Random versus Selective Membrane Phospholipid Oxidation in Apoptosis: Role of Phosphatidylserine†

James P. Fabisiak,*,‡,§ Yulia Y. Tyurina,‡,|| Vladimir A. Tyurin,‡,|| John S. Lazo,§ and Valerian E. Kagan‡,§

Department of Environmental and Occupational Health, School of Public Health, RIDC Park, 260 Kappa Drive, University of Pittsburgh, Pittsburgh, Pennsylvania 15238, and Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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ABSTRACT: The formation of reactive oxygen species has been associated with apoptosis. To assess the role of lipid peroxidation in apoptosis, we used 2,2′-azobis(2,4-dimethylisovaleronitrile) (AMVN) to generate peroxyl radicals within cellular membranes of HL-60 cells. *cis*-Parinaric acid (*cis*-PnA) metabolically integrated into phospholipids of HL-60 cells was used as a probe to assess the extent of lipid peroxidation within specific phospholipid classes. Within 2 h, AMVN (500 μM) randomly oxidized more than 85% of *cis*-PnA contained in all major classes of phospholipids. AMVN-induced lipid peroxidation was followed by apoptosis as determined by nuclear condensation, DNA fragmentation, and annexin V binding to externalized phosphatidylserine (PS). Fluorescamine derivatization of external aminophospholipids revealed that PS, but not phosphatidylethanolamine, was externalized. The vitamin E analogue, 6-hydroxy-2,2,5,7,8-pentamethylchromane (PMC), inhibited overall oxidation of *cis*-PnA in phospholipids by more than 85%. Not all phospholipids, however, were equally protected. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin were nearly completely protected by PMC, while oxidation of PS was unaffected in whole living cells. The insensitivity of PS to PMC was not an intrinsic property because PMC protected all lipids equally during AMVN oxidation of liposomes prepared from *cis*-PnA-labeled cells. The potential role for PS oxidation in apoptosis was further suggested by the faithful execution of apoptosis following coexposure of cells to AMVN and PMC.

Apoptosis or programmed cell death arises from the active initiation and propagation of a series of precise biochemical events that result in the elimination of specific cells when they are damaged or no longer functionally required. Apoptosis is characterized morphologically by cell shrinkage, membrane surface blebbing, nuclear condensation and fragmentation, and eventual dissolution of the cell into a series of membrane-bound fragments termed apoptotic bodies (*1*). Biochemically, these features are associated with activation of specific proteases termed caspases (*2*), DNA fragmentation (*3*), and loss of membrane phospholipid asymmetry with resultant phosphatidylserine (PS)[1] externalization (*4*).

Intracellular oxidation is an attractive candidate mediator for apoptosis because it is produced by stimuli associated with the initiation of apoptosis and can generate irreparable damage (*5*−*7*). Intracellular oxidation, however, occurs in multiple aqueous and hydrophobic cellular compartments, which makes tracking an oxidant signaling pathway complex because there are many substrates for oxidation. Furthermore, oxidation could be the result rather than an essential mediator of apoptosis.

One attractive target for oxidative signaling in apoptosis is lipid peroxidation, in part, because polyunsaturated membrane phospholipids are extremely sensitive to oxidant attack. Indeed, a number of studies have suggested a general connection between lipid peroxidation and apoptosis (*7*−*9*). Selective outward translocation of a specific phospholipid, namely, phosphatidylserine (PS), within the plasma membrane accompanies apoptosis and may be critical for recognition and phagocytosis of apoptotic cells by macrophages (*10*, *11*). We have recently demonstrated that selective oxidation of PS preceded its translocation and apoptosis following exposure of 32D cells to the prototypic oxidant paraquat (*12*, *13*). It is not known, however, whether selective PS oxidation is critical for other agents and in other cells, and whether oxidation of other phospholipids is important. To probe this, we induced random oxidation of multiple phospholipid classes in HL-60 cells using the lipid-soluble azo initiator 2,2′-azobis(2,4-dimethylisovaleronitrile) (AMVN) that generates carbon-centered and peroxyl radicals (*14*) localized primarily within the lipid membrane environment (*15*). The isoprenoid-depleted vitamin E homologue (*16*), 6-hydroxy-2,2,5,7,8-pentamethylchromane (PMC), sup-

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* To whom correspondence should be addressed.

‡ School of Public Health, University of Pittsburgh.

§ School of Medicine, University of Pittsburgh.

|| On leave from Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Science, St. Petersburg, Russia 194223.

[1] Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylisovaleronitrile); *cis*-PnA, *cis*-parinaric acid; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; DPG, diphosphatidylglycerol; lyso-PC, lysophosphatidylglycerol; NL, neutral lipid; FFA, free fatty acids; HPLC, high-pressure liquid chromatography; HP-TLC, high-performance thin-layer chromatography; PMC, 6-hydroxy-2,2,5,7,8-pentamethylchromane; P_i, inorganic phosphorous.

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pressed peroxidation of all measured phospholipids except PS. Furthermore, both AMVN-induced apoptosis and PS externalization were resistant to PMC protection.

MATERIALS AND METHODS

Materials. All tissue culture media, additives, and antibiotics were obtained from GIBCO BRL (Gaithersburg, MD) with the exception of fetal bovine serum (FBS), which was from HyClone (Logan, UT). Proteinase K, ribonuclease (RNase) T1, and RNase A were from Boehringer Mannheim (Indianapolis, IN). *cis*-Parinaric acid (*cis*-PnA) [(9*Z*,11*E*,-13*E*,15*Z*)-octadecatetraenoic acid] was obtained from Molecular Probes (Eugene, OR). HPLC grade solvents were from Fisher Scientific (Pittsburgh, PA). 2,2′-Azobis(2,2-dimethylvaleronitrile) (AMVN) and 6-hydroxy-2,2,5,7,8-pentamethylchromane (PMC) were supplied by Wako Chemicals (Richmond, VA) and Eisai Co. (Tokyo, Japan), respectively. Fluorescamine was obtained from Sigma (St. Louis, MO). All other chemicals and reagents were molecular biology grade.

Cell Culture and Treatments. Stock HL-60 cells were routinely split at 3−4 day intervals and cultured in RPMI 1640 containing 15% fetal bovine serum (FBS) and supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), glutamine (2 mM), and Fungizone (1.25 μg/mL). For experiments, cells obtained from stock cultures were counted, centrifuged, and resuspended in the indicated media at a density of 1×10^6 cells/mL. Apoptosis experiments were carried out in serum-free RPMI or serum-free Leibovitz's L-15 medium (without phenol red) adjusted to pH 7.4 as indicated. L-15 medium was supplemented with glucose to a 2 mg/mL final concentration to match that present in RPMI 1640. The extent and time course of apoptosis following AMVN appeared to be similar for these two media. AMVN was applied at the indicated concentrations from a 100 mM stock prepared in DMSO. PMC stock solution (10 mM) was also made in DMSO. To assess the protective effects of vitamin E analogue, we applied PMC to cells 15 min prior to addition of AMVN.

Lipid Peroxidation. These experiments exploited the ability of cells to metabolically incorporate the fluorescent, oxidant-sensitive fatty acid, *cis*-parinaric acid (*cis*-PnA), into total cellular phospholipids (*17*) to measure lipid peroxidation in selected phospholipids of live cells. We have previously described the details of this assay (*12*, *13*, *17*). Briefly, HL-60 cells were labeled with *cis*-PnA (2 μg/mL final concentration) in 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose, and 25 mM HEPES (pH 7.4) at a density of 1×10^6 cells/mL. *cis*-PnA-labeled HL-60 cells were washed and resuspended in serum-free buffer at 1×10^6 cells/mL. Aliquots were taken for phospholipid analyses representing the amount of *cis*-PnA incorporated immediately after labeling. Cells were then treated with AMVN and/or PMC at the indicated concentrations. Cells were preincubated for 15 min with or without PMC prior to addition of AMVN. Cells were incubated for 2 h at 37 °C, centrifuged, and lysed in 0.5 mL of ice-cold methanol containing butylated hydroxytoluene (0.1 mg). Total lipids were immediately separated by high-performance liquid chromatography (HPLC) as previously described (*12*, *17*). The amount of *cis*-PnA fluorescence in individual phospholipid classes was normalized to the amount of inorganic phosphorus (P_i) contained in the total lipid extract (relative PnA oxidation), as well as the P_i content of the individual phospholipid class (specific PnA oxidation) determined from parallel HP-TLC plates (see below). The P_i content was determined spectrophotometrically using the method of Chalvardjian and Rubnicki (*18*).

Preparation of cis-PnA-Labeled Liposomes. *cis*-PnA-labeled phospholipids were isolated from HL-60 cells prelabeled with *cis*-PnA as described. Lipids were extracted by the Folch procedure (*19*) and dissolved in toluene; the mixture was evaporated, and the cells were resuspended in incubation buffer [115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose, and 25 mM HEPES (pH 7.4)] to achieve a concentration of lipids identical to that used in the above-mentioned cellular experiments. The resulting suspension was sonicated (four 15 s pulses on ice) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer-Instrument Co., Chicago, IL). *cis*-PnA-labeled liposomes were incubated for 2 h in the presence or absence of AMVN and/or PMC at 37 °C in the dark. Oxidation in individual phospholipids was then analyzed as described above.

Apoptosis. Nuclear morphology was assessed using Hoescht 33342 fluorescent staining as previously described (*12*, *13*). The percentage of apoptosis was determined by counting at least 300 cells scored as either normal or apoptotic. The extent of low-molecular weight DNA fragmentation was determined using conventional gel electrophoresis as described previously (*12*, *13*). Briefly, aliquots of 1×10^6 cells were collected at various times after treatment by centrifugation at 400*g* for 10 min. Washed cell pellets were lysed and digested overnight at 50 °C with proteinase K (1 mg/mL final concentration). Samples were incubated with 10 μg of RNase A and 200 units of RNase T1 at 37 °C for 1 h. Samples were electrophoresed in 2% agarose gels (60 V) for approximately 4 h. Gels were then stained with ethidium bromide (1 μg/mL) and evaluated under UV illumination.

Annexin V Binding. Annexin V binding to cells was measured using flow cytometry essentially as previously described (*12*) with a commercially available staining kit (R & D Systems, Minneapolis, MN). Briefly, HL-60 cells were placed in serum-free RPMI 1640 (1×10^6 cells/mL), and then the mixture was treated with or without AMVN. Cells (7.5×10^5) were recovered at the indicated times and washed twice in ice-cold PBS. Cells were incubated with the annexin V−fluorescein conjugate (1 μg/mL final concentration) and propidium iodide (5 μg/mL) for 20 min at room temperature. Cells were analyzed with a FACSTAR flow cytometer (Becton-Dickenson) with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V−fluorescein and red fluorescence (585 nm, 42 nm band-pass filter) associated with propidium iodide. Quadrant limits were set to be 40-fold greater than the mode red and green fluorescence of the control untreated cell population.

Fluorescamine Labeling of Externalized Aminophospholipids. Labeling of cells with fluorescamine was achieved according to a modification of the method described by Fadok et al. (*11*). HL-60 cells (4×10^7) were suspended in 2 mL of fluorescamine labeling buffer [150 mM NaCl, 5

Table 1: Effect of 25 μM PMC on the Relative Extent of *cis*-PnA Oxidation in Various Phospholipids in Live Cells after AMVN Treatment[a]

	control			25 μM PMC			
	without AMVN	with AMVN	*cis*-PnA oxidized	without AMVN	with AMVN	*cis*-PnA oxidized	% of the control
PC	560.6 ± 84.1	77.2 ± 7.6	483.4 ± 84.4	550.0 ± 74.8	500.0 ± 24.7	50.0 ± 78.8	10.3
PEA	115.6 ± 10.6	14.5 ± 2.6	101.1 ± 10.9	116.0 ± 9.1	101.7 ± 8.2	14.3 ± 12.2	14.1
PI	33.3 ± 5.0	3.7 ± 0.6	29.6 ± 5.0	29.2 ± 7.0	24.4 ± 1.7	4.8 ± 7.2	16.2
PS	17.1 ± 1.4	4.6 ± 0.6	12.5 ± 1.5	28.2 ± 9.2	12.5 ± 1.8	15.7 ± 9.4	125.6
SPH	1.9 ± 1.0	0.2 ± 0.1	1.7 ± 1.0	1.9 ± 0.7	1.9 ± 0.3	0	0
total			628.3			84.8	13.5

[a] Data expressed as relative *cis*-PnA content (nanograms per microgram of total phospholipid P_i).

mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 5 mM glucose, and 20 mM HEPES (pH 8.0)]. Fluorescamine was dissolved in DMSO and added to cells (200 μM final concentration) and the mixture gently shaken for 15 s at room temperature. The final DMSO concentration was 0.05%. Three milliliters of 40 mM Tris-HCl (pH 7.4) was then added. Cells were centrifuged (1000*g* for 10 min) and resuspended in 2 mL of 40 mM Tris-HCl (pH 7.4) followed by lipid extraction in the dark (*19*).

Lipids were analyzed by two-dimensional HP-TLC using a solvent system of chloroform/methanol/28% ammonium hydroxide (65:35:5, v/v/v) in the first direction and chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5, v/v/v/v/v) in the second. The location of each of the phospholipids was confirmed by comparison to authentic standards (Avanti Polar Lipids, Alabaster, AL). Fluorescamine-derivatized PS and PEA standards were prepared by incubating each aminophospholipid with an excess of fluorescamine in chloroform/triethylamine (40:0.2, v/v). Individual phospholipids were scraped off the plate, and the total phosphorus amount was determined. Phosphatidylcholine was used as an internal standard. Results were expressed as the ratio of derivatized to underivatized aminophospholipid, based of total phosphorus analysis.

RESULTS

AMVN Induced cis-PnA Oxidation in HL-60 Cells. HL-60 cells readily incorporated *cis*-PnA into the major phospholipid classes. Figure 1 shows the specific *cis*-PnA content in five major phospholipid classes, namely, phosphatidylcholine (PC), phosphatidylethanolamine (PEA), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPH) after a 2 h labeling and removal of free *cis*-PnA, followed by a 2 h control incubation. The specific content of *cis*-PnA normalized to the amount of P_i in each phospholipid ranged from 1108 ng/μg of lipid P_i for PC to 20 ng/μg of lipid P_i for SPH. This corresponds to labeling of 12.5 mol % of PC and 0.22 mol % of SPH. Hence, only a fraction of each class of phospholipid becomes labeled with *cis*-PnA (see also Table 2). Differences in the extent of incorporation of *cis*-PnA into each phospholipid class most likely reflect the metabolic turnover rate of each phospholipid, as well as the overall composition of endogenous saturated and polyunsaturated fatty acids within individual phospholipid classes. Table 1 shows the *cis*-PnA content of control cells normalized relative to the amount of total lipid phosphorus and indicates that the majority of *cis*-PnA incorporated into cells was found in PC (77%) and PEA (16%), reflecting the overall abundance of these two phospholipids within cells.

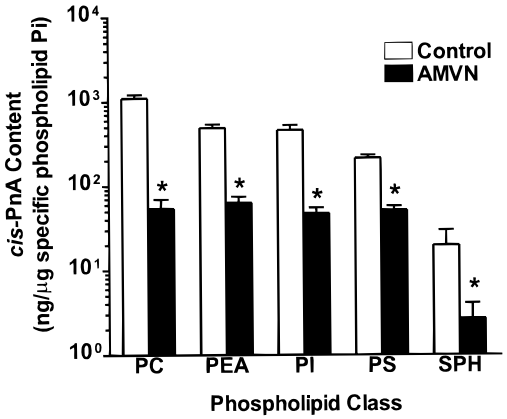

FIGURE 1: AMVN induced lipid peroxidation in all major phospholipid classes. Phospholipids of HL-60 cells were metabolically labeled with *cis*-parinaric acid (*cis*-PnA) at 37 °C for 2 h as described in Materials and Methods. Cells were then exposed to AMVN (500 μM) or left untreated for 2 h, after which phospholipids were extracted and resolved by HPLC. The unoxidized *cis*-PnA content of each phospholipid was quantified using fluorescence detection and normalized to the inorganic phosphate content of each individual phospholipid on the basis of the percentage of total phospholipid P_i determined from parallel HP-TLC plates. Data represent the mean ± standard deviation (SD) of four observations. The asterisks denote a significant difference compared to the untreated control by the Student's *t* test ($p < 0.0.1$). Note the log scale of the *y*-axis and the dramatic loss of *cis*-PnA fluorescence in all phospholipid classes reflecting rapid and extensive lipid peroxidation following AMVN exposure.

Table 2: Phospholipid Composition of HL-60 Cells after *cis*-PnA Labeling and the Effect of AMVN Treatment[a]

	% of total phospholipid		*cis*-PnA content (mol of
phospholipid	control	AMVN	PnA/mol of phospholipid)
PC	49.6 ± 2.5	50.0 ± 2.8	1:8
PEA	28.9 ± 1.6	28.2 ± 1.9	1:18
PS	6.3 ± 1.2	5.8 ± 1.1	1:19
PI	6.4 ± 1.1	6.1 ± 0.9	1:41
SPH	6.2 ± 0.8	6.2 ± 1.0	1:444
DPG	2.0 ± 0.9	2.4 ± 0.8	ND[b]
LPC	0.6 ± 0.5	1.3 ± 0.5	ND

[a] Data are expressed as the mean ± SEM of four observations. [b] ND means it contained too little *cis*-PnA to accurately calculate.

AMVN was chosen as an inducer of lipid peroxidation because of its unique ability to generate both carbon-centered and peroxyl radicals exclusively within the hydrophobic environment of membranes (*15*). Although 500 μM AMVN extensively oxidized *cis*-PnA in all HL-60 phospholipid classes, Table 2 shows that AMVN treatment did not alter the phospholipid composition of HL-60 cells. The rank order of abundance for phospholipids was PC (50%), PEA (28%), PS (6%), PI (6%), and SPH (6%). Diphosphatidylglycerol

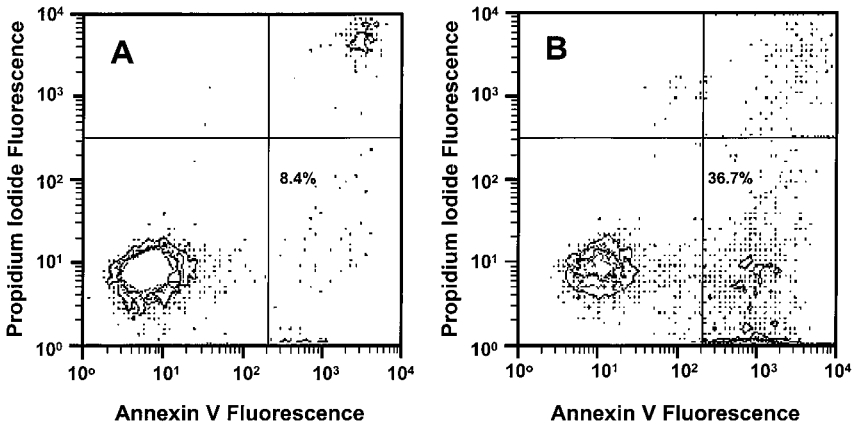

FIGURE 2: AMVN induced annexin V binding measured by flow cytometry in HL-60 cells. HL-60 cells were incubated in serum-free RPMI with or without AMVN (500 μM) at 37 °C for 5 h. The extent of annexin V binding and propidium iodide uptake were measured using flow cytometry as described in Materials and Methods. Plots show the occurrence of events as a function of both propidium iodide (*y*-axis) and annexin V−fluorescein (*x*-axis) fluorescence intensity. Panel A shows data for control untreated cells and panel B data for cells treated with AMVN. Accumulation of cells into the bottom right quadrant (control, 8.4%; and AMVN, 36.7%) represents enhanced appearance of PS on the cell surface in cells that maintain membrane integrity.

(DPG) and lyso-PC (LPC) comprised very little of the total phospholipids, and the *cis*-PnA content could not be reliably measured in these classes. Thus, *cis*-PnA-labeled phospholipids represented only a relatively minor fraction of the total phospholipid pool in HL-60 cells (approximately 8% of total phospholipids were labeled with *cis*-PnA), making it a highly useful, minimally invasive, probe for lipid oxidation. Figure 1 shows that AMVN induced pronounced oxidation in all phospholipid classes that produced loss of *cis*-PnA fluorescence ranging from 95% in PC to 75% in PS. No selective oxidation in any particular class of phospholipid was observed; the extent of *cis*-PnA loss was roughly proportional to its content in a phospholipid class.

AMVN Induced Apoptosis in HL-60 Cells. We next tested the hypothesis that lipid peroxidation could serve as a stimulus for programmed cell death by measuring multiple markers of apoptosis following AMVN treatment, including PS externalization, which can be specifically determined by annexin V binding. It is important to point out that, in contrast to our assays of the extent of total cell lipid peroxidation, measurements of the extent of PS externalization by annexin binding and fluorescamine derivatization are specific for changes in the plasma membrane pool of PS.

Figure 2 compares representative flow cytometry plots obtained from control HL-60 cells and cells treated with 500 μM AMVN for 5 h. AMVN exposure produced significant accumulation of HL-60 cells in the bottom right quadrant, which represents cells possessing intact cellular membranes and enhanced annexin V binding to externalized PS. Control cells incubated in media alone contained less than 10% annexin V positive/propidium iodide negative cells, whereas nearly 40% of the cells in this experiment expressed externalized PS after AMVN treatment. AMVN-induced PS externalization was time-dependent (Figure 3). While the percentage of annexin V positive/propidium iodide negative cells was not significantly different from that of control cells after treatment with AMVN for 1.5 h, the proportions of these cells were elevated at 3 (21.8 ± 5.8%) and 5 h post-treatment (27.8 ± 6.4%).

Annexin V characterizes the fraction of cells expressing externalized PS; however, it provides no information regarding the amount of externalized PS. To quantify the amount

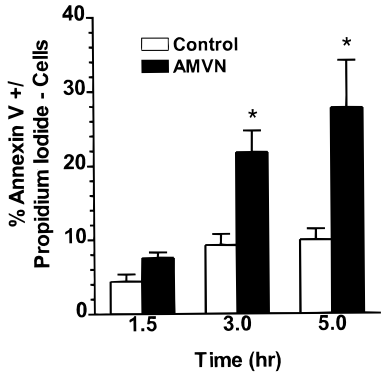

FIGURE 3: Time course of annexin V binding following AMVN. HL-60 cells were incubated in serum-free RPMI with or without AMVN (500 μM) at 37 °C. At the indicated times, cells were assessed for annexin V binding by flow cytometry as described in Materials and Methods either by nuclear morphology or by Hoescht 33342 fluorescent microscopy. The time course for the induction of PS externalization is shown [mean ± standard error of the mean (SEM), $n = 4$] represented by the percentage of annexin V positive/propidium iodide negative cells. The asterisks denote a significant difference ($p < 0.05$) for the untreated control at the same time point determined by the Student's paired *t* test.

of externalized PS and to determine if PEA was similarly translocated, we performed chemical modification of both aminophospholipids by fluorescamine, a cell-impermeable fluorescent reagent capable of reacting with primary amines (*20*). Figure 4 shows representative HP-TLC plates of phospholipids extracted from control untreated HL-60 cells (panel A) and cells treated with AMVN (panel B) following treatment of cells with fluorescamine. PS and PEA modified by fluorescamine (mPS and mPEA) migrated as distinct spots and were confirmed using detection of UV fluorescence and comigration with purified standards. The amounts of mPS and mPEA were then quantified relative to the total PS or PEA amount with determination of P_i content in individual spots after scraping. Figure 5 shows the percentage of each aminophospholipid available for derivatization by fluorescamine after a 2 h treatment with AMVN. In control untreated cells, very little PS was exposed on the cell surface (2.3%) and AMVN produced a 6-fold increase in the amount of PS available to fluorescamine (Figure 5A). The amount of PEA on the surface of control cells was greater compared to that

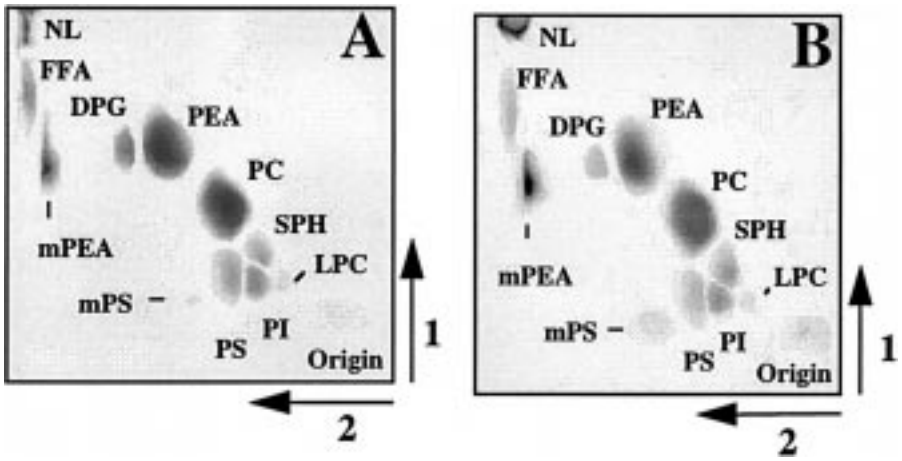

FIGURE 4: Representative HP-TLC plates of fluorescamine-modified and unmodified phospholipids from control and AMVN-treated HL-60 cells. Following AMVN treatment, cells were reacted with fluorescamine and lipids extracted as described in Materials and Methods. The location of individual species was verified using purified lipid standards. Lipids were as follows: FFA, free fatty acids; NL, neutral lipids; DPG, diphosphatidylglycerol; PEA, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; and LPC, lysophosphatidylcholine. mPEA and mPS represent PS and PEA that have been derivatized by fluorescamine, respectively.

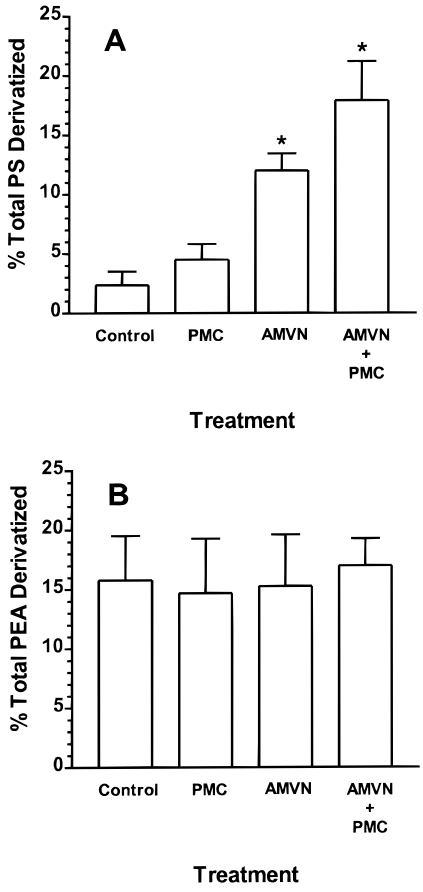

FIGURE 5: Comparison of the PS and PEA accessible to fluorescamine following AMVN and PMC treatment. HL-60 cells were treated with AMVN and/or PMC and then reacted with fluorescamine as described in Materials and Methods. The percentage of total PS and PEA modified by fluorescamine was determined by phosphorus analysis of each phospholipid spot after two-dimensional HP-TLC. Data represent the mean ± SEM for between four and six samples for each group. The asterisks denote a statistically significant difference compared to untreated cells by one-way ANOVA and Dunnett's multiple comparisons to control ($p < 0.01$).

of PS, and no significant changes were observed in PEA following AMVN or PMC treatment (Figure 5B).

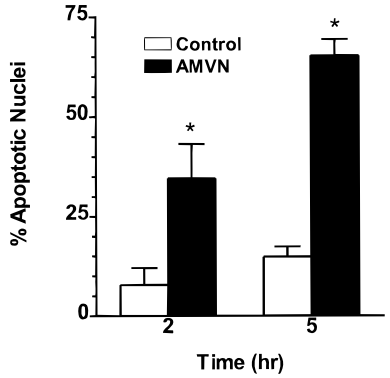

FIGURE 6: AMVN induced the formation of apoptotic nuclei in HL-60 cells. Cells were incubated in serum-free RPMI in the presence or absence of AMVN (500 μM) for the indicated times. Cells were then stained using Hoescht 33342 and nuclei visualized as described in Materials and Methods. The percentage of cells with apoptotic nuclei characterized by chromatin condensation and fragmentation was determined in at least 300 cells/sample. Data represent the mean ± SEM of five individual experiments. The asterisks denote a significant difference compared to the untreated control by the Student's *t* test.

Chromatin condensation and fragmentation is another established biomarker of apoptosis. Figure 6 shows that AMVN-treated HL-60 cells acquired a nuclear phenotype characterized by chromatin condensation and fragmentation into discrete fluorescent bodies typical of apoptosis. This nuclear morphology was observed in 35% of the cells as early as 2 h following AMVN and increased to more than 60% by 5 h post-treatment. Furthermore, Figure 7 shows that treatment with 500 μM AMVN for 2 h also induces internucleosomal DNA cleavage (180−200 bp "ladders"), which is considered a hallmark of apoptosis.

PS Oxidation Was Resistant to PMC Antioxidant Action. To test the relative importance of lipid peroxidation for apoptosis after exposure to the lipid-soluble azo initiator of peroxyl radicals, AMVN, we next used an antioxidant, PMC, a homologue of vitamin E, the major lipid-soluble antioxidant of membranes (*21*). We examined the relative extent of *cis*-PnA oxidation induced by AMVN in PC, PEA, PI, PS, and SPH in the absence and presence of 25 μM PMC (Table 1). PMC alone had little or no effect on the amount of

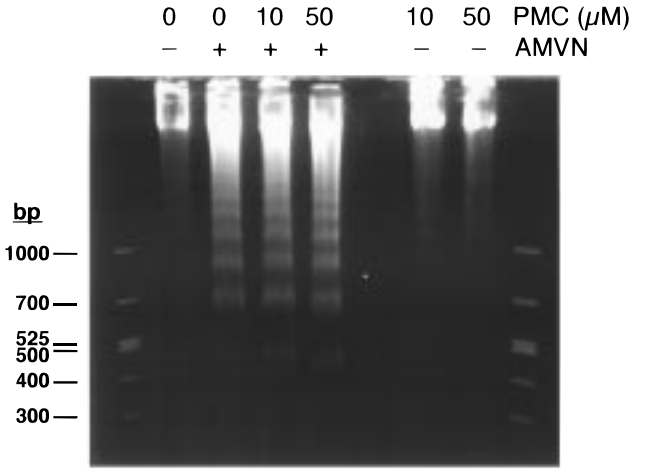

FIGURE 7: Internucleosomal DNA fragmentation following AMVN and PMC treatment. HL-60 cells were treated like those described in Figure 6. DNA was extracted from 1×10^6 cells 2 h after exposure to AMVN (500 μM) and/or PMC (10 or 50 μM) and electrophoresed in a 2% agarose gel. The formation of DNA cleavage corresponding to internucleosomal fragments (180−200 bp ladders) was clearly observed following AMVN treatment. AMVN-induced DNA ladders were unaffected in the presence of 10 or 50 μM PMC. PMC alone did not produce internucleosomal DNA fragmentation.

unoxidized *cis*-PnA contained in each phospholipid at the end of the 2 h control incubation in the absence of AMVN. However, the inclusion of PMC during AMVN treatment greatly attenuated the loss of *cis*-PnA fluorescence in PC, PEA, and PI where the amount of *cis*-PnA oxidized was reduced to between 10.3 and 16.2% of that seen following AMVN treatment alone. Sphingomyelin appeared to be totally protected from AMVN-induced oxidation by inclusion of PMC. Because of the abundance of PC, PEA, and PI, the overall antioxidant action of PMC was significant with more than 85% of the total AMVN-induced lipid peroxidation blocked. Most notable, however, was the significant resistance of PS oxidation to protection by PMC. The absolute amount of *cis*-PnA oxidized by AMVN in PS was 12.5 ± 1.5 ng/mg of total lipid P_i as compared to 15.7 ± 9.4 in the presence of PMC (25 μM).

Figure 8 compares the relative rates of *cis*-PnA oxidation in individual phospholipid classes at various concentrations of PMC. Note that essentially complete protection of PC, PE, PI, and SPH was observed at PMC concentrations from 25 to 75 μM and that each of these four phospholipids showed similar concentration−response relationships for protection by PMC. In contrast, PS oxidation following AMVN remained completely unaffected in the presence of PMC up to 75 μM.

To compare the intrinsic ability of PMC to protect PS against AMVN oxidation, we prepared liposomes using *cis*-PnA-labeled phospholipids extracted from HL-60 cells prelabeled with *cis*-PnA. *cis*-PnA liposomes were incubated with 500 μM AMVN in the presence and absence of PMC at various concentrations. Figure 9 illustrates the fact that AMVN produced substantial oxidation of *cis*-PnA in all phospholipids, similar to that seen in intact live cells. In this cell-free system, however, PMC is fully able to protect all *cis*-PnA-labeled phospholipids, including PS. A small statistically significant degree of oxidation remained in PEA in the presence of 10 μM PMC, but was not observed when

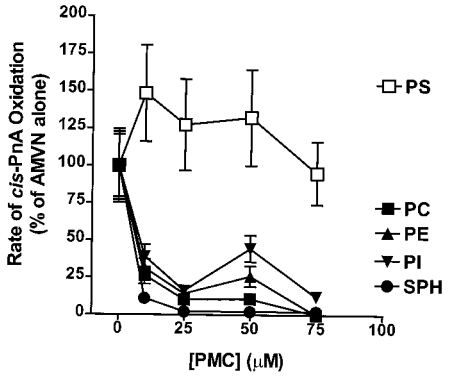

FIGURE 8: AMVN-induced PS oxidation in cells was selectively resistant to PMC. HL-60 cells were metabolically labeled with *cis*-PnA as described in Materials and Methods. Cells were then preincubated with various concentrations of 6-hydroxy-2,2,5,7,8-pentamethylchromane (PMC), a short chain analogue of α-tocopherol, for 15 min followed by exposure to 500 μM AMVN for 2 h. Control cells were pre-exposed to PMC alone and incubated in the absence of AMVN. Phospholipids were then extracted and subjected to HPLC as described in Materials and Methods for quantification of the amount of fluorescent *cis*-PnA. The rate of AMVN-induced oxidation of *cis*-PnA was taken as the difference between the rates of AMVN-treated and control cells at any given concentration of PMC. Data are expressed as the rate of *cis*-PnA oxidation in a specific phospholipid class relative to the rate observed in the absence of PMC. Absolute rates of AMVN-induced *cis*-PnA oxidation for each phospholipid class in the absence of PMC were as follows: PC, 242 ng (μg of P_i)$^{-1}$ h^{-1}; PE, 51 ng (μg of P_i)$^{-1}$ h^{-1}; PI, 15 ng (μg of P_i)$^{-1}$ h^{-1}; PS, 6.2 ng (μg of P_i)$^{-1}$ h^{-1}; and SPH, 1 ng (μg of P_i)$^{-1}$ h^{-1}. Note the marked protection of all major phospholipids except PS by PMC. Data represent the mean ± SD of four observations per time point.

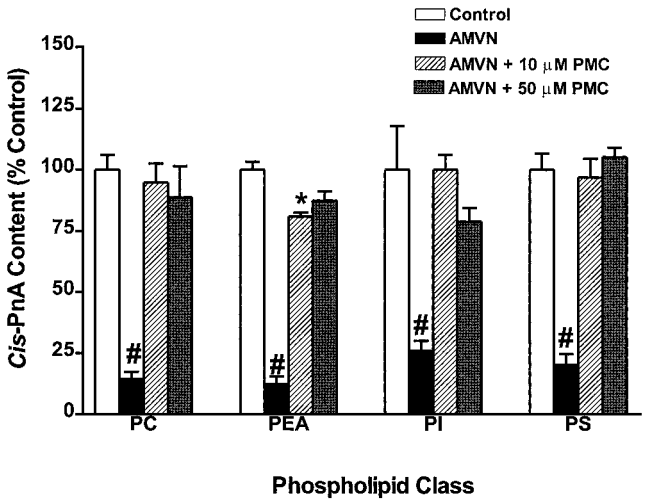

FIGURE 9: PMC protected all major phospholipids, including PS, from AMVN-induced oxidation in liposomes. Liposomes were prepared from HL-60 cells prelabeled with *cis*-PnA as described in Materials and Methods. Liposomes were suspended in incubation buffer to a lipid concentration similar to that achieved with experiments using intact cells and incubated with AMVN under conditions identical to those used with cells in the presence of PMC (10 and 50 μM). *cis*-PnA fluorescence in each phospholipid class was then determined. Data are expressed as the unoxidized *cis*-PnA content as a percent of that in control untreated liposomes and represent the mean ± SEM of three observations. Significant differences compared to control cells were determined using one-way ANOVA and Dunnett's multiple comparisons to control (#, $p < 0.01$; and *, $p < 0.05$).

the PMC concentration was increased to 50 μM. In addition, 2 μM PMC similarly provided approximately 75% protection

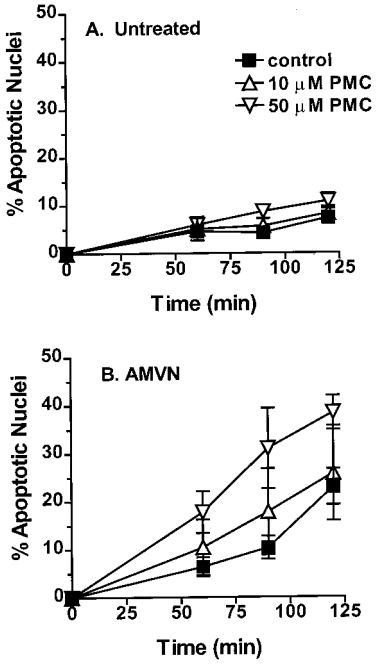

FIGURE 10: PMC failed to prevent apoptosis following AMVN treatment. HL-60 were treated with AMVN (500 μM) in serum-free L-15 medium in the presence or absence of PMC (10 and 50 μM). PMC was applied to cells 15 min prior to AMVN. Cells were harvested at various times after AMVN treatment and stained with Hoescht 33342. The percentage of cells showing condensed and fragmented nuclei characteristic of apoptosis was determined during fluorescent microscopy. Panel A shows the effect of PMC treatment alone, while panel B shows the rate of apoptosis following AMVN treatment. Data represent the mean ± the range of duplicate experiments. Note that PMC failed to attenuate apoptosis following AMVN treatment. PMC essentially had no effect in the absence of AMVN.

to all phospholipid classes (data not shown). Thus, PS resistance to the antioxidant actions of PMC was observed only with intact living cells and did not arise from an intrinsic inability of PMC to protect PS from oxidation.

AMVN-Induced PS Externalization and Apoptosis Were Resistant to PMC. We next measured the ability of PMC to modulate externalization of PS as assessed by its accessibility to fluorescamine. Figure 5A shows that PS accessibility remained high (17.9% of total) even when 50 μM PMC was included with AMVN during treatment. Thus, PS externalization following AMVN was resistant to PMC. PEA whose accessibility to fluorescamine was not affected by AMVN was also unaffected by either PMC alone or PMC and AMVN. Similarly, flow cytometry measurements revealed that AMVN-induced annexin V binding to externalized PS was unaffected by PMC (data not shown).

Figure 7 shows that the presence of PMC (10 and 50 μM) during the treatment with AMVN failed to block the formation of DNA ladders in HL-60 cells. Essentially no difference was observed between control cells and cells treated with PMC at these two concentrations alone in the absence of AMVN.

The inability of PMC to block AMVN-induced apoptosis was also manifest by its failure to inhibit the changes in nuclear morphology following treatment with the azo initiator. Figure 10 (panel B) shows that 10 and 50 μM PMC failed to block the time-dependent formation of apoptotic nuclei following AMVN treatment. Similar to the effect observed on internucleosomal DNA fragmentation, PMC treatment alone was not different from untreated control cells.

DISCUSSION

Although oxidative stress is frequently associated with the initiation and execution of apoptosis, the relative importance and cellular location of various targets for these effects are not clear. Because transmembrane movements of specific phospholipids, such as PS, could be key components of apoptosis, we have focused our studies on the relationship between specific and random membrane phospholipid oxidation and several markers of apoptosis, including PS translocation.

AMVN-Induced Random Lipid Peroxidation and Apoptosis in HL-60 Cells. We have previously described apoptosis following treatment with the prototypic oxidant paraquat, which is converted into its radical form by NADPH−cytochrome P450 reductase and subsequently generates superoxide radicals within the cytosol (*12*). PS oxidation, and to a lesser extent PI oxidation, preceded frank morphological evidence of paraquat-induced apoptosis. Moreover, neither PC nor PEA was oxidized in this model. Our current studies with AMVN and HL-60 cells further substantiate a critical role for PS oxidation in apoptosis. The precise cellular location and role for PS oxidation, however, remain to be described.

Loss of *cis*-PnA fluorescence is irreversible and describes low levels of lipid peroxidation in total cellular phospholipids in a sensitive and cumulative way independent of phospholipid deacylation and/or reacylation repair reactions. We observed no smearing or "tailing" of individual phospholipids on HP-TLC plates, especially PC, which contained a relatively high content of *cis*-PnA, as would be expected if the phospholipids contained appreciable levels of oxidized fatty acids. It is also important to point out that we observed no change in the percentage of lyso-PC, a product of PC deacylation, after treatment with AMVN for 2 h. Thus, it appears that the degree of lipid peroxidation following AMVN treatment did not exceed the capacity of the cell to repair the oxidized phospholipids. Furthermore, the level of lipid peroxidation reported by *cis*-PnA here did not induce or arise from necrosis since cells remained intact and continued to exclude Trypan Blue after treatment of HL-60 with 500 μM AMVN for 2 h (data not shown). The level of lipid peroxidation, on the other hand, was sufficient to induce apoptosis in HL-60 cells. Since the initiating stimulus is restricted in action to the membrane compartment, we conclude that relatively little oxidative damage to membrane lipids can serve as a stimulus for the initiation of apoptosis.

In contrast to DNA damage and p53-mediated apoptosis, we can only speculate at this time how damage to cellular membranes is sensed and transmitted into the apoptotic response. Lipid peroxidation could alter Ca^{2+} distribution and eicosanoid synthesis, as well as perturb various membrane-dependent signal transduction systems. These, in turn, could trigger the biochemical responses of apoptosis such as mitochondrial dysfunction, cytochrome *c* release, and caspase activation. Thus, oxidation of membrane lipids could subsequently generate molecular events at points distant from the initial site of damage.

It is well-established that azo initiators generate radicals with a relatively low first-order rate constant (R_i = 1.36−

2.39[AMVN] × 10^{-6} M/s) (*14*, *15*); therefore, under our conditions, we expect radical generation and subsequent lipid peroxidation to have occurred at essentially a linear rate throughout the initial 2 h incubation. We observed more than 85% oxidation of *cis*-PnA by this time, which suggests that substantial oxidation occurred throughout this time period. Consistent with this, we found all markers of apoptosis, including nuclear condensation, DNA fragmentation, and PS externalization, were detectable 2 h after AMVN treatment. These results support a role for lipid peroxidation as a mediator of apoptosis.

PS Oxidation in Live Cells and Apoptosis Were Resistant to PMC. The ability of antioxidants and antioxidant enzymes to protect cells from apoptosis has been suggested previously (*5*, *7*, *8*). Vitamin E represents the major lipoprotective antioxidant within cell membranes (*21*). Several groups have reported that the vitamin E analogue, Trolox, can inhibit apoptosis (*22*−*24*), but the relationship between the anti-apoptotic action and its lipid-based antioxidant activity is questionable due to the hydrophilicity of Trolox and the relatively high concentrations (10 mM) required for protection. The idea of protection against apoptosis with more lipophilic analogues, such as α-tocopherol, is contentious since protection has been absent in some studies (*25*−*28*). Indeed, DL-α-tocopherol induced apoptosis in a number of transformed cell lines (*29*).

We have previously shown that PMC is an attractive vitamin E-based lipid antioxidant because it partitions randomly and uniformly throughout the lipid membrane and possesses exceptional antioxidant potential (*16*). Nevertheless, PS was uniquely spared protection by PMC compared to other phospholipids. The asymmetrical distribution of PS in membranes cannot account for its resistance to PMC protection. We have observed that PMC has a much faster transbilayer mobility than other vitamin E derivatives (*16*) and should have distributed throughout the membrane within this time. In addition, PMC fully protected PEA despite its asymmetric distribution, similar to the case with PS. It is possible that the selective distribution of PS occurs within a cellular compartment that is inaccessible to PMC; however, if oxidation was random in this compartment, we would have expected to see that at least some portion of the other phospholipids were resistant to PMC protection. PS has been shown to be concentrated into an annulus surrounding a number of integral membrane proteins, including acetylcholine receptor (*30*), cytochrome P450 (*31*), and NADPH−cytochrome reductase (*32*). In addition, intracellular proteins such as spectrin (*33*) and annexin (*34*) bind to PS. Since PS is a relatively nonabundant phospholipid, it is possible that by association with specific membrane proteins, PS becomes concentrated into microdomains that are physically restricted from interaction with PMC and, hence, resistant to its antioxidant activity.

Another possibility is that a significant portion of the PS oxidation following AMVN treatment is not the direct result of AMVN-induced radical generation, but is followed by another independent mechanism, perhaps related to apoptotic execution. PS may serve as a molecular target for cytochrome *c* following its release from mitochondria during apoptosis (*35*), since cytochrome *c* appears to have a high affinity for anionic phospholipids (*36*) and possesses a redox-active heme center containing iron (*37*). This notion is supported by our observation that PMC-resistant PS oxidation was observed only with intact, living cells, and not in the cell-free liposome preparations.

It is somewhat surprising that PMC completely failed to inhibit apoptosis despite its ability to inhibit the bulk of AMVN lipid oxidation. If PS oxidation is the direct result of AMVN treatment, perhaps this is sufficient to initiate apoptosis. If, however, PS oxidation follows the initiation of apoptosis, then AMVN-dependent lipid oxidation below our level of detection may be sufficient to induce apoptosis. It also needs to be considered that the lipoprotective action of vitamin E is maintained at the expense of generating tocopheroxyl radicals. These species are recycled back to their unoxidized forms by reactions with several cytosolic hydrophobic antioxidants such as ascorbate, glutathione, and lipoic acid. Thus, extensive recycling of PMC following AMVN treatment could lead to depletion of important intracellular antioxidants and produce redox changes triggering apoptosis.

Specific PS Oxidation Was Associated with AMVN-Induced Apoptosis. One of our most important observations was that PS oxidation alone was associated with apoptosis. In the presence of PMC, only PS remained uniquely sensitive to oxidation during exposure to AMVN, yet the extent of apoptosis was not attenuated by the antioxidant. These data reinforce our previous observations with paraquat (*12*) and neocarzinostatin treatment (N. F. Schor, Y. Y. Tyurina, J. P. Fabisiak, V. A. Tyurin, J. S. Lazo, and V. E. Kagan, unpublished observations). It is possible, therefore that PS oxidation is a part of an apoptotic signaling pathway after AMVN exposure by a novel and, as yet, undefined mechanism. It is not clear at this time, however, if PS oxidation serves to specifically inititate the apoptotic cascade or represents a product of apoptotic activation. Examining the temporal relationships of PS oxidation to cytochrome *c* release and caspase activation will be of interest. In addition, studies aimed at establishing the sensitivity of PS oxidation to caspase inhibitors are currently underway.

Since our methods measured the extent of lipid peroxidation in total cellular phospholipids, we cannot directly assign the cellular location where PS is oxidized. If, however, a portion resides in plasma membrane, then externalization of PS may be functionally linked to its oxidation. The extent of transmembrane movement of phospholipids, such as PS, is determined by the concerted action of two lipid-transporting activities. Aminophospholipid translocase transports PS and, with less affinity, PEA from the external to the inner membrane leaflet, and phospholipid scramblase bidirectionally transports phospholipids in a nonspecific manner (*10*, *38*). The accumulation of externalized PS in our studies clearly supports the failure of aminophospholipid translocase to perform its normal surveillance function following AMVN treatment. It is tempting to speculate that PS oxidation is mechanistically related to this inability of aminophospholipid translocase to internalize PS. Externalized PS may therefore accumulate as the result of inactivation of the enyzme, whose activity is sensitive to oxidative stress and maintenance of reduced sulfhydryls (*39*−*41*). One could speculate that oxidative modification of the APT protein during its interaction with a reactive PS oxidation product (such as a lipid hydroperoxide) may result in loss of enzyme function. It is also

possible that oxidized PS appearing in the external bilayer may not be efficiently recognized by the aminophospholipid translocase and, hence, not be transported back to the internal surface. Oxidation of phospholipids decreases fatty acid unsaturation and may produce shorter chain lengths, both of which have been shown to negatively influence aminophospholipid translocase activity (*42*, *43*).

It is clear, however, that inhibition of aminophospholipid translocase alone cannot fully account for our results. Bratton et al. (*44*) indicated that activation of scramblase and inactivation of aminophospholipid translocase were both required for efficient accumulation of aminophospholipids on the cell surface during apoptosis. In contrast to Fadok et al. (*11*), we found no increase in the accessibility of PEA to fluorescamine at times when PS had become externalized. It should be pointed out, however, that even they observed a greater relative increase in the amount of derivatized PS (≈5-fold) compared to that of PEA (≈1.5−2-fold). Because phospholipid scramblase presumably recognizes PS and PEA similarly (*45*), it is likely that selective PS externalization measured 2 h after AMVN treatment was not primarily due to scramblase activation. This is not to say that activation of scramblase may not occur at later times following AMVN treatment. It remains to be determined why PS alone, and not PEA, appears on the surface to begin with. Inhibition of aminophospholipid translocase alone would be expected to produce similar increases in the amount of PEA and PS if both phospholipids appeared on the external surface at similar rates. Lipid peroxidation induces rapid transbilayer movement of phospholipids in protein-free liposomes (*46*). It is possible that the rate of spontaneous "flop" for oxidized PS is substantially higher than those for other phospholipids, including oxidized PEA. Future studies will be performed to identify the oxidation status of externalized PS and directly measure specific phospholipid translocation activities during oxidant-induced apoptosis to clarify these issues.

In conclusion, we have found that AMVN induced random lipid peroxidation and subsequent apoptosis in HL-60 cells. The vitamin E analogue, PMC, suppressed lipid peroxidation in all phospholipid classes except PS, but did not protect against AMVN-induced apoptosis. These results reveal the potentially important role of PS oxidation in modulating various aspects of programmed cell death.

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