylserine appears to serve as an important signal for targeting recognition and elimination of apoptotic cells by macrophages, however, the molecular mechanisms responsible for phosphatidylserine translocation during apoptosis remain unresolved. Studies have focused on the function of aminophospholipid translocase and phospholipid scramblase as mediators of this process. Here we present evidence that unique oxidative events, represented by selective oxidation of phosphatidylserine, occur during apoptosis that could promote phosphatidylserine externalization. We speculate that selective phosphatidylserine oxidation could affect phosphatidylserine recognition by aminophospholipid translocase and/or directly result in enzyme inhibition. The potential interactions between the anionic phospholipid phosphatidylserine and the redox-active cationic protein effector of apoptosis, cytochrome c, are presented as a potential mechanism to account for selective oxidation of phosphatidylserine during apoptosis. Thus, cytochrome c-mediated PS oxidation may represent an important component of the apoptotic pathway.

## Introduction.

The "raison d'etre" for the plethora of different molecular species of phospholipids in membranes is not clearly understood at the molecular level. Neither are cellular functions of individual phospholipid species well described (with perhaps the notable exceptions of phosphoinositol-derived signaling products and precursors of bioactive eicosanoids). Despite that, asymmetric distribution of major phospholipid classes across membranes has been established as a fundamental feature of all cells whose disturbance is incompatible with physiological functions of membranes and with cell viability [1,2]. Although membrane lipid asymmetry has been known for many years, the mechanisms for maintaining or regulating the transbilayer lipid distribution are still not completely understood. Recently, three major enzymatic pathways involved in phospholipid transbilayer asymmetry have been identified: (1) An inward-directed pump, an ATP-dependent transporter specific for aminophospholipids (phosphatidylserine (PS) and phosphatidylethanolamine (PE), known as aminophospholipid translocase or "flippase"; (2) A phospholipid scramblase, which facilitates bi-directional migration of all phospholipid classes independent of the polar headgroup across the bilayer; and (3) An outward-directed pump referred to as "floppase" with little selectivity for the polar headgroup of the phospholipid [3]. The actual participation of the latter in transport of phospholipids has not been firmly established.

The concerted action of aminophospholipid translocase and "floppase" is believed to account for the maintenance of lipid asymmetry in intact cells [3]. The surveillance function of aminophospholipid translocase (APT) provides for the rapid inward translocation of aminophospholipids, while "floppase" may facilitate the transbilayer movement of phospholipids to replace aminophosphospholipids. APT inhibition by itself, however, does not lead to spontaneous redistribution of lipids [4,5]. Conditions of cellular activation characterized by elevated levels of intracellular $Ca^{2+}$ can cause collapse of lipid asymmetry by activation of an ATP-independent scramblase and subsequent bi-directional movement of all phospholipid classes [6]. Thus, both inhibition of the APT and activation of the scramblase are necessary for a collapse of lipid asymmetry, manifested by exposure of PS on the cell surface [3]. The latter phenomenon, known as PS externalization, has been identified as one of early and prominent features of programmed cell death or apoptosis [7,8]. PS exposure on the outer leaflet of the plasma membrane is a surface change common to many apoptotic cells and has several potential biological consequences, one of which is recognition and removal of the apoptotic cell by phagocytes [9]. It is still not clear which receptors mediate PS recognition on apoptotic cells; however, several candidates have been proposed. These include the Class B scavenger and thrombospondin receptor (CD36), an oxidized LDL receptor (CD68), CD14, annexins, $beta_2$ glycoprotein I, gas-6, and a novel protein expressed on macrophages stimulated with digestible particles such as beta-glucan. Whether PS is the sole ligand recognized by phagocytes or whether it is associated with other molecules to form a complex ligand is unknown [10].

If inhibition of aminophospholipid translocase (along with the activation of scramblase) is critical for aminophospholipid externalization, then one would expect that both PS and PE should be exposed on the outer surface of plasma membrane in apoptotic cells. Numerous data, however, indicate that PS, rather than PE, is predominantly externalized on plasma membranes of apoptotic cells. To some extent, this conclusion may be based on the specificity of the assay for PS externalization. A negatively charged PS can specifically bind fluorescently-labeled Annexin V the most commonly used reagent for flow cytometric measurements of apoptosis [11,12]. In other words, Annexin V binding assay ignores any potential contribution of PE to aminophospholipid externalization. High-performance thin-layer chromatography assays of aminophospholipids chemically labeled on the cell surface by non-permeating reagents, however, demonstrated that the degree of PS externalization far exceeds that of PE during apoptosis in different cells [9,13,14]. This suggests that some, as yet unidentified, factor(s) may be responsible for predominant translocation of PS in plasma membranes of apoptotic cells.

**Measurements of peroxidation in different classes of phosphlipids in live cells.**

Oxidative stress is a frequent trigger of apoptosis in a variety of cells and is also thought to be involved as a component of the common pathway in execution of apoptosis [15-21]. While effects of oxidative stress on apoptotic machinery, such as caspases [22,23] have been well characterized, the information on selective oxidation of specific classes of phospholipids is scarce. This is mainly due to the fact that quantitative assays for oxidation of different classes of phospholipids are not readily available. One of the major reasons for this is a very effective system of remodeling and repair of oxidatively modified phosholipids [24] that interferes with their accurate measurement.

In our attempts to characterize phospholipid oxidation during oxidative stress-induced apoptosis we metabolically-labeled cellular phospholipids with a natural oxidation-sensitive and highly fluorescent fatty acid, *cis*-parinaric acid (PnA). This reagent has been extensively used in its free (non-esterified) form for structural measurements in membranes as well as for assays of oxidative stress in simple model systems [25,26]. We recently developed and optimized conditions that yielded cells containing the major phospholipid classes (phosphatidylcholine (PC), PE, PS, phosphatidylinositol (PI), diphosphatidylglycerol (DPG), and sphingomyelin (SPH)) fluorescently labeled with PnA and extremely low intracellular concentration of free PnA [27]. The level of PnA-labeling of endogenous phospholipids (=1-3mol%) was low enough to have minimal effects on cell viability and functions yet sufficient to permit quantitative detection of oxidative stress [27]. Since free PnA was not available for phospholipid repair resolution of major phospholipid classes by fluorescence HPLC can be used to quantify their oxidative damage (as a decreased content of fluorescent PnA-residues in respective phospholipid classes). Importantly, the PnA-based assay can identify the selectivity of phospholipid oxidation based on their polar head groups and it

is obviously independent of the fatty acid composition of phospholipids [27,28]. In addition, the proximity of PnA-labeled phospholipids to the sites of radical generation will also determine the degree of oxidation.

**Non-random oxidation of different classes of phospholipids during oxidative stress-induced apoptosis.**

We have found that apoptosis is associated with selective oxidation of specific phospholipid classes, most notably PS. For example, paraquat - a known inducer of oxidative stress via $CYP_{450}$ reductase-mediated redox-cycling and subsequent production of reactive oxygen species – caused apoptosis in 32D cells as evidenced by characteristic changes in nuclear morphology, DNA fragmentation, and PS externalization revealed by Annexin V binding. When 32D cells containing PnA-labeled phospholipids were exposed to paraquat only two classes of phospholipids, PS and PI, underwent significant peroxidation during 2 hr incubation [13]. Importantly, neither apoptosis nor oxidation of PS and PI was induced by paraquat in 32D cells overexpressing the bcl-2 gene product. These initial experiments posed several questions: (i) whether selective oxidation of PS is typical of apoptotis induced by other agents, (ii) what is the major intracellular compartment where PS is oxidatively modified during apoptosis, (iii) whether PS oxidation is an early or late event in executive pathways of apoptosis, (iv) whether PS oxidation is required for PS externalization (and other apoptotic mechanisms), (v) what may act as an endogenous catalyst for selective PS oxidation during apoptosis?

**Oxidation of PS is selective and preceeds its externalization in cells during apoptosis.**

Our initial experiments with paraquat-treated 32D cells clearly showed that PS oxidation preceded PS externalization as measured by Annexin V binding and DNA fragmentation [13]. Similarly, in keratinocytes exposed to cumene hydroperoxide, selective PS oxidation occurred prior to its detection on the cell surface by either non-permeant amino-reagents or Annexin V and in the absence of DNA laddering [29]. Figure 1 compares the oxidation of PnA labeled PS and PC in intact cells (panel A) and in keratinocyte-derived liposomes (panel B) treated with cumene hydroperoxide. Note the preferential oxidation of PS is observed only in intact living cells and not in the cell-free liposome preparation. In several other cell types such as human leukemia HL-60 cells [18], pheochromocytoma PC12 cells [30], rat cardiomyocytes [31] selective oxidation of PS preceded or coincided with the appearance of very early biomarkers of apoptotic execution pathway – caspase-3 activation and PS externalization. A summary of these and other studies is presented in Table 1.

The fundamental association of PS oxidation with apoptosis was strengthened by experiments in which we used a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxy-chromane (PMC). Here we employed the lipophillic azo-initiator of radicals, 2,2'-azobis(2,4-dimethylisovaleronitrile) (AMVN), to

generate membrane-confined oxidative stress and induce apoptosis in HL-60 cells [18]. As an effective radical scavenger, PMC was able to completely protect all phospholipids against oxidation with the remarkable exception of PS. Furthermore, PMC failed to protect HL-60 cells against apoptosis following AMVN (Table 1). An exclusive resistance of PS to antioxidant protection by PMC implies a unique molecular mechanism responsible for its oxidation during apoptosis.

**Oxidation of PS is predominantly confined to plasma membrane during oxidative stress-induced apoptosis.**

The temporal sequence of PS oxidation and externalization is compatible with a casual link between these two events. If so, PS oxidation should occur within the plasma membrane where PS translocation events during apoptosis are known to occur We performed subcellular fractionation experiments in PnA-labeled cells challenged with tert-butylhydroperoxide (t-BuOOH). We recently documented that t-BuOOH induced apoptosis and prominent PS oxidation in whole cell lipid extracts. Most importantly, we found that plasma membrane PS was the largest source of oxidized PS compared to other organelles such as mitochondria, microsomes, nuclei, and lysosomes [32]. In these cells, we also established that apoptosis resulted in a selective externalization of PS as compared to PE. Thus, time course and intracellular location of PS oxidation support our hypothesis regarding the mechanistic link between PS oxidation and subsequent externalization.

**The hypothetical role of PS oxidation in its externalization.**

There are several potential pathways through which PS oxidation may mediate its externalization. As has been pointed out, maintenance of PS asymmetry is mainly due to the activity of APT. Inhibition of APT and activation of phospholipid scramblase are required for apoptotic PS externalization (see above). Therefore, it is of interest to explore the relationships between PS oxidation and externalization and these enzymatic activities. APT has been reported to be sensitive to oxidative stress [4,41,42]. In particular, oxidation of APT cysteines are targets for reactive oxygen species resulting in amelioration of the enzyme activity. While scramblase is not activated directly by oxidants, influx and elevations of cytosolic $Ca^{2+}$ (that could indirectly follow oxidant challenge [43,44]) is responsible for enzyme activation during apoptosis ([45-47]). The failure of APT to internalize PS may be caused by either direct enzyme inhibition or the enzyme's inability to recognize oxidized PS. In the latter case, oxidized PS has to be located in the outer leaflet of plasma membrane. It is noteworthy that oxidized phospholipids undergo spontaneous "flip-flop" more readily than their non-oxidized counterparts [48]. Thus, the requirement of having oxidized PS on the cell surface does not seem to be unrealistic. In our experiments, however, direct inhibition of APT, as measured by 7-nitro-2,1,3-benzoxadiazol-4-yl-PS (NBD-PS) internalization, occurred at the same time as PS oxidation [14]. This implies that enzyme inhibition itself can play a role in PS externalization.

The activity of the enzyme towards oxidized PS is not known and further studies are necessary to determine the relative affinity of APT for oxidized and non-oxidized PS, as well as the nature of the reactive species responsible for enzyme modification.

It should be also mentioned that PS oxidation may be sufficient but not necessary for APT inhibition and PS externalization during apoptosis. Indeed, we have observed that AMVN-induced apoptosis and PS externalization in HL-60 cells occurred even after exposure to nitric oxide, which completely blocked oxidation of all membrane phospholipids, including PS [14]. APT cysteines, however, are potential targets for S-nitrosylation and enzyme inhibition by NO [49,50]. In fact, we observed decreased content of SH-groups in the molecular weight region corresponding to APT (115 kD) on SDS PAGE gels of HL-60 proteins stained by a fluorescent maleimide thiol reagent [14]. It should be also noted that direct caspase-driven inactivation of APT is not likely. Using different inhibitors of mitochondrial electron transport, Zhuang *et al.* could dissociate PS externalization from caspase activation in THP-1 cells [51]. Similarly, Fadeel *et al.* showed that oxidants generated via NADPH oxidase were essential for PS exposure during neutrophil apoptosis despite their potential to inhibit caspase activity [52]. Recent studies demonstrated that overexpression of scramblase in Raji cells, which exhibit low constitutive expression of this enzyme, by retroviral transduction of phospholipid scramblase or treatment of the cells with interferon-α, failed to confer the capacity to expose PS in response to apoptotic stimuli [53]. However, PS exposure in cells over-expressing scramblase could be reconstituted only in the presence of thiol reactive agents, such as N-ethylmaleimide, disulfiram and diamide [53]. Thus, oxidative and/or nitrosative modification of APT is likely to be critical for PS externalization and subsequent recognition of apoptotic cells by phagocytes. This poses the question as to what potential molecular mechanisms are responsible for PS oxidation/externalization.

**Cytosolic cytochrome c as a catalyst for plasma membrane PS peroxidation during apoptosis.**

It is generally accepted that release of cytochrome c from mitochondria is one of very early events in the development of apoptotic program that is a prerequisite for subsequent engagement of caspase cascades and PS externalization [54-56] (although some workers reported that PS exposure during apoptosis preceded release of cytochrome c and decrease in mitochondrial transmembrane potential [57]). Using green fluorescent protein (GFP)-tagged cytochrome c, Goldstein et al. [58] found that the release of cytochrome-c-GFP *always* precedes exposure of PS and the loss of plasma-membrane integrity - characteristics of apoptotic cells. Moreover, p53 activates the apoptotic machinery through induction of the release of cytochrome c from the mitochondrial intermembrane space [58].

The role of cytochrome c in the initiation of caspase cascades is well established and is presumed to be independent of its redox-acitivity [54-56,59,60]. In fact, cytochrome c containing Zn or Cu

in place of Fe is sufficient for interactions within procaspase protein complex and subsequent caspase activation [61]. However, the redox repercussions of cytochrome c release from mitochondria into cytosol cannot be ignored. Recently, Cai and Jones [21] demonstrated that departure of cytochrome c from electron transport chains in mitochondria is accompanied by a dramatic increase in production of reactive oxygen species. This is in keeping with the antioxidant role for cytochrome c during electron transport demonstrated by Skulachev [62,63]. It is well known, however, that antioxidants may readily be subverted to prooxidants when highly-orchestrated redox conditions in the environment are dysregulated, e.g., during apoptosis. For example, one of the major water-soluble antioxidants, vitamin C (ascorbate) is routinely used for induction of oxidative stress in conjunction with free transition metals (such as Cu or Fe) or with hemoproteins (such as hemoglobin, cytochrome c) [44]. Indeed, cytochrome $c^{2+}$ is approximately 20 times more effective at catalyzing hydroxyl radical production from $H_2O_2$ than is free iron [64].

A series of papers have recently described the molecular nature of the apoptosome, the multimeric molecular complex of Apaf1/cytochrome c/procaspase 9 [65-67]. Cytochrome c appears necessary for the initial binding to Apaf-1, which in the presence of ATP/dATP assembles into a multimeric complex. Once this multimeric complex is formed, the recruitment and cleavage of caspase 3 appear independent of cytochrome c or ATP hydrolysis, however, some cytochrome c can still be found associated with the apoptosome and could serve to further stabilize this structure. The affinity of Apaf-1 for cytochrome c is extremely high ($\approx 10^{11}$ $M^{-1}$) [68] implying that only a few molecules of cytochrome c per cell are necessary for apoptotic execution, especially in light of the amplifying function of the apoptosome. Yet, cytochrome c is fairly abundant and presumably released into cytosol in excess of that needed for Apaf-caspase interaction. In addition, it may not be required for apoptosome function once formed. Therefore, cytochrome c is potentially available for interactions with other molecular targets. It should be kept in mind that cytochrome c is a basic protein (pI 10.3) [69]. As a result of this, it would more readily interact with negatively charged molecules. Outside of mitochondria PS is one of the two (along with PI) negatively charged phospholipids. Physiological localization of PS to the inner leaflet of plasma membranes renders it particularly suitable for interaction with cationic cytochrome c in cytosol. In fact, apocytochrome c/PS interactions have been implicated in the mitochondrial import of cytochrome c [70,71]. Moreover, apocytochrome c has been shown to have a very high affinity for PS-containing vesicles (dissociation constant Kd less than 1 $\mu$M) [72,73]. The initial electrostatic interaction of apocytochrome c with PS is followed by penetration of the protein in between the acyl chains [74]. $^{31}P$ NMR studies demonstrated that conformational changes occur in the protein on binding to PS [75]. Numerous studies [76-78] demonstrated the potential of cytochrome c to catalyze peroxidation of different phospholipids in both liposomes and membranes fragments. Combined these data suggest that cytosolic cytochrome c may be involved in specific interaction with PS located in the cytosolic leaflet of plasma membrane and be responsible for selective PS oxidation during apoptosis. In fact, incorporation of cytochrome c into PnA-labeled HL-60 cells by gentle sonication resulted in preferential oxidation of PS as

compared to other phospholipids [32]. Furthermore, in cell-free model systems, PS proved to be selectively oxidized by a cytochrome c/ascorbate/$H_2O_2$ catalytic system as compared to PC [32]. Figure 2 shows that PS is preferentially oxidized relative to PC when intact c HL-60 cells were loaded with cytochrome c (panel A) and when multilamellar dispersions of PS or PC are incubated with cytochrome c/ascorbate/$H_2O_2$ (panel B).

**Concluding remarks**

Since APT inhibition is a prerequisite for PS externalization during apoptotsis, it is important to understand the mechanisms by which the inhibition occurs. We speculate that PS oxidation acts as a sufficient (if not necessary) component of apoptotic machinery ultimately contributing to PS externalization. Our proposed model for the lipid oxidation signaling pathway in apoptotsis is shown on Scheme 1. A variety of apoptotic stimuli cause mitochondrial permeability transition and release of cytochrome c from mitochondria into cytosol. While a fraction of cytochrome c is transiently involved in interactions with Apaf-1 within the apoptosomal complex to initiate the caspase cascade, non-bound cytochrome c or that released from the apoptosome is free to interact with other membrane and cytosolic targets. Among those, the electrostatic interaction of cationic cytochrome c with anionic phospholipids, such as PS, would be predicted. The extreme juxtapositon of PS fatty acid residues with a redox-active heme-site would favor selective oxidation of PS as opposed to other phospholipids. Oxidized PS then can be externalized spontaneously and/or enzymatically (via scramblase-assisted mechanism). Oxidized PS on the cell surface may be recognized as a signal by macrophage scavenger receptor and serve to direct phagocytosis of apoptotic cells. This may only happen if oxidized PS, in contrast to non-oxidized PS, is not recognized by APT. Alternatively, oxidized PS could be utilized by APT as a substrate but the electrophilicity and high reactivity of the hydroperoxy-group on oxidized PS make it a potential suicidal substrate resulting in enzyme inactivation. Thus, cytochrome c-catalyzed oxidation of PS may represent an important component of the final common pathway associated with signaling and recognition of apoptotic cells and their safe elimination by professional phagocytes.

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Table 1. Comparison of PS oxidation, PS externalization and apoptosis in various cells exposed to oxidants.

| Cell line | Stimuli | Apoptosis | PS Externalization | PS Oxidation | References |
|---|---|---|---|---|---|
| HL-60 | AMVN | + | + | + | [18] |
| HL-60 | AMVN + PMC | + | + | + | [18] |
| HL-60 | AMVN + NO* | + | + | - | [14] |
| HL-60 | t-BuOOH | + | + | + | [32] |
| HL-60 | $H_2O_2$ | + | N.D. | + | [33] |
| HL-60 | Cu-NTA + NO | + | + | + | Liu, *et al.*, (unpublished) |
| | Cu-NTA | + | + | + | [34] Kawai, *et al.* (unpublished) |
| 32D | Paraquat | + | + | + | [13] |
| 32D/bcl-2 | Paraquat | - | - | - | [13] |
| PC12 | Neocarzinostatin | + | + | + | [30] |
| PC12/bcl-2 | Neocarzinostatin | - | - | - | [30] |
| PC12 | AMVN | + | N.D. | + | [35] |
| PC12/bcl-2 | AMVN | - | N.D. | + | [35] |
| PC12 | Glutamate | - | N.D. | - | [36] |
| MCF-7 | AMVN | + | N.D. | + | [37] |
| NHEK | Phenol | - | N.D. | - | [38] |
| NHEK | Hydroperoxide | + | + | + | [29] |
| RCm | Hydroperoxide | + | N.D. | + | [31,39,40] |

HL-60 = human leukemia cells, 32D = mouse hematopoietic progenitor cells, PC12 = rat pheochromacytoma cells, NHEK = normal human epidermal keratinocytes, RCm = rat cardiomyocytes.

N.D. signifies not done.

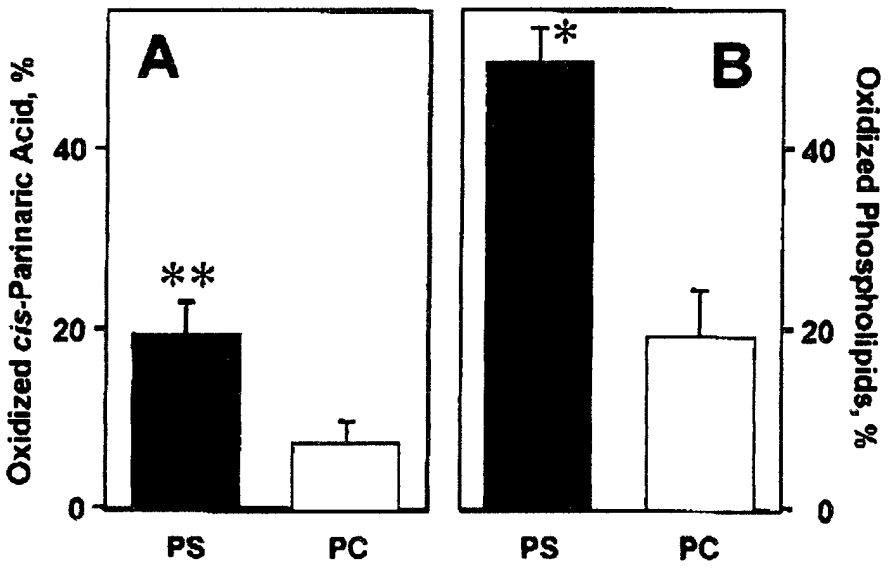
Asterik denotes that APT activity was also inhibited by both AMVN and NO.

## Figure Legends

Figure 1. Cumene hydroperoxide-induced oxidation of PnA-labeled PS and PC in intact live normal human epidermal keratinocytes (NHEK) (Panel A) and cell-free PnA-labeled liposomes derived from NHEK. PnA was incorporated into normal human epidermal keratinocyte phospholipids as described earlier [27]. Intact living PnA-labeled NHEK (Panel A) were exposed to cumene hydroperoxide (200 μM) for 1h at 37°C. PnA-labeled liposomes (Panel B) were prepared from PnA-loaded NHEK as described earlier [38] and similarly treated with 200 μM cumene hydroperoxide at 37°C for 1 h. At the end of the incubations, total lipid were extracted by Folch procedure and resolved by HPLC [27]. PS, phosphatidylserine; PC, phosphatidylcholine. Data represent means ± SEM, *n*=3, *p<0.02.

Figure 2. Cytochrome *c*-induced oxidation of PS and PC in intact living HL-60 cells (A) and liposomes (B). PnA was incorporated into HL-60 cell phospholipid as previously described [27] and then incubated in the presence of cytochrome *c* for 20 min at 37°C Panel A). At the end of this time total lipids were extracted by Folch procedure and resolved by HPLC [27] and analyzed for fluorescent content of PnA. Cytochrome *c* was incorporated into PnA labeled cells by mild sonication. The amount of incorporated cytochrome *c* was estimated spectrophotometrically and estimated as 0.44 ± 0.03 nmol/mg protein. PS, phosphatidylserine; PC, phosphatidylcholine. Data are means ± SEM, n=14, **p<0.04. Multilamellar dispersions of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (PC) or 1-palmitoyl-2-arachidonyl-sn-glycero-3-phospho-L-serine (PS) (2 mg/ml) in 50 mM phosphate buffer pH 7.4, were incubated in the presence of cytochrome *c* (5 μM), ascorbate (500 μM), $H_2O_2$ (400 μM), desferoxamine (100 μM) during 1 h at 37°C (Panel B). At the end of incubation phospholipids were extracted and HP-TLC was performed [27]. Oxidized phospholipid products could be resolved as a distinct "tailing" following the native unoxidized lipid spot. The percent of oxidation was estimated by determining the phosphorus content of the tail relative to the total lipid phosphorus available. Data are means ± SEM, n=3, *p<0.02.

Scheme 1: Proposed mechanism for oxidative signaling of PS externalization during apoptosis. The left side of scheme illustrates the common pathways of apoptotic program via mitochondrial permeability transition, cytochrome c release, apoptosome formation, and caspase activation. This leads to a number of apoptotic endpoints including nuclear fragmentation and PS externalization. The right side details our proposed model by which redox-active and positively-charge cytochrome c interacts with negatively-charged PS on the cytosolic side of plasma membrane. Cytochrome c catalyzed reactive oxygen species attack PS to form its hydroperoxide. Oxidatively-modified PS then undergoes spontaneous and/or scramblase-assisted externalization. The surveillance function of APT then is disrupted either by direct inhibition of enzyme activity by reactive electrophilic PS oxidation products or failure to recognize the oxidatively-modified PS. Recognition and phagocytosis of apoptotic cells is then facilitated by interaction of PS with the macrophage scavenger receptor, which may preferentially bind oxidized PS.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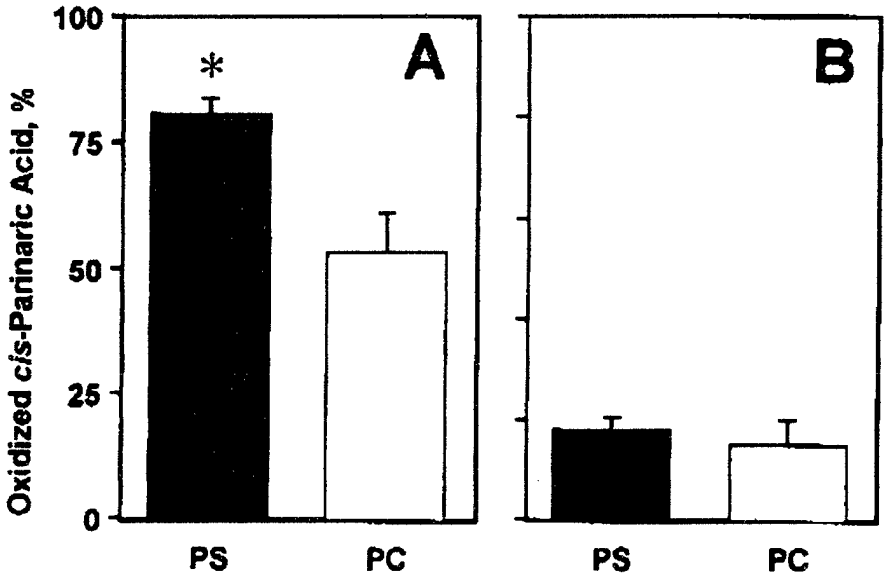
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