concentration of H_2O_2 (2 m*M*) does not induce an intracellular drop in pH, but conversely the pH_i increases, as shown in Fig. 5a.

Conclusions

The intracellular concentration of ROI must be tightly regulated during homeostasis, and a significant deviation from this may tilt the balance between oxidative or reductive stress-induced death. We have shown that H_2O_2-induced apoptosis results in a significant drop in the intracellular pH and O_2^- concentration, irrespective of whether H_2O_2 is added exogenously or induced intracellularly using drugs.[5,11] The reduced intracellular milieu then provides an environment permissive for the execution of the death signal.[11] Therefore, it is important to differentiate between oxidative stress, induced by high concentrations of ROI that cause necrosis of the cells, from reductive stress-induced cell death that involves ROI production associated with a drop in the pH_i and subsequent activation of the apoptotic cell death pathway.

Acknowledgments

The authors thank J.L. Hirpara for technical assistance. This work was supported by Grants R-185-000-019-213 and R-185-000-009-112 from the NRMC, Singapore, and ARF, National University of Singapore, respectively, to S.P. and Grant R-364-000-008-213 from the NMRC, Singapore to M-V.C.

[14] Peroxidation of Phosphatidylserine in Mechanisms of Apoptotic Signaling

By YULIA Y. TYURINA, VLADIMIR A. TYURIN, ANNA A. SHVEDOVA, JAMES P. FABISIAK, and VALERIAN E. KAGAN

Introduction

Asymmetric distribution of major phospholipid classes across membranes is a fundamental characteristic of all cells whose disturbance is incompatible with physiological functions of membranes and with cell viability.[1,2] Under normal conditions, phosphatidylcholine (PC) and sphingomyelin (SPM) are located primarily in the outer leaflet of plasma membrane, whereas aminophospholipids—phosphatidylethanolamine (PE) and phosphatidylserine (PS)—are found almost

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entirely in the inner leaflet.[1–3] Transmembrane migration and externalization of PS by apoptotic cells are considered to be the triggering events for their recognition by cognate "scavenger" receptors of macrophages.[4] This is followed by phagocytosis and safe digestion of apoptotic cells, thus preventing development of an inflammatory response.[5]

Normal maintenance of PS asymmetry is mainly due to the constitutive activity of aninophospholipid translocase (APT), an ATP-dependent enzyme that transports aminophospholipids from the external to the internal surface of the plasma membrane lipid bilayer.[6] Activation of a Ca^{2+}-dependent phospholipid scramblase, which promotes random redistribution of all phospholipids in a bidirectional manner, is involved in the initiation of PS externalization during apoptosis. It is also clear, however, that downregulation of the surveillance function of APT is required to maintain PS externalization.[7] Previous work has demonstrated that apoptosis is associated with selective oxidation of specific phospholipid classes, most notably PS.[8,9] Site-specific oxidation of PS in plasma membrane may be an important early step in the mechanisms leading to its externalization[8,10,11] and binding of (oxidized) PS with scavenger receptors.[12,13] We speculated that externalization of (oxidized) PS might be due to the failure of APT to internalize it via either direct enzyme inhibition or the inability of the enzyme to recognize oxidized PS. It is also noteworthy that oxidized phospholipids undergo spontaneous "flip-flop" more readily than their nonoxidized counterparts.[14]

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 the execution

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of apoptosis.[10,11,15–17] While effects of oxidative stress on protein components of apoptotic machinery, such as caspases,[18,19] have been well characterized, information on selective oxidation of specific classes of phospholipids in live cells is limited. This results mainly from the paucity of sensitive and specific quantitative assays for measuring the oxidation of different classes of phospholipids. The direct measurement of oxidized lipid products is problematic, as cells possess a very effective system for remodeling and repairing oxidatively modified phospholipids[20] that interferes with their accurate measurement. We have developed a sensitive, specific, and reliable procedure for the assessment of oxidative stress in different classes of membrane phospholipids in intact live cells based on their metabolic acylation with an oxidation-sensitive and fluorescent fatty acid, *cis*-parinaric acid (*cis*-PnA), as a reporter molecule. Basic procedures and some applications of the technique are described in this chapter.

Reagents and Cells

cis-Parinaric acid [(9Z, 11E, 13E, 15Z)-octadecatetraenoic acid] is from Molecular Probes (Eugene, OR). Chloroform, methanol, glacial acetic acid, hexane, 2-propanol (HPLC grade), Tween 20, butylated hydroxytoluene (BHT), malachite base green, phospholipase A_2 from bee venom, and mellitin from bee venom are from Sigma (St. Louis, MO). Ammonium hydroxide is from Fisher Scientific (Pittsburgh, PA). α-Tocopherol acetate is from Aldrich Chemical (Milwaukee, WI). KGM-2 medium is from Clonetics (San Diego, CA). AMVN, 2.2′-azobis (2,4-dimethylisovaleronitrile) is from Wako Chemicals (Richmond, VA). Silica G HPTLC plates (5 × 5 cm) are from Whatman (Clifton, NJ).

We have, to date, utilized a number of diverse cell lines in conjunction with the metabolic incorporation of *cis*-PnA to measure lipid peroxidation in various phospholipid classes following oxidative stress (see Tables I and II). Our collective experience with multiple cell types includes those that grow as suspension cultures, as well as attached monolayers. The following detailed methods describe the use of HL-60 cells and normal human epidermal keratinocytes (NHEK) as representative example of suspension and monolayer cells, respectively. NHEK from adults are from Clonetics (San Diego, CA). Cells are plated at a density of 6.25×10^4 cells per 75-ml tissue culture flask (Greiner Laboratories, GmbH, Germany) and grown in KGM-2 medium until confluent monolayers are obtained. Repeated counts

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TABLE I
INTEGRATION OF *cis*-PARINARIC ACID INTO PHOSPHOLIPIDS OF DIFFERENT CELL TYPES[a]

Cell line	*N*	*cis*-Parinaric acid integrated into cell phospholipids (ng/μg total lipid phosphorus)			
		PI	PE	PS	PC
Rat vascular smooth muscle cells	7	13.4 ± 0.4	66.2 ± 2.4	11.6 ± 1.1	237.0 ± 4.8
Rat cardiomyocytes	3	N.D.	73.7 ± 4.8	6.5 ± 0.7	384.5 ± 27.4
Rat myoblasts	3	5.6 ± 0.6	46.3 ± 4.6	4.2 ± 0.7	165.5 ± 16.6
Rat hepatocytes	3	0.9 ± 0.6	6.8 ± 0.4	2.2 ± 0.7	43.8 ± 2.1
Rat spinal cord neurons	3	9.4 ± 0.4	85.8 ± 1.6	8.8 ± 0.6	361.9 ± 4.2
Rat pheochromocytoma PC-12 Cells	7	0.7 ± 0.1	8.4 ± 2.0	1.7 ± 0.3	105.5 ± 15.0
Murine myeloid 32D cells	7	12.7 ± 2.0	35.4 ± 1.8	5.2 ± 0.4	180.2 ± 9.2
Mouse lung fibroblasts	5	N.D.	1.6 ± 0.1	0.3 ± 0.1	9.4 ± 0.6
Sheep pulmonary artery endothelial cells	3	1.8 ± 0.3	33.7 ± 4.0	13.3 ± 2.6	141.2 ± 10.0
Bovine aorta endothelial cells	6	1.4 ± 0.2	7.7 ± 0.4	3.7 ± 0.3	118.8 ± 4.8
Normal human epidermal keratinocytes	10	2.0 ± 0.2	32.4 ± 1.9	3.9 ± 0.3	50.8 ± 2.9
MCF-7 breast cancer cells	13	4.4 ± 1.8	29.8 ± 3.1	4.6 ± 0.8	135.9 ± 14.4
Human leukemia HL-60 cells	5	12.8 ± 3.5	62.7 ± 12.0	6.4 ± 2.3	212.0 ± 39.6

[a] All data are means ±SEM.

of NHEK reveal a concentration of 1–2 × 10^6 cells per flask at the time of harvest. HL-60 human promyelocytic leukemia cells from American Type Culture Collection (Manassas, VA) are cultured in RPMI 1640 medium supplemented with 12.5% fetal bovine serum. Routine passages (1 : 5–1 : 10 splits) are performed when cells reach a density of between 1 and 1.5 × 10^6 cells/ml.

Integration of *cis*-PnA Into Cell Phospholipids

cis-PnA has been used as a fluorescent probe in physical–chemical studies of model membranes and as a reporter for the assessment of peroxidation in chemical systems, lipoproteins, and simple membrane systems[29] and in total cell lipids.[30] *cis*-PnA is a natural 18 carbon fatty acid with four conjugated double bonds. The four conjugated double bonds confer highly fluorescent properties to *cis*-PnA and render it highly susceptible to peroxidation. Upon peroxidation, fluorescence is irreversibly lost as mammalian cells do not synthesize fatty acids with conjugated double bond systems. *cis*-PnA can be metabolically incorporated into phospholipids similar to endogenous free fatty acids. If care is taken to remove free *cis*-PnA after metabolic integration so that it is no longer available for

TABLE II
EFFECT OF OXIDATIVE STRESS ON PHOSPHOLIPID COMPOSITION AND OXIDATION OF *cis*-PNA-LABELED PHOSPHOLIPIDS IN CELLS

Cell line	Oxidants	Changes in phospholipid composition	Oxidation of *cis*-parinaric acid-labeled phospholipids	References
Rat vascular smooth muscle cells	*tert*-BuOOH	−	+	21
Rat vascular smooth muscle cells	AMVN	−	+	22
Rat cardiomyocytes	*tert*-BuOOH	−	+	23
Rat spinal cord neurons	AMVN	−	+	
Sheep pulmonary artery endothelial cells	AMVN	−	+	
Normal human epidermal keratinocytes	AMVN	−	+	24
	Phenol	−	+	24
	Cumene-OOH	−	+	25
MCF-7 breast cancer cells	AMVN	−	+	26
Leukemia HL-60 cells	AMVN	−	+	11
	Cu-NTA	−	+	27
	tert-BuOOH	−	+	
Murine myeloid 32D cells	Paraquat	−	+	8, 10
Pheochromocytoma PC-12 cells	AMVN	−	+	28
	Neocarzinostatin	−	+	9

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phospholipid repair, then resolution of major phospholipid classes by fluorescence HPLC can be used to quantify their oxidative damage (as a decreased content of fluorescent *cis*-PnA residues in respective phospholipid classes). Importantly, the *cis*-PnA-based assay can identify the selectivity of phospholipid oxidation based on polar head groups and is independent of the fatty acid composition of phospholipids.[30,31]

cis-PnA is incorporated into HL-60 cell suspensions or NHEK monolayers by addition of its complex with human serum albumin (hSA). The purity of each lot of *cis*-PnA is determined by UV spectrophotometry using the molar extinction $\varepsilon_{304\text{ nm ethanol}} = 80 \times 10^3\ M^{-1}\text{cm}^{-1}$. The complex is prepared by adding *cis*-PnA (500 μg, 1.8 μmol) in 25 μl of dimethyl sulfoxide to hSA (50 mg, 750 nmol) in 1 ml of phosphate-buffered serum (PBS).

HL-60 cells (10^6/ml) or NHEK (80–90% confluent monolayers) are incubated in the presence of *cis*-PnA/hSA complex (2 or 5 μg *cis*-PnA/10^6 cells/ml medium, respectively) in serum-free RPMI 1640 medium or KGM-2 medium, respectively, at 37° in 5% CO_2 atmosphere for 2 hr. Preliminary studies reveal that the integration of *cis*-PnA into cellular phospholipids under these conditions is time dependent and reaches maximum after 1–2 hr of incubation. At the end of incubation, cells are washed once with PBS containing fatty acid-free hSA (0.5 mg/ml) and again without hSA. This step is necessary to remove excess free *cis*-PnA and to exclude its potential interference by a reacylation reaction that might mask the degree of actual oxidation of *cis*-PnA-labeled phospholipids. *cis*-PnA-labeled HL-60 cells or NHEK are then exposed to various oxidative stimuli: *tert*-butyl hydroperoxide (*tert*-BuOOH) (150 μM) for 20 min in the case of HL-60 cells or cumene hydroperoxide (cumene-OOH) (200 μM) for 2 hr (NHEK cells) at 37°. At the end of treatment, NHEK are scraped and HL-60 cells are collected by centrifugation. Lipids are extracted by the Folch procedure.[32]

HPLC Analysis of *cis*-PnA-Labeled Phospholipids

An HPLC procedure is used to separate and detect *cis*-PnA integrated into various cellular phospholipid classes. The lipid extracts are applied to a 5 μm Microsorb-MV column (4.5 × 250 mm, Rainin, Woburn, MA) equilibrated with a mixture of one part of solvent A [2-propanol : hexane : water (56 : 42 : 2, by volume)] and nine parts of solvent B [2-propanol : hexane : 40 m*M* aqueous ammonium acetate (54 : 41 : 10, by volume)] pH 6.7. The column is eluted during the first 3 min with a linear gradient from 10% solvent B to 37% solvent B, for 3–15 min

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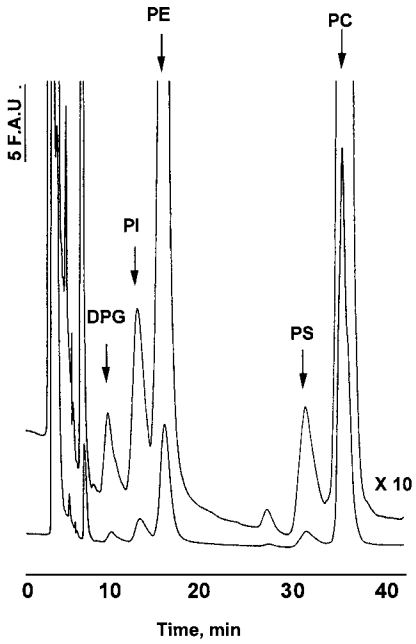

FIG. 1. A normal phase HPLC chromatogram of total *cis*-PnA-labeled phospholipids extracted from HL-60 cells. Fluorescence emission intensity, excitation at 324 nm, emission at 420 nm. PI, phosphatidylinositol; PE, phosphatidylethanolamine, PS, phosphatidylserine, PC, phosphatidylcholine; DPG, diphosphatidylgycerol.

with an isocratic gradient at 37% solvent B, for 15–23 min with a linear gradient to 100% solvent B, and for 23–45 min with an isocratic gradient at 100% solvent B. The solvent flow rate is maintained at 1 ml/min. Separations are performed using a high-performance liquid chromatograph (Shimadzu Model LC-600) equipped with an in-line configuration of fluorescence (Model RF-551) and UV-VIS (Model DPD-10AV) detectors. The effluent is monitored by absorbance at 205 nm to detect lipids; the fluorescence of *cis*-PnA is measured by its emission at 420 nm after excitation at 324 nm.

A typical fluorescence emission profile of the HPLC column eluate of total lipids extracted from HL-60 cells is shown in Fig. 1. Major fluorescence peaks were identified using authentic phospholipid standards and include diphosphatidylgycerol (DPG), phosphatidylinositol (PI), PE, PS, and phosphatidylcholine (PC). Under the indicated conditions, retention times for DPG, PI, PE, PS, and PC were determined as 11.3, 15.1, 17.3, 32.1, and 36.2 min, respectively. The identity of the fluorescence peaks was also confirmed by HPTLC of the individual collected HPLC fractions. Lipid phosphorus was determined using a micro method.[33] Data

[33] A. Chavardjian and E. Rubbnicki, *Anal. Biochem.* **36,** 225 (1970).

are expressed as nanograms of *cis*-PnA per microgram of total lipid phosphorus. Values for relative incorporation of *cis*-PnA into membrane phospholipids of examined cells are presented in Table I. This shows that incorporation of *cis*-PnA in the various phospholipids was differential, and the amount of *cis*-PnA incorporated was in the following order PC > PE > PI ≥ PS. In general, this parallels the relative abundance of each of these phospholipid classes within cells.

HPTLC of Cell Phospholipids during Apoptosis Induced by Oxidative Stimuli

Table II shows our collective experience at measuring *cis*-PnA oxidation following exposure of a variety of cell types to numerous oxidants. In all cases, *cis*-PnA oxidation (assessed by comparing *cis*-PnA fluorescent content in oxidant-treated cells compared to untreated controls) could be measured in individual classes of phospholipids following oxidative stress. Importantly, these changes were observed early following exposure to low level oxidants at a time when overall cell viability was unchanged by oxidant treatment. In live cells, oxidative modifications of only a relatively small fraction of membrane phospholipids are compatible with the preservation of cellular functions. Therefore, lipid oxidation as measured here represents relatively small levels of oxidation, and it is doubtful that significant changes of phospholipid composition would be detectable on oxidative challenge to cells within their limits of survival. It was important to determine if changes in phospholipid composition arose as a result of these oxidative challenges. In addition, information on phospholipid composition, i.e., distribution of phospholipids between their different classes, may be useful for determinations of changes in phospholipid peroxidation on a specific basis (per unit of a given class of phospholipids). To these ends, HPTLC separation and quantification of phospholipid distribution were performed as follows.

Cells after exposure to oxidative stimuli are collected by centrifugation and resuspended in 2 ml of 0.1 *M* NaCl. To extract lipids, methanol (3 ml) containing butylated hydroxytoluene (100 μM, as an antioxidant blocking peroxidation during work-up of samples) and chloroform (2 ml) are added to cell suspensions. After phase separation, the lower phase is collected, and the solvent is evaporated under nitrogen. The film of lipids is dissolved in hexane : propanol (4 : 3, by volume) and used for phospholipid analysis. Individual phospholid classes in lipid extracts are separated by two-dimensional HPTLC on silica G plates (5 × 5 cm, Whatman). The plates are first developed with a solvent system consisting of chloroform : methanol : 28% ammonium hydroxide (65 : 25 : 5, by volume). After drying the plates with a forced air blower to remove the solvent, plates are developed in the second dimension with a solvent system consisting of chloroform : methanol : glacial acetic acid : water (50 : 20 : 10 : 10 : 5, by volume). The phospholipid spots are visualized by exposure to iodine vapor and identified by

comparison with the migration of authentic phospholipid standards. The spots identified by iodine staining are scraped and transferred on silica to glass tubes. Lipid phosphorus is determined by a micro method as described by Bottcher *et al.*[34] In all cell lines studied (Table II), PC and PE are the two major phospholipids, which represent 43.4–60.9% and 18.1–29.9% of total phospholipid, respectively. SPH, PI, DPG, and PS are also detectable on HPTLC plates, and their amount is dependent on the cell line (data not shown).

Expectedly, no significant difference in phospholipid distribution was detected after exposure of any examined cells to different oxidative stimuli (Table II). The lack of HPTLC-detectable changes in the phospholipid composition of different cells exposed to oxidative stimuli might be due to the effective repair of phospholipids via deacylation/reacylation pathways.[35,36] Oxidatively modified phospholipids are known to undergo rapid and effective remodeling that involves phospholipase A_2-catalyzed hydrolysis with subsequent acyltransferase-catalyzed reacylation of peroxided phospholipids.[37] Thus, in live cells, direct analysis of phospholipid composition may not be used for purposes of detection or quantitation of oxidatively modified phospholipids. The information from such determinations, however, may be utilized for more complete characterization of specific oxidative stress in different classes of membrane phospholipids using *cis*-PnA as a reporting molecule (see later).

Positional Distribution of *cis*-PnA Integrated in Cell Phospholipids

In mammalian cells, the *sn*-2 position in phospholipid molecules is usually occupied by a polyunsaturated fatty acid residue. We were anxious to determine whether *cis*-PnA, with its four conjugated double bonds, was similarly metabolically integrated into phospholipids as well, thus representing a positionally "normal" target for oxidative stress. To analyze the positional distribution of *cis*-PnA in membrane phospholipids, we treated homogenates of *cis*-PnA-prelabeled HL-60 cells with phospholipase A_2 whose catalytic action is enhanced in the presence of mellitin. Because phospholipase A_2 specifically hydrolyzes phospholipids in *sn*-2 position fluorescence HPLC, as well as HPTLC, analysis of the products permits detrmination of the relative amounts of *cis*-PnA esterified in the *sn*-1 and *sn*-2 positions of membrane phospholipids.

Homogenates (2.5×10^7 cells/ml) are prepared by freezing ($-80°$) and thawing *cis*-PnA-loaded cells treated with phospholipase A_2 from bee venom (20 U/ml)

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and mellitin (10 μM) in 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ at 37° for 30 min. The reaction is terminated by the extraction of lipids by the Folch procedure.[32] HPTLC results demonstrate that >95% phospholipids underwent hydrolysis under the conditions used (data no shown). Our HPLC data showed that >99% of *cis*-PnA was confined to the *sn*-2 position in all major classes of phospholipids (99.3, 99.5, 99.2, and 95.6% for PC, PE, PS, and PI, respectively) (Table III). Thus, *cis*-PnA containing four conjugated double bonds was predominantly integrated into the *sn*-2 position of phospholipids in HL-60 cells in line with the positional distribution of endogenous polyunsaturated fatty acid residues in mammalian phospholipids.[38] This indicates that an oxidative attack on *cis*-PnA-labeled cells would also be occurring at the *sn*-2 position. In fact, our fluorescence HPLC measurements of HL-60 cell phospholipids after exposure to *tert*-BuOOH revealed no peaks corresponding to fluorescently labeled lysophospholipids (see later). This confirms that *cis*-PnA esterified in the *sn*-2 position was by far the major substrate for peroxidation in cells following oxidant exposure.

Thus our developed and optimized protocol yields cells containing the major classes of membrane phospholipids—PC, PE, PS, PI—fluorescently labeled with *cis*-PnA (Table I) and extremely low intracellular concentration of free *cis*-PnA. The level of *cis*-PnA labeling of endogenous phospholipids (≈1–3 mol%) was low enough to have minimal effects on cell viability and functions, yet sufficient to permit quantitative detection of oxidative stress. Using the assay we were able to reliably and sensitively detect phospholipid peroxidation in different cell lines induced by a variety of oxidants at sublethal levels of oxidative stress (Table II). Importantly, in none of these cases was conventional analysis of phospholipids sensitive enough to detect oxidation-induced changes in phospholipid composition.

Site-Selective Oxidation of PS Induced by Apoptotic Oxidative Stimuli

Because the *cis*-PnA-based assay permits quantification of the amount of oxidative stress in different classes of phospholipids in live cells, it can be utilized for revealing the roles that oxidative modification of specific phospholipids may play in cell function and signaling. In particular, oxidative stress-induced apoptosis represents an exciting area of research to discover specific mechanisms and pathways through which peroxidation of different classes of phospholipids participates in execution of the apoptotic program. Externalization of phosphatidylserine has been identified as a critical event in macrophage recognition of apoptotic cells. Therefore, we were interested in determining whether oxidant-induced apoptosis was also associated with selective oxidation of PS.

[38] A. L. Lehninger, D. L. Nelson, and M. M. Cox, *in* "Principles of Biochemistry," 2nd Ed. Worth Publishers Inc., 1993.

TABLE III

EFFECT OF PHOSPHOLIPASE A_2 ON PHOSPHOLIPID COMPOSITION AND CONTENT OF *cis*-PNA-LABELED PHOSPHOLIPIDS IN HL-60 CELLS[a]

	Content of phospholipids, % of total phospholipids,				*cis*-PnA-labeled phospholipids, ng PnA/μg total lipid Pi,			
	without phospholipase A_2		with phospholipase A_2		without phospholipase A_2		with phospholipase A_2	
Phospholipid	Control	*tert*-BuOOH	Control	*tert*-BuOOH	Control	*tert*-BuOOH	Control	*tert*-BuOOH
Phosphatidylcholine	45.9 ± 3.9	46.4 ± 1.1	0.5 ± 0.1	0.4 ± 0.4	314.2 ± 57.4	244.1 ± 27.5	2.2 ± 0.1	1.3 ± 0.1
Phosphatidylethanolamine	31.4 ± 5.4	27.9 ± 1.4	0.3 ± 0.2	0.3 ± 0.1	56.2 ± 14.1	40.4 ± 4.9	0.3 ± 0.1	0.2 ± 0.1
Phosphatidylserine	6.5 ± 0.5	5.8 ± 0.5	Tr.	Tr.	13.3 ± 5.1	4.1 ± 0.8	0.1 ± 0.1	0.1 ± 0.1
Spingomyelin	6.1 ± 0.5	7.9 ± 0.7	8.6 ± 1.2	8.4 ± 1.6	N.D.	N.D.	N.D.	N.D.
Phosphatidylinositol	7.7 ± 0.5	8.3 ± 0.8	Tr.	Tr.	17.3 ± 5.9	11.3 ± 1.8	0.8 ± 0.2	0.7 ± 0.1
Diphosphatidylglycerol	1.9 ± 0.9	3.2 ± 0.8	3.8 ± 0.4	3.7 ± 0.9	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Lysophospholipids[b]	0.5 ± 0.2	0.5 ± 0.2	86.8 ± 1.9	87.2 ± 4.0	N.D.	N.D.	78.7 ± 6.8	70.5 ± 1.5
Free *cis*-parinaric acid	N.D.	N.D.	N.D.	N.D.	1.6 ± 0.7	1.2 ± 0.1	318.2 ± 10.9	245.5 ± 3.1

[a] All values are means ± SD ($n = 3$). N.D., not detectable. Tr., trace—less than 0.1%.

[b] Lysophospholipids: lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol.

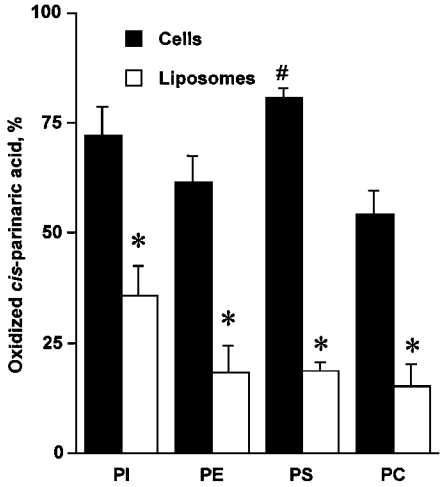

FIG. 2. Cumene hydroperoxide-induced oxidation of *cis*-PnA-labeled phospholipids in intact live normal human epidermal keratinocytes and cell-free *cis*-PnA-labeled liposomes derived from normal human epidermal keratinocytes. Intact living *cis*-PnA-labeled NHEK were exposed to cumene hydroperoxide (200 μM) for 1 hr at 37°. *cis*-PnA-labeled liposomes were prepared from *cis*-PnA-loaded NHEK and treated similarly with 200 μM cumeme hydroperoxide at 37° for 1 hr. At the end of the incubations, total lipids were extracted and resolved by HPLC. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Data represent means ± SEM, $n = 3$, $^*p < 0.01$.

Similar to our earlier studies observing the selective oxidation of PS as an early biomarker of apoptosis,[8,11] we documented that treatment of NHEK and HL-60 cells with organic hydroperoxides results in selective oxidation of PS that precedes PS externalization and activation of caspases in these cells.[25,39] NHEK are treated with cumene-OOH (200 μM) in phenol red-free KGM-2 medium for 1 hr at 37° in the dark. Data presented in Fig. 2 show that cumene-OOH (200 μM) causes substantial oxidation of all *cis*-PnA-labeled phospholipids in NHEK. At this concentration, however, cumene-OOH-induced oxidation of PS is significantly greater than oxidation of the two major phospholipids PC and PE.

To reveal whether this oxidation of PS was specific for the execution of apoptosis or simply reflected PS as a preferential target of cumene-OOH-induced oxidation of phospholipids, we performed studies to measure cumene-OOH-dependent oxidation of *cis*-PnA-labeled phospholipids in a cell-free system containing *cis*-PnA-labeled liposomes. Lipids are extracted from *cis*-PnA-loaded cells, the solvent is evaporated under N_2, and the film of lipids is suspended in 20 mM HEPES buffer,

[39] K. Kawai, Y. Y. Tyurina, V. A. Tyurin, V. E. Kagan, and J. P. Fabisiak, *Toxicologist* **54,** 165 (2000).

pH 7.4, to achieve the lipid concentration equivalent to that used in the experiments with live cells. Liposomes are prepared by sonication of lipid suspension (four 15-sec pulses on ice) using a tip sonicator (Ultrasonic Homogenizer 4710 series, Cole-Palmer Instrument Co., Chicago, IL). Liposomes containing *cis*-PnA-labeled phospholipids are incubated for 1 hr in the presence of cumene-OOH (200 μM) at 37° in the dark. At the end of incubation, lipids are extracted and resolved by HPLC. In liposomes, the oxidation of all classes of phospholipids was significantly less pronounced than that observed in live NHEK (Fig. 2). Importantly, preferential oxidation of PS observed in live NHEK cells was not detectable in the phospholipid-containing cell-free system. This suggests that nonrandom preferential oxidation of PS is characteristic of oxidant-induced apoptosis in NHEK cells.

The plasma membrane is the site where major events associated with PS signaling during apoptosis, including its externalization and subsequent recognition by macrophages, take place. This implies that selective oxidation of PS—if it is a part of apoptotic signaling—should occur within the plasma membrane compartment.[40,41] To establish whether plasma membrane PS undergoes peroxidation during apoptosis, we performed experiments in which we isolated different subcellular fractions and determined the amounts of oxidative stress in different classes of phospholipids in these organelles.[39] Importantly, the sensitivity of our PnA-based assay for phospholipid peroxidation permits us to conduct the study using practical amounts of cell material. Figure 3 compares the oxidation of *cis*-PnA-labeled PS, PE, and PC derived from whole HL-60 cells treated with *tert*-BuOOH to that observed within the plasma membrane of similarly treated cells. After a 20-min incubation of HL-60 cells with *tert*-BuOOH (150 μM) in serum-free RPMI medium 1640 without phenol red, oxidation of all phospholipids is observed. PS is remarkably more sensitive to oxidation than PC, PE, and PI (Fig. 3, left). We next determined the amounts of oxidative stress in plasma membrane phospholipids. Plasma membranes are isolated from *tert*-BuOOH-treated HL-60 cells as described by Storrie and Madden.[42] Figure 3 (right) shows that in HL-60 cells challenged with *tert*-BuOOH, plasma membrane PS was the largest source of oxidized PS (58%) and it was almost completely accountable for *tert*-BuOOH-induced PS oxidation in HL-60 cells. Notably, lower rates of PS oxidation were detected in other subcellular fractions, such as mitochondria, microsomes, lysosomes, and nuclei[39] (data not shown).

[40] V. E. Kagan, J. P. Fabisiak, A. A. Shvedova, Y. Y. Tyurina, V. A. Tyurin, N. F. Schor, and K. Kawai, *FEBS Lett.* **477,** 1 (2000).

[41] Y. Y. Tyurin, A. A. Shvedova, K. Kawai, V. A. Tyurin, C. Kommineni, P. J. Quinn, N. F. Schor, J. P. Fabisiak, and V. E. Kagan, *Toxicology* **148,** 93 (2000).

[42] B. Storrie and E. A. Madden, *Methods Enzymol.* **182,** 203 (1990).

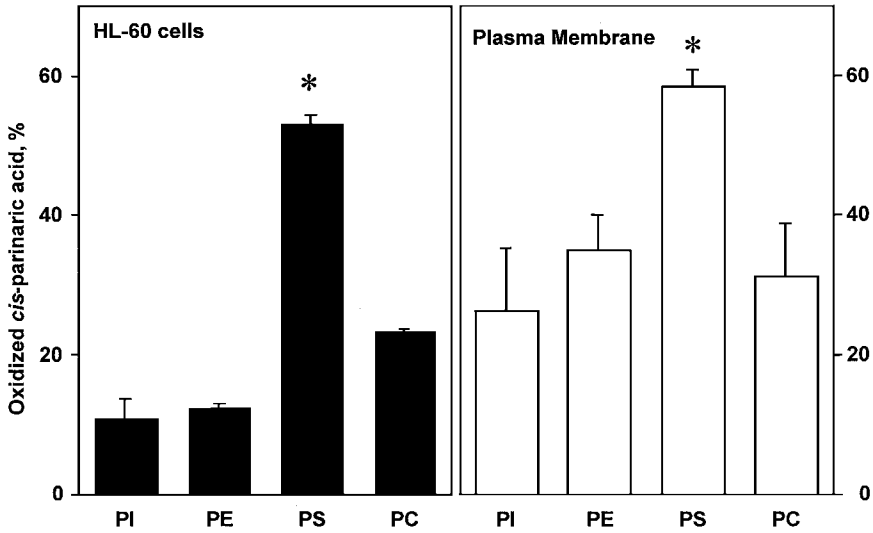

FIG. 3. tert-Butyl hydroperoxide-induced oxidation of *cis*-PnA-labeled phospholipids in intact live HL-60 cells and plasma membrane of HL-60 cells. *cis*-PnA-labeled HL-60 cells (left) were exposed to *tert*-butyl hydroperoxide (150 μM) for 20 min at 37°. The plasma membrane (right) was isolated from *cis*-PnA-labeled HL-60 cells treated with *tert*-butyl hydroperoxide (150 μM) at 37° for 20 min. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Data represent means ± SEM, $n = 3$, $^*p < 0.02$.

Note: The levels of *cis*-PnA-labeled phospholipids after incubation of cells without oxidants were used as controls for comparisons. During incubation in the absence of oxidants, the content of *cis*-PnA-labeled phospholipids was essentially unchanged (within 10% of the initial levels).

Antioxidants Protect against Phospholipid Peroxidation

To further prove that *cis*-PnA-labeled cells represent a good model for the quantitative assay of oxidative stress, we investigated the protective effects of vitamin E on the peroxidation of *cis*-PnA-labeled phospholipids induced by AMVN in NHEK and HL-60 cells. *cis*-PnA-loaded cells are incubated in the presence or in the absence of a lipid-soluble azoinitiator, AMVN [2,2′-azobis(2,4-dimethylisovaleronitrile], which generates peroxyl radicals within the lipid bilayer at a constant rate[43] and induces apoptosis in cells.[8,28,44] After incubation, NHEK

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[44] J. P. Fabisiak, V. A. Tyurin, Y. Y. Tyurina, A. Sedlov, J. S. Lazo, and V. E. Kagan, *Biochemistry* **39,** 127 (2000).

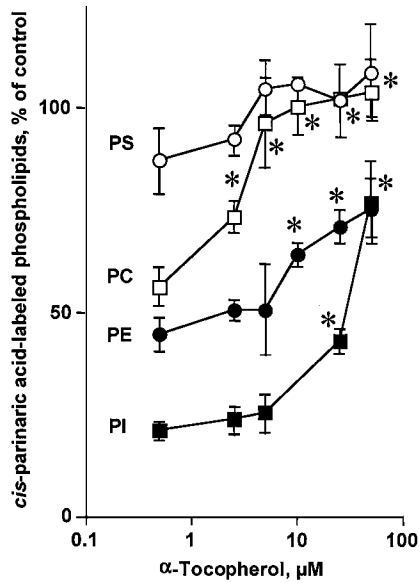

FIG. 4. Effect of α-tocopherol on oxidation of *cis*-PnA-labeled phospholipids induced by AMVN in normal human epidermal keratinocytes. NHEK ($\approx$100% confluency) were cultured in the presence or in the absence of α-tocopherol (2.5–50 μM) for 24 hr at 37° in KGM-2 medium. Unloaded NHEK and NHEK loaded with α-tocopherol were exposed to AMVN (500 μM, 1 hr). At the end of incubation, cells were scraped and lipids were extracted and resolved by HPLC. PI, phosphatidylinositol; PE, phosphatidylethanolamine, PS, phosphatidylserine, PC, phosphatidylcholine. $^*p < 0.03$ vs AMVN. Data represent means $\pm$ SEM.

and HL-60 cells are collected and washed twice with PBS, and total lipids are extracted according to Folch *et al.*[32] in the presence of BHT (100 μM) to prevent subsequent oxidation. The acyl chains of four classes of phospholipids in NHEK and HL-60 cells, namely PE, PC, PS, and PI, were the major targets for AMVN-induced peroxidation (Fig. 4 for NHEK and Fig. 5 for HL-60).

The sensitivity of this oxidation to the lipo-protective antioxidant, vitamin E, is then assessed. NHEK ($\approx$100% confluence) and HL-60 cells (0.5×10^6) are cultured in the presence of α-tocopherol acetate (2.5–50 μM) for 24 hr before *cis*-PnA labeling and exposure to oxidants. Vitamin E (α-tocopherol acetate) is added to the growth medium. Excess vitamin E that has not been integrated into cells is removed by washing cells with medium. The effect of α-tocopherol on the AMVN-induced oxidation of *cis*-PnA-labeled phospholipids is presented for NHEK in Fig. 4 and for HL-60 cells in Fig. 5. The protective effect of α-tocopherol on AMVN-induced oxidation of phospholipids is concentration dependent. As an effective radical scavenger, α-tocopherol (50 μM) is able to completely protect all phospholipids against oxidation.

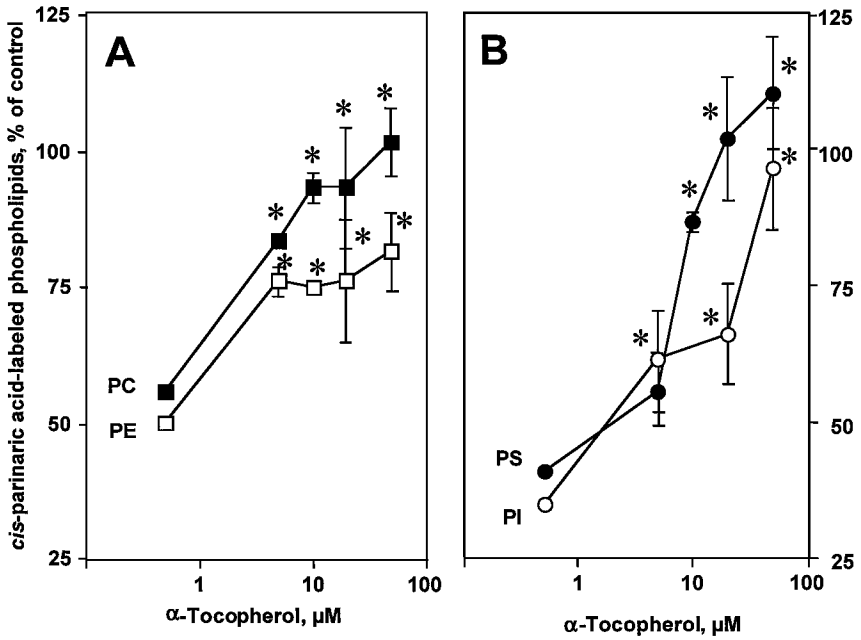

FIG. 5. Effect of α-tocopherol on oxidation of *cis*-PnA-labeled phospholipids induced by AMVN in HL-60 cells. HL-60 cells (0.5×10^6) were cultured in the presence or in the absence of α-tocopherol (5–50 M) for 24 hr at 37° in RPMI medium 1640. Unloaded HL-60 and HL-60 cells loaded with α-tocopherol were exposed to AMVN (500 μM). At the end of incubation, cells were collected by centrifugation, and lipids were extracted and resolved by HPLC. (A) PC, phosphatidylcholine; PE, phosphatidylethanolamine. (B) PS, phosphatidylserine; PI, phosphatidylinositol. $^*p < 0.01$ vs AMVN. Data represent means ± SEM.

Conclusion

In conclusion, our results clearly indicate that our model of *cis*-PnA-labeled cells offers a unique model system for quantitative studies of oxidative stress and selective oxidation of specific classes of phospholipids under normal physiological conditions, as well as during cell injury and apoptotic death.

Acknowledgments

Supported by grants from NIH 1RO1HL64145-01A1, EPA STAR Grant R827151, the NCI Oncology Research Faculty Development Program and Magee-Womens Reseach Institute (V.A.T.), and Leukemia Research Foundation and the International Neurological Science Fellowship Program (F05 NS 10669) administered by NIH/NINDS in collaboration with WHO, Unit of Neuroscience, Division of Mental Health and Prevention of Substance Abuse (Y.Y.T.).