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Non-random peroxidation of different classes of membrane phospholipids in live cells detected by metabolically integrated *cis*-parinaric acid

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Abstract

Quantitative assays of lipid peroxidation in intact, living cells are essential for evaluating oxidative damage from various sources and for testing the efficacy of antioxidant interventions. We report a novel method based on the use of *cis*-parinaric acid (PnA) as a reporter molecule for membrane lipid peroxidation in intact mammalian cells. Using four different cell lines (human leukemia HL-60, K562 and K/VP.5 cells, and Chinese hamster ovary (CHO) fibroblasts), we developed a technique to metabolically integrate PnA into all major classes of membrane phospholipids, i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin, that can be quantified by HPLC with fluorescence detection. Integrated PnA constituted less than 1% of lipid fatty acid residues, suggesting that membrane structure and characteristics were not significantly altered. Low concentrations (20–40 μM) of *tert*-butyl hydroperoxide (t-BuOOH) caused selective oxidation of PnA residues in phosphatidylserine and phosphatidylethanolamine of K562 cells and K/VP.5 cells while cell viability was unaffected. At higher t-BuOOH concentrations (exceeding 100 μM), however, a progressive, random oxidation of all major phospholipid classes occurred and was accompanied by significant cell death. In HL-60 cells, phosphatidylethanolamine, phosphatidylserine and cardiolipin were sensitive to low concentrations of t-BuOOH, while phosphatidylcholine and phosphatidylinositol were not affected. Phosphatidylinositol was the only phospholipid that responded to the low concentrations of t-BuOOH in CHO cells. At high t-BuOOH concentrations, again, all phospholipid classes underwent extensive oxidation. All phospholipids were nearly equally affected by peroxidation induced by a initiator of peroxyl radicals, 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN), in K562 cells. In gamma-irradiated (4–128 Gy) CHO cells, phosphatidylserine was the most affected phospholipid class (34% peroxidation) followed by phosphatidylinositol (24% peroxidation) while the other three phospholipid classes were apparently unaffected. Since loss of PnA fluorescence is a direct result of irreparable oxidative loss of its conjugated double bond system, the method described allows for selective and sensitive monitoring of oxidative stress in live cells without interference from cell repair mechanisms.

Keywords: Oxidative stress; *tert*-Butyl hydroperoxide; Phospholipid; *cis*-Parinaric acid; Fluorescence probe; HPLC

Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, butylated hydroxytoluene; CL, cardiolipin; DHLA, dihydrolipoic acid; HPLC, high pressure liquid chromatography; hSA, human serum albumin; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PnA, *cis*-parinaric acid; PS, phosphatidylserine; SPH, sphingomyelin; t-BuOOH, *tert*-butyl hydroperoxide; VP-16, etoposide.

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1. Introduction

Oxygen and lipid free radicals are implicated in the etiology of a host of degenerative diseases, such as cardiovascular disease and neurodegenerative disease, in acute conditions such as trauma and infection, and in aging (see [1] and references therein). Numerous investigations at the cell, tissue, whole animal, and human population levels support the general concept that the probability of certain diseases is inversely correlated with levels of antioxidants in the body [2]. Of the various types of oxidative damage that cells experience, lipid peroxidation is considered to be one of the major contributors to oxidative injury [3]; thus, inhibition of lipid peroxidation is extremely important in the maintenance of cell integrity. Quantitative assays of lipid peroxidation are essential for evaluating oxidative damage from various sources, and in testing the efficacy of antioxidant interventions.

A major obstacle in studies of lipid peroxidation in intact cells is the lack of probes sensitive enough to allow detection of peroxidation before significant levels of damage activate cellular repair systems. While a variety of techniques have been used successfully for analysis of lipid peroxidation in membrane suspensions and cell homogenates, there has been no report of a quantitative assay that accurately reflects the degree of peroxidation induced by oxidants in living cells. The major hurdle for such an assay is the rapid turnover and repair of membrane lipids in live cells. Presently applied assays of peroxidative damage often require relatively large doses of oxidants in order for effects to become apparent. Abnormally high loads of oxidants, however, make it difficult or impossible to distinguish the direct oxidative damage caused by an agent from its toxic, cell-killing effects. Dead cells are subject to further, degradative oxidative attack, leading to artifactual oxidative damage.

In this paper, we report a novel method based on the use of *cis*-parinaric acid (PnA) as a reporter molecule for lipid peroxidation in living mammalian cells. PnA is a natural, 18-carbon fatty acid containing four conjugated double bonds. The conjugated π-orbital system of PnA is highly susceptible to peroxidation and makes it a sensitive fluorescent probe since its fluorescence is lost upon peroxidation [4,5]. PnA has proven to be useful as a fluorescent probe in physical-chemical studies of model membranes, and as a probe of lipid peroxidation in chemical systems, including lipoproteins and simple cell membrane systems (erythrocyte ghosts) [5–10]. In these model systems, PnA was incorporated into membranes or lipoproteins by physical, non-physiological means, i.e., by partitioning. These studies provide much valuable data, but suffer from the major drawback that PnA was not incorporated in a physiologically-relevant manner, and thus may have had an unnatural distribution and orientation.

PnA is a natural fatty acid that can be incorporated by normal metabolism into intact cell membrane phospholipids as shown by Rintoul and Simoni [11]. In the present study, we developed a physiological procedure for integration of PnA into membrane phospholipids of cultured cells. We demonstrated that PnA incorporated into different classes of phospholipids was sensitive to low levels of oxidative stress as measured by loss of PnA fluorescence. We further demonstrated that selective oxidation of PnA-incorporated phospholipids occurred under different experimental oxidative stress conditions. Because the fluorescence of PnA is irreversibly lost upon oxidation and lipid repair mechanisms will not mask this loss in live cells, our results support the hypothesis that PnA may be a useful tool for the analysis of non-toxic oxidative stress events in intact cellular membranes in living cells.

2. Materials and methods

2.1. Chemicals

Cis-parinaric acid (PnA) (*Z*-9,*E*-11,*E*-13,*Z*-15-octadecatetraenoic acid) was purchased from Molecular Probes (Eugene, OR). The purity of each lot of purchased PnA was determined by UV spectrophotometry (Shimadzu UV160U spectrophotometer) using the molar extinction $\varepsilon_{304nm,\ EtOH} = 80 \cdot 10^3\ M^{-1}\ cm^{-1}$ [4]. Alamethicin, containing peptide F30 as the major component, was prepared by V.R. (at Moscow State University, Russian Federation). HPLC grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA). Deferoxamine methanesulfonate was purchased from CIBA-Geigy (Basel, Switzerland). AMVN was a kind gift from Wako Chemicals USA (Richmond, VA). All other chemicals, including fatty acid-free human serum albumin (hSA) and phospholipids, were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

HL-60, K562 and K/VP.5 cells were grown in RPMI 1640 medium. CHO cells were grown in Eagle's minimal essential medium. Medium in each case was supplemented with 10% fetal calf serum and cultures were maintained at 37°C in a 5% CO_2 atmosphere. Cell viability was determined microscopically on a hemacytometer using the Trypan blue exclusion assay.

2.3. Incorporation of PnA into cell phospholipids

PnA was incorporated into cells by addition of its hSA complex (PnA-hSA) to cell suspensions (monolayers for CHO cells). The complex was prepared by adding PnA (500 μg, 1.8 μmol) in 25 μl of dimethylsulfoxide to hSA (50 mg, 760 nmol) in 1 ml of phosphate-buffered normal saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4 (pH 7.4). Cells in log phase growth were rinsed twice with L1210 buffer contain-

ing 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). Cells were diluted to a density of $1.0 \cdot 10^6$ cells/ml, then incubated with different added volumes of PnA-hSA complex (final concentrations of 1–8 μg/ml PnA) in L1210 buffer at 37°C for 2 h, or for the time periods indicated in experiments in which the time-course of incorporation into cell phospholipids was examined. Control samples were incubated in the presence of hSA without PnA under the same conditions. After the incubation period, cells were pelleted by centrifugation then washed twice with L1210 or PBS buffer with or without hSA (0.5 mg/ml). Aliquots were taken for determination of cell viability.

2.4. tert-Butyl hydroperoxide (t-BuOOH) or 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced peroxidation

Cells loaded with PnA were incubated in the presence of either t-BuOOH (0.02–5.0 mM) or AMVN (0.5 mM) in L1210 buffer at 37°C for 1 h. After incubation, cells were pelleted by centrifugation then washed twice with PBS. Aliquots were taken for determination of cell viability and cell pellets were used for lipid extraction.

2.5. Irradiation procedures

A ^{137}Cs source (J.L. Shepherd Model 143) with a dose rate of 4.76 ± 0.143 Gy/min was used for irradiation experiments. The cell suspension in PBS was irradiated at room temperature in a cumulative dose design in which samples of the suspension where removed at appropriate intervals up to 26.8 min. Some experiments used separate cell samples for each dose without any detectable difference from the cumulative method.

2.6. Extraction of cell lipids

Total lipid extracts were obtained using a slightly modified Folch procedure [12]. Cells were pelleted by centrifugation, medium was removed, and cells were suspended in methanol (0.5 ml) containing butylated hydroxytoluene (BHT, 0.1 mg). The suspension was mixed with chloroform (1 ml) and, to insure complete extraction, kept for 1 h under a nitrogen atmosphere on ice in the dark. After addition of 0.1 M NaCl (0.3 ml) and vortex mixing (still under a nitrogen atmosphere), the chloroform layer was separated by centrifugation ($1500 \times g$, 5 min). The chloroform was evaporated with a stream of nitrogen and the lipid extract was dissolved in 4:3:0.16 (v/v/v) isopropanol/hexane/water (0.2 ml). Control experiments demonstrated that the procedure recovered more than 95% of cell phospholipids.

2.7. HPLC analysis of cell lipids

Lipid extracts were separated with an ammonium acetate gradient by normal phase HPLC as per [13] with the minor modifications described below. A 5-μm Supelcosil LC-Si column (4.6×250 mm) was employed with the following mobile phase flowing at 1 ml/min: solvent A (57:43:1 isopropanol/hexane/water, v/v/v), solvent B (57:43:10 isopropanol/hexane/40 mM aqueous ammonium acetate, pH 6.7, v/v/v), 0–3 min linear gradient from 10% B to 37% B, 3–15 min isocratic at 37% B, 15–23 min linear gradient to 100% B, 23–45 min isocratic at 100% B. A Shimadzu high performance liquid chromatograph (model LC-600) equipped with an in-line configuration of fluorescence (model RF-551) and UV-VIS (model SPD-10AV) detectors was employed. The effluent was monitored at 205 nm to gauge separation of lipids. Fluorescence of PnA in eluates was monitored by emission at 420 nm after excitation at 324 nm. Ammonium acetate was used for reproducibility and to separate cardiolipin from the solvent front. UV and fluorescence data were processed and stored in digital form with Shimadzu EZChrom software. In some experiments phospholipid fractions were collected manually for subsequent analysis of unsaturated fatty acids as described below.

2.8. Saponification and HPLC analysis of free fatty acids

Total lipids or phospholipids were dissolved in ethanol (5 ml) and treated with deferoxamine methanesulfonate (0.1 ml of a 25 mg/ml aqueous solution), aqueous ascorbic acid (1 ml of a 25% w/v solution), and KOH (1 ml of 10 M solution). The solutions were kept in the dark at room temperature for 14 h, then extracted with HCl-acidified n-hexane (10 ml, apparent pH 3–4). The hexane phase was collected by centrifugation, evaporated to dryness, and the resulting residue was dissolved in acetonitrile containing 0.14% acetic acid (v/v). Reversed phase C_{18} HPLC analysis with diode array detection was performed as previously described [14].

2.9. Measurements of PnA fluorescence in cell suspensions and total lipid extracts

Fluorescence emission spectra of PnA in cell suspensions and total lipid extracts were measured from 350–550 nm using a Shimadzu RF5000U spectrofluorometer equipped with 5 nm resolution slits at a scan speed 1800 nm/min and an excitation wavelength of 324 nm. PnA fluorescence in cell suspensions was determined in 3 ml of PBS at $3.3 \cdot 10^5$ cells/ml in the presence of the membrane pore-forming peptide alamethicin (0.1 μg/ml) that was employed to lyse cells and to reduce turbidity [15]. PnA fluorescence in total lipid extracts was determined in isopropanol/hexane/water (4:3:0.16, v/v/v).

3. Results

3.1. Effect of cis-parinaric acid on cell viability

Four different cell lines, Chinese hamster ovary (CHO) fibroblasts and three human leukemia cell lines: human promyelocytic leukemia HL-60 cells, erythroleukemia K562 cells, and an etoposide (VP-16)-resistant subclone of K562 cells, K/VP.5 [16], used in the study were incubated with PnA-hSA (1–8 μg/ml PnA) for 2 h at 37°C. This treatment had no measurable effects on cell viability, which was > 90% for all PnA-treated cells and their controls that were incubated in PBS-containing hSA.

3.2. Uptake of cis-parinaric acid by cells

The uptake of PnA by the four cell lines was determined by measurement of its fluorescence in suspensions of washed cells and in total lipid extracts from the cells. Fig. 1 shows the fluorescence emission spectrum recorded from a suspension of CHO cells after their incubation with and without hSA-PnA, respectively. Cells treated with hSA-PnA and then washed twice with PBS possessed a fluorescence spectrum characteristic of PnA. No significant fluorescence could be detected from suspensions of cells incubated with hSA only.

Quantitatively, the fluorescence intensity recorded from suspensions of $1 \cdot 10^6$ CHO cells (incubated for 2 h with 4 μg PnA per ml in the form of the hSA-PnA complex) corresponded to 15–25% of the amount of total PnA added to the cell suspension. Essentially the same results were

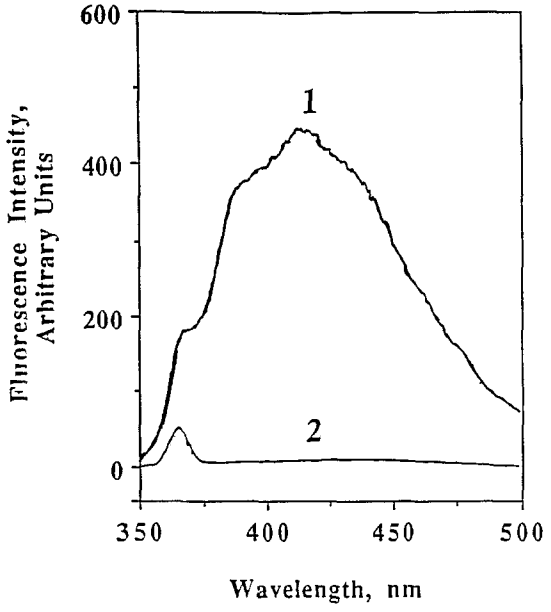

Fig. 1. Fluorescence emission spectra (excitation at 324 nm) from CHO cells incubated with (1) or without (2) *cis*-parinaric acid. CHO cells were incubated with PnA (4 μg/ml) or without PnA for 2 h at 37°C. PnA-loaded cells were washed twice with PBS. Fluorescence was measured in PBS at a cell concentration of $3.3 \cdot 10^5$ cells/ml. Alamethicin (100 μg/ml) was added to lyse the cells and to reduce turbidity.

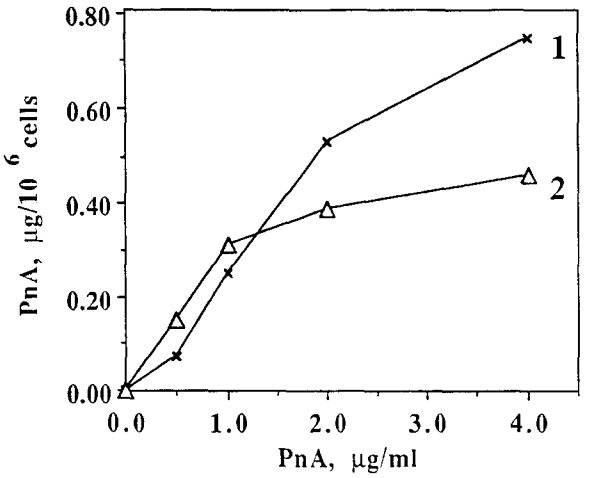

Fig. 2. Dependence of fluorescence from total lipid extracts of CHO (1) and K562 (2) cells on the concentration of *cis*-parinaric acid in the incubation medium. Cells ($1 \cdot 10^6$/ml) were incubated with different concentrations of the hSA-PnA complex (0.5–4 μg of PnA/ml of medium) for 2 h at 37°C. PnA-loaded cells were washed twice with PBS. Cell lipids were extracted with chloroform-methanol, dried under a stream of nitrogen, and fluorescence was measured in 3 ml of 4:3:0.16 (v/v) isopropanol/hexane/water by excitation at 324 nm and monitoring emission at 420 nm. This experiment was repeated two times; the one shown is representative.

obtained when total lipid extracts from cells were examined fluorometrically. Since more than 95% of total PnA fluorescence was found in the chloroform phase after a single extraction, only less than 5% of PnA (its fluorescence) might be accounted for as PnA associated with proteins in the aqueous phase. Repeated extraction of the methanol/water layer with chloroform did not yield any additional fluorescence in the chloroform phase. Comparison of the fluorescence intensity of the cell suspension to that of total lipid extract from the cells showed that the content of PnA was identical in both cases, i.e., 15–25% of the added amount of PnA was incorporated. The PnA fluorescence intensity measured from total lipid extracts of CHO and K562 cells was dependent on the concentration of PnA in the incubation medium (Fig. 2). At a PnA concentration of 4 μg/ml the fluorescence intensity of total lipid extracts from $1 \cdot 10^6$ cells corresponded to 0.7 ± 0.3 μg (2.5 + 1.1 nmol) PnA/10^6 cells. Thus, only a fraction of added PnA was integrated into cells and the remaining 75–85% was bound to hSA and/or underwent metabolic or oxidative degradation during the incubation.

3.3. Incorporation of cis-parinaric acid into cell lipids

We used an HPLC procedure to separate and detect PnA integrated into the various lipid classes in the cells. Fig. 3 shows the typical UV- and fluorescence-detected chromatograms of lipids from K562 cells incubated in the presence or absence of hSA-PnA. Five phospholipid peaks were well-resolved and identified by comparison with commercial standards as cardiolipin (CL, retention time

12.0 min), phosphatidylinositol (PI, retention time 13.2 min), phosphatidylethanolamine (PE, retention time 14.6 min), phosphatidylserine (PS, retention time 29.1 min), and phosphatidylcholine (PC, retention time 32.5 min), respectively. All were apparent by both UV (Fig. 3A) and fluorescence (Fig. 3B) detection. Fluorescent HPLC components, under the excitation and emission limits used, were not detected in extracts from cells incubated with hSA in the absence of PnA.

Previous work has demonstrated that HPLC separation of phospholipids results in two poorly resolved peaks of PC as detected by UV absorbance [13,17]. In addition, two species of SPH are identified with this methodology: one incompletely resolved from the PC peak and another that is distinct and follows the PC peak [13]. Accordingly, we observed splitting of the PC peak and an SPH peak in UV tracings of the chromatograms plus two more partially resolved PC peaks and two SPH peaks in fluorescence tracings of the chromatograms (Fig. 3B and Fig. 4). The two PC and two SPH peaks have previously been tentatively identified as different molecular species of PC and SPH, respectively [13]. In our experiments, we always calculated total PC as the sum of the area of both PC peaks. Since one of the SPH peaks was not sufficiently resolved from the PC peak, we chose not to quantitate the areas of SPH peaks. A better normal phase separation of PC and SPH can be achieved using acetonitrile/methanol/water mixtures as described earlier [18].

Fluorescence detection allowed identification of free PnA in HPLC chromatograms (retention time 4.8 min, Figs. 3 and 5). The magnitude of the free PnA peak could be significantly decreased by washing the cells with a fatty acid-free hSA solution in PBS subsequent to the initial incubation with hSA-PnA as shown for CHO cells in Fig. 5, inset. Because unincorporated PnA could be utilized in lipid repair reactions occurring as a consequence of oxidative stress, cells were washed with hSA in all subsequent experiments. While phospholipid patterns were not markedly different in the four cell lines studied, the metabolic integration of PnA into membrane phospholipids was cell type-specific (Figs. 3 and 5, Table 1).

PnA incorporation into different membrane phospholipids in the four cell lines was dependent on the concentration of PnA in the incubation buffer (Fig. 6). Incorporation of PnA into CL, PS, PEA, and PC in HL-60, K562 and K/VP.5 cells displayed saturation kinetics in the range of PnA concentrations used (from 1 to 8 μg/ml). The integration of PnA into PI in CHO cells increased linearly over this range of concentrations.

The time-course of PnA incorporation into cellular phospholipids is shown in Fig. 7. At a PnA concentration of 4 μg/ml, maximal incorporation into all phospholipid

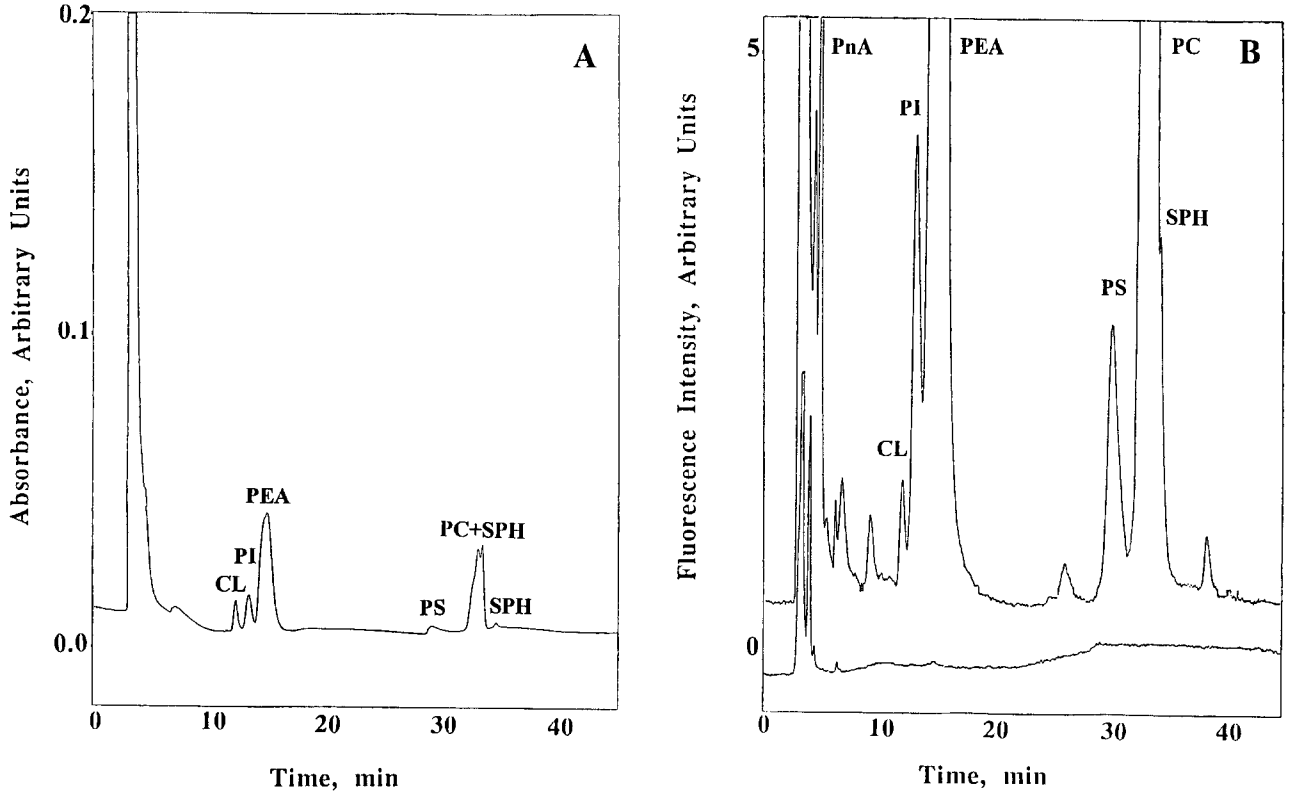

Fig. 3. Normal phase HPLC chromatogram of total lipids extracted from $1 \cdot 10^6$ K562 cells. (A) UV detection, 205 nm; (B) fluorescence detection, excitation at 324 nm, emission at 420 nm; (1), cells incubated with the hSA-PnA complex (4 μg of PnA/0.4 mg hSA/ml of medium) for 2 h at 37°C then washed twice with PBS; (2), cells incubated with hSA (0.4 mg/ml) only under the same conditions. CL, cardiolipin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PnA, free *cis*-parinaric acid; SPH, sphingomyelin.

Table 1
Incorporation of *cis*-parinaric acid into phospholipids of four different mammalian cell lines

Cell line	ng of PnA incorporated per 10^6 cells [a]						% Incorporation into phospholipids
	CL [b]	PI	PEA	PS	PC	total lipids	
CHO ($n = 5$)	0.28 ± 0.1	14 ± 6.0	22 ± 4.0	8 ± 2.0	95 ± 15	1000 ± 200	14
HL-60 ($n = 5$)	2.9 ± 0.9	12 ± 4.4	41 ± 9.6	4.4 ± 1.6	163 ± 27	700 ± 200	35
K562 ($n = 5$)	1.0 ± 0.3	12 ± 1.6	166 ± 13.7	12 ± 1.6	433 ± 48	1100 ± 100	57
K/VP.5 ($n = 3$)	2.3 ± 0.9	11 ± 1.5	175 ± 25	16 ± 4.0	336 ± 34	940 ± 146	57

[a] Cells ($1 \cdot 10^6$ cells/ml) were incubated for 2 h at 37°C in L1210 medium (without calf fetal serum and phenol red) in the presence of 4 μg/ml *cis*-parinaric acid. The cells were isolated by centrifugation, washed once with PBS containing 0.5 mg/ml human serum albumin and once with PBS, then extracted with chloroform-methanol (see Section 2). Values are means ± S.D.
[b] CL, cardiolipin; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine.

classes, except PI in K562 and CHO cells, was reached after 1–2 h of incubation (Fig. 7).

The HPLC procedure used for separation of cell lipids enabled the recovery of essentially all of the PnA present (as detected by its characteristic fluorescence) in the total lipid extracts from cells. The total amount of PnA integrated into cell lipids in the 2 h loading experiments was estimated from the sum of all peak areas in fluorescence HPLC tracings and averaged between 700 and 1100 ng per 10^6 cells (Table 1). Nearly the same amount of PnA (700 ± 300 ng PnA/10^6 cells) was found in total lipid extracts from the cells (as estimated by the fluorescence intensity of the extracts). Among the cell lines studied, the amounts of PnA in phospholipids varied from 100 to 500 ng/10^6 cells. Importantly, the fluorescence of PnA in phospholipids constituted 15–60% of the total fluores-

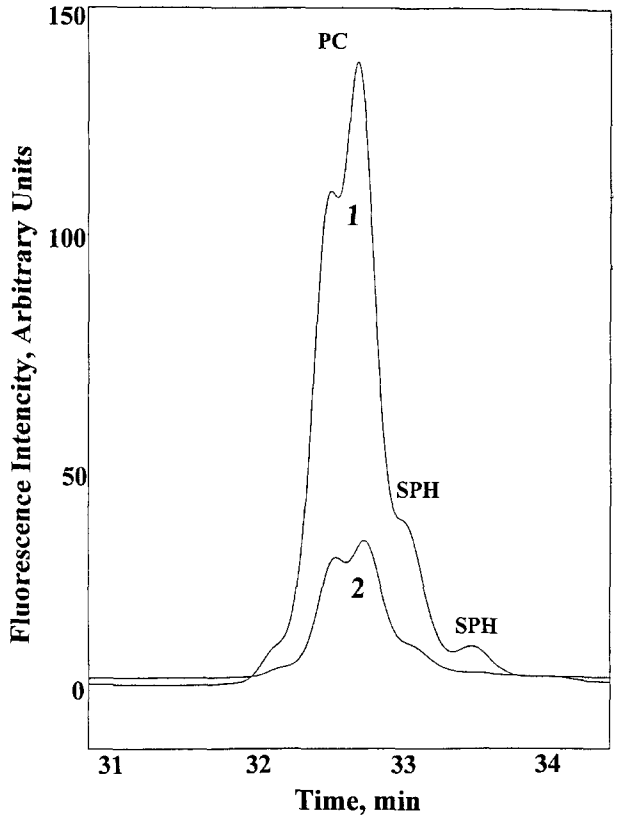

Fig. 4. t-BuOOH-induced oxidation of PnA-phosphatidylcholine and PnA-sphingomyelin in K562 cells. Fluorescence detection: excitation at 324 nm, emission at 420 nm; (1), PnA-loaded cells incubated without t-BuOOH for 1 h at 37°C; (2), PnA-loaded cells incubated with 5 mM t-BuOOH under the same conditions. PC, phosphatidylcholine; SPH, sphingomyelin.

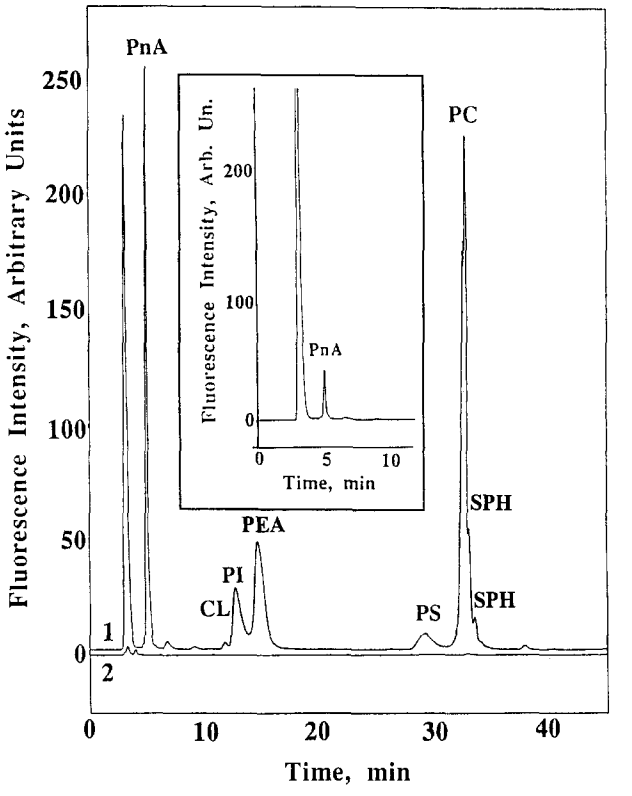

Fig. 5. Normal phase HPLC chromatogram of total lipids extracted from $1 \cdot 10^6$ CHO cells. Fluorescence detection, excitation at 324 nm, emission at 420 nm; (1), cells incubated with the hSA-PnA complex (4 μg of PnA/0.4 mg hSA/ml of medium) for 2 h at 37°C then washed twice with PBS; (2), cells incubated with hSA (0.4 mg/ml) only under the same conditions. CL, cardiolipin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PnA, free *cis*-parinaric acid; SPH, sphingomyelin. Inset: A part of HPLC chromatogram of total lipids extracted from $1 \cdot 10^6$ CHO cells incubated with hSA-PnA complex (4 μg of PnA/0.4 mg hSA/ml of medium) for 2 h at 37°C then washed once with fatty-acid free hSA (0.5 mg/ml) and once with PBS.

cence recovered in all fluorescently-detected HPLC peaks (Table 1). A significant fraction of PnA was not integrated into phospholipids. We suggest that this unaccountable portion of PnA fluorescence in non-phospholipid HPLC peaks might be due to PnA in neutral lipids that could not be separated (except for free PnA) under the HPLC conditions used.

Average values for PnA incorporated into the five major phospholipid classes are presented in Table 1. On an absolute scale, PC constituted the major pool of integrated PnA in all of the cell lines, followed by PEA, PI, PS and CL. The distribution of PnA between different classes of phospholipids among cell lines differed significantly. It is also notable that the fluorescence intensity within peaks of minor phospholipids – CL, PS, and PI – did not exceed 2–3% of the total fluorescence intensity of lipid extracts. In relative units, calculated as the ratio of the fluorescence-detected peak area of PnA in a given phospholipid to the UV-detected peak area of that phospholipid, maximum integration of PnA occurred into PC and PS (Table 2).

To evaluate the fraction of PnA-acylated phospholipids in the overall pool of membrane phospholipids in the cells, we quantified unsaturated fatty acid residues in total lipid extracts from K562 and K/VP.5 cells. A comparison of the total amounts of unsaturated fatty acid residues with those of PnA in lipid extracts showed that PnA accounted for less than 1% of membrane phospholipid unsaturated fatty acid residues (Table 3).

3.4. Effects of oxidants on cis-parinaric acid-labelled phospholipids in cells

We studied the effects of three different kinds of oxidative stress on metabolically incorporated PnA in membrane phospholipids. Oxidation in the cells was initiated with the organic hydroperoxide, t-BuOOH, the azo free radical-initiator, AMVN, or gamma radiation. These three oxidants were chosen because they are commonly used to induce oxidative stress both *in vitro* and *in vivo*.

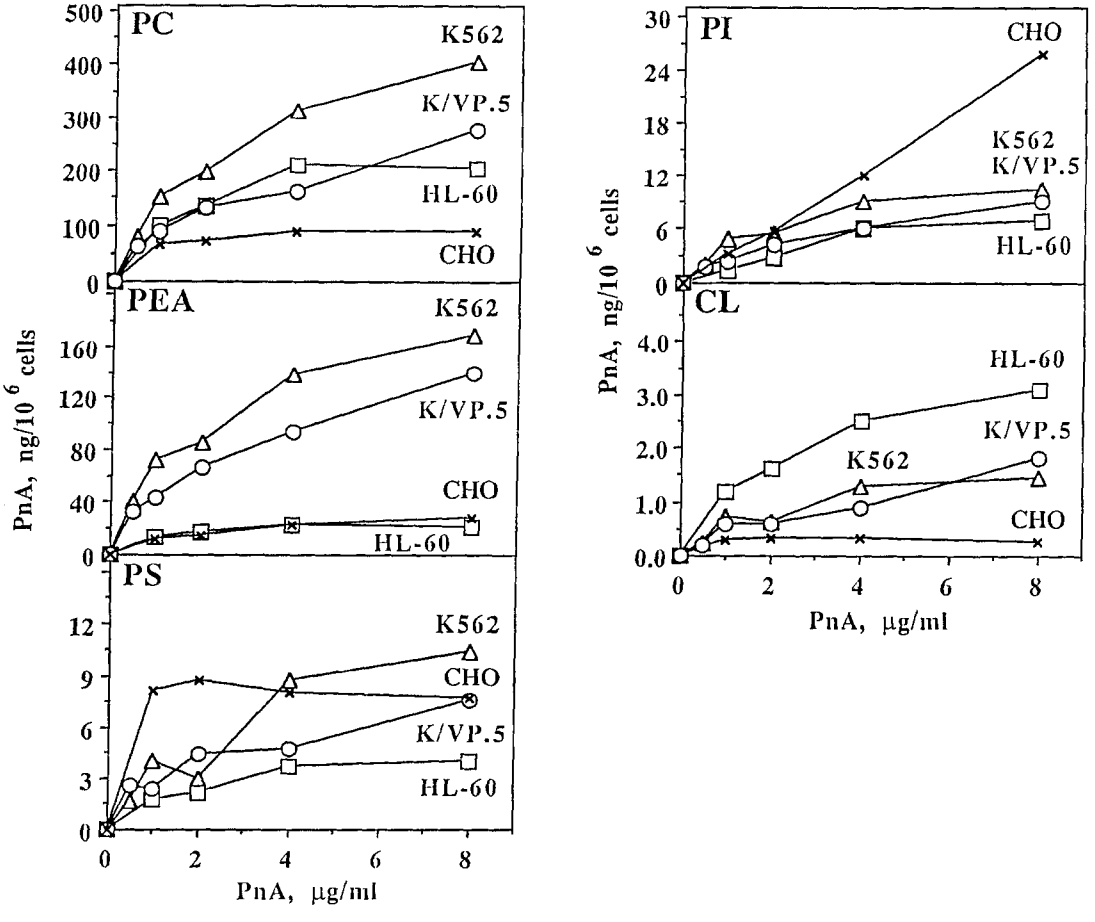

Fig. 6. Dependence of fluorescence from individual phospholipids of K562, K/VP.5, CHO and HL-60 cells on the concentration of *cis*-parinaric acid in the incubation medium determined by normal phase HPLC with fluorescence detection. Cells ($1 \cdot 10^6$/ml) were incubated with different concentrations of the hSA-PnA complex (0.5–4 μg of PnA/0.05–0.4 mg hSA/ml of medium) for 2 h at 37°C. PnA-loaded cells were washed with fatty-acid free hSA (0.5 mg/ml) and then with PBS. Cell lipids were extracted with chloroform-methanol, dried under a stream of nitrogen, and HPLC separation was performed. CL, cardiolipin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. This experiment was repeated three times; the one shown is representative.

Table 2
Ratios of the fluorescence to UV peak areas of phospholipids in CHO and K562 cells loaded with *cis*-parinaric acid

Cell line	Ratio of fluorescence/UV peak areas [a]				
	CL [b]	PI	PEA	PS	PC
CHO ($n = 5$)	0.04 ± 0.01	0.5 ±0.04	0.15 ± 0.01	0.84 ± 0.16	0.8 ± 0.1
K562 ($n = 3$)	0.07 ± 0.02	0.36 ± 0.04	1.6 ±0.5	12.0 ±6.0	5.8 ± 2.0

[a] Cells ($1 \cdot 10^6$ cells/ml) were incubated for 2 h at 37°C in L1210 medium (without calf fetal serum and phenol red) in the presence of 4 μg/ml *cis*-parinaric acid. The cells were isolated by centrifugation, washed once with PBS containing 0.5 mg/ml human serum albumin and once with PBS, then extracted with chloroform-methanol (see Section 2). Values are means ± S.D.
[b] CL, cardiolipin; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine: PC, phosphatidylcholine.

3.4.1. *Effects of t-BuOOH*

Initially, we compared the effects of oxidants on the fluorescence of PnA-labelled cells in suspensions with that of individual phospholipids (by HPLC) from cells labelled with PnA. Results of measurements using K562 and K/VP.5 cells exposed to t-BuOOH are presented in Table 4. Incubation of PnA-labelled cells with increasing concentrations of t-BuOOH (in the range from 0.02 mM to 0.1 mM) did not significantly change the fluorescence response from the suspensions (Table 4). Extracts of these same PnA-labelled K562 and K/VP.5 cells were analyzed by HPLC with fluorescence detection to determine if there were any differences in the oxidation of PnA incorporated into the individual phospholipid classes. Incubation of cells with t-BuOOH at concentrations as low as 0.02–0.04 mM induced significant oxidation of PnA in specific phospho-

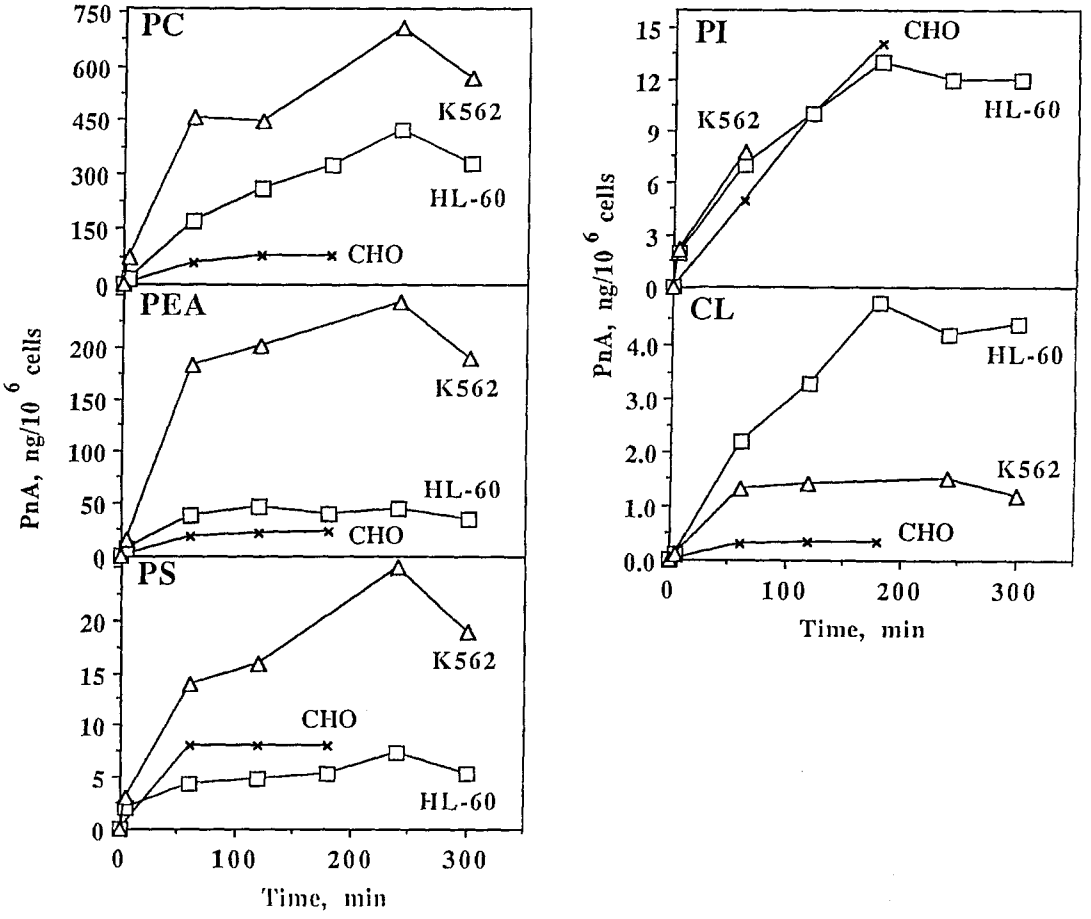

Fig. 7. Time dependence of incorporation of *cis*-parinaric acid into phospholipids of K562, K/VP.5, CHO and HL-60 cells. Cells ($1 \cdot 10^6$/ml) were incubated with 4 μg of PnA/0.4 mg hSA/ml of medium for 2 h at 37°C. PnA-loaded cells were washed with fatty-acid free hSA (0.5 mg/ml) and then with PBS, lipids were extracted, and normal phase HPLC separation with fluorescence detection was performed. CL, cardiolipin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. This experiment was repeated three times; the one shown is representative.

Table 3
Effects of *tert*-butyl hydroperoxide on the composition of unsaturated fatty acids in total lipids of K562 and K/VP · 5 cells

Cells/Conditions [a]	Fatty acid (nmol/10^6 cells)				
	22:6	20:4	18:2	18:1	PnA
K562	0.98	7.61	11.94	33.78	0.00
K562 + t-BuOOH	0.80	3.81	12.69	41.67	0.00
K562 + PnA	0.85	3.34	11.42	37.56	0.53
K562 + PnA + t-BuOOH	0.41	2.21	9.37	33.44	0.35
KJVP · 5	1.38	3.20	10.77	32.48	0.00
K/VP · 5 + t-BuOOH	1.01	2.53	10.86	33.41	0.00
K/VP · 5 + PnA	1.18	2.78	10.49	28.19	0.26
K/VP · 5 + PnA + t-BuOOH	0.42	1.74	9.56	26.86	0.12

[a] Cells ($1 \cdot 10^6$/ml) were incubated with or without 4 μg/ml *cis*-parinaric acid (PnA) in L1210 buffer for 2 h at 37°C, then incubated with t-BuOOH (5 mM) in L1210 for 60 min at 37°C. Cell lipids were extracted with chloroform-methanol and dried under a stream of nitrogen gas. Determinations of the unsaturated fatty acid compositions were performed by C_{18} HPLC as described in Section 2. These experiments were repeated three times; those shown are representative.

lipids (Fig. 8, Table 5). At these low t-BuOOH concentrations, the oxidation was highest in PnA-acylated PS, while it was significantly less pronounced in PEA and PC and was almost undetectable in CL and PI (except for K/VP.5 cells). At higher t-BuOOH concentrations (0.1–1.0 mM), however, all phospholipid classes were oxidized in both cell lines. PnA-acylated PS was completely oxidized by low concentrations of t-BuOOH such that higher t-BuOOH concentrations produced no additional effects. Importantly, 1 h incubations of K562 or K/VP.5 cells with the low concentrations of t-BuOOH did not affect cell viability as determined by trypan blue exclusion. Higher t-BuOOH concentrations, 0.1 and 1.0 mM, caused 15–20% and greater than 75% decreases, respectively, in the cell survival of K562 cells. In K/VP.5 cells, the effects of t-BuOOH were stronger: 55–60% decrease of cell survival at 0.1 mM and greater than 75% at 1.0 mM. These results demonstrated that t-BuOOH at low concentrations specifically induced oxidation of PnA residues incorporated into PS (and to a much lesser extent in PEA and PC) of *live cells*. t-BuOOH at higher concentrations caused significant cell death and thus random oxidation of PnA residues in all phospholipids.

Table 4
Effect of *tert*-butyl hydroperoxide on the fluorescence of K562 and K/VP · 5 cells loaded with *cis*-parinaric acid

t-BuOOH (mM)	PnA (μg) detected/$1 \cdot 10^6$ cells [a]	
	K562	K/VP.5
0.00	0.9	0.7
0.02	1.2	0.7
0.04	1.2	0.6
0.08	0.9	0.7
0.10	0.7	0.6

[a] Cells ($1 \cdot 10^6$ cell/ml) were incubated for 2 h at 37°C in L1210 buffer (without calf fetal serum and phenol red) in the presence of 4 μg/ml *cis*-parinaric acid. The cells were isolated by centrifugation, washed once with PBS containing 0.5 mg/ml human serum albumin and once with PBS, then extracted with chloroform-methanol (see Section 2). These experiments were repeated three times; those shown are representative.

In HL-60 cells, PnA-acylated PEA, PS and CL were sensitive to low concentrations of t-BuOOH (0.02 and 0.04 mM), while PC and PI were not affected by these concentrations (Fig. 8). PI was the only phospholipid that responded to the low concentrations of t-BuOOH in CHO cells. At high t-BuOOH concentrations, again, all phospholipid classes (except PS in CHO cells) underwent extensive oxidation.

3.4.1.1. Effects of antioxidants on t-BuOOH-induced oxidation of cis-parinaric acid. The t-BuOOH-induced decrease of PnA-PS fluorescence in K562 cells was prevented by a thiol antioxidant, dihydrolipoic acid (DHLA) [19]. At a concentration of 0.5 mM, DHLA completely protected PS-PnA against oxidation induced by 100 μM t-BuOOH (data not shown). Another known free radical scavenger, the hindered phenol VP-16 [20], was also effective in protecting PS-PnA in K562 cells against t-BuOOH-induced (100 μM) oxidation (60% protection at 0.1 mM VP-16; data not shown).

Table 5
Efffect of *tert*-butyl hydroperoxide and 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN) on the fluorescence of phospholipids in K562 cells loaded with *cis*-parinaric acid

Phospholipid	*cis*-Parinaric acid, % of control [a]	
	t-BuOOH	AMVN
CL [b]	73.5 ± 9.4	57.0 ± 7.4
PI	56.3 ± 7.3	15.9 ± 4.3
PEA	60.0 ± 11.1	21.1 ± 0.9
PS	15.7 ± 3.0	29.4 ± 0.9
PC	62.8 ± 4.4	20.0 ± 1.8

[a] Cells ($1 \cdot 10^6$ cells/ml) were incubated for 2 h at 37°C in L1210 buffer (without calf fetal serum and phenol red) in the presence of 4 μg/ml *cis*-parinaric acid then incubated with t-BuOOH (0.1 mM) or AMVN (0.5 mM) in L1210 buffer for 60 min at 37°C. Lipids were extracted with chloroform-methanol as described in Section 2. Data are means ± S.D., $n = 3$.

[b] CL, cardiolipin; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine.

3.4.2. Effects of AMVN on K562 cells

AMVN, a thermally-activated lipid-soluble azo-initiator of peroxyl radicals [21], produced a pattern of oxidative stress in phospholipids of K562 cells different from that induced by t-BuOOH (Table 5). We found that all phospholipids were nearly equally affected by 0.2 mM AMVN in the course of 2 h incubation at 37°C.

3.4.3. Effects of gamma irradiation

In CHO cells exposed to gamma-irradiation (4–128 Gy), PS was the most affected phospholipid class. The radiation-induced oxidation destroyed 34% of the PnA incorporated into PS. PI was the other phospholipid affected by irradiation, and 24% of its PnA was lost (Table 6). The other three phospholipid classes were apparently

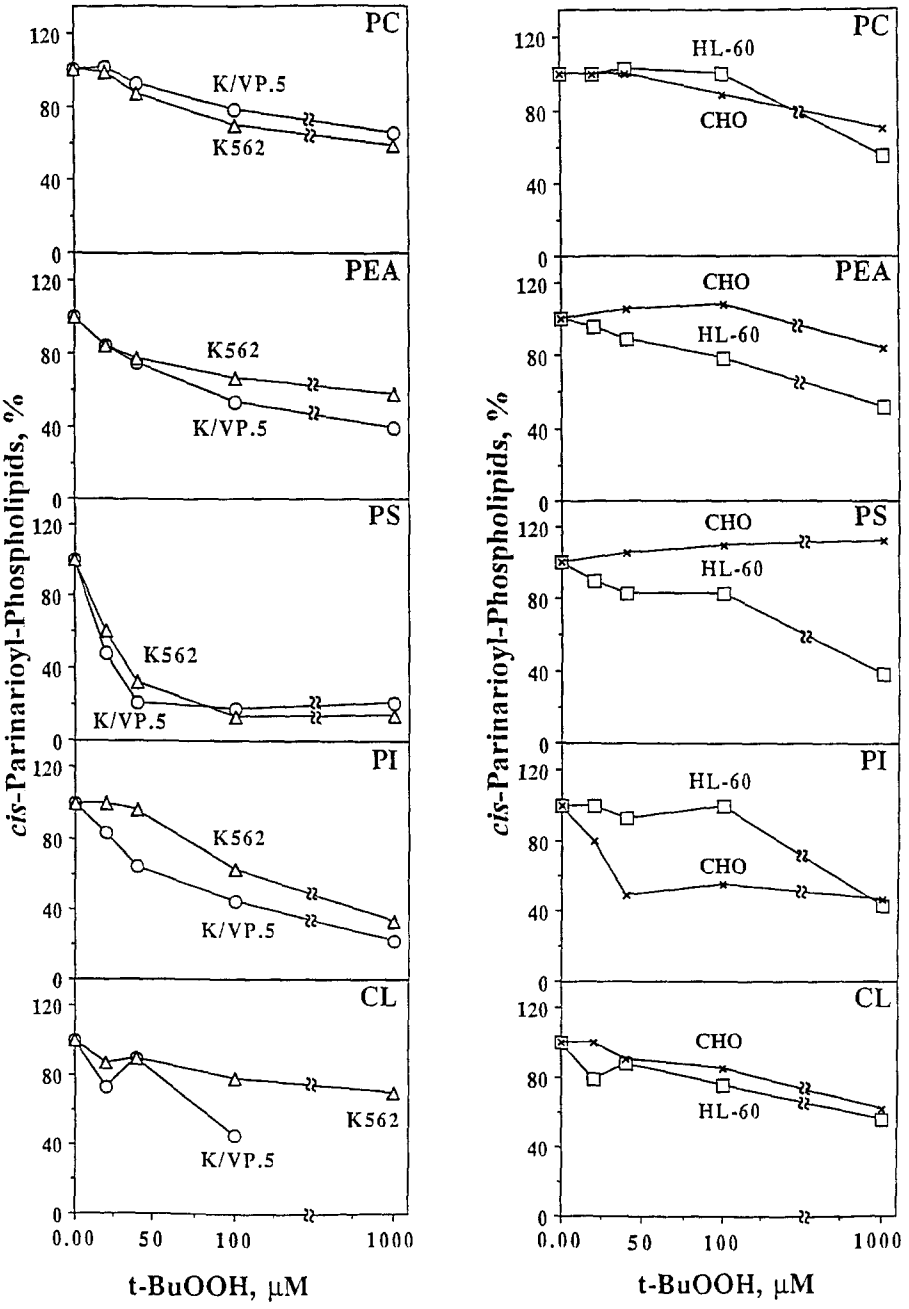

Fig. 8. Effects of *tert*-butyl hydroperoxide on *cis*-parinaric acid-integrated phospholipids in K562, K/VP.5, CHO and HL-60 cells. Cells ($1 \cdot 10^6$/ml) were incubated with 4 μg of PnA/0.4 mg hSA/ml in L1210 medium for 2 h at 37°C, washed with L1210 medium containing fatty-acid free hSA (0.5 mg/ml) and then with L1210 medium. PnA-loaded cells were then incubated for 1 h with the indicated concentrations of t-BuOOH in L1210 medium. The cells were isolated by centrifugation, washed twice with PBS, lipids were extracted, and normal phase HPLC separation with fluorescence detection was performed. CL, cardiolipin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Experiments were repeated three times with K562 and CHO cells and two times with K/VP.5 and HL-60 cells; those shown are representative.

Table 6
Effect of gamma irradiation on *cis*-parinaric acid-loaded phospholipids in CHO cells

Exposure	Ratio of fluorescence/UV peak areas [a]				
(Gy)	CL [b]	PI	PEA	PS	PC
0	0.04	0.42	0.12	0.93	0.98
4	0.03	0.37	0.11	0.88	0.92
16	0.03	0.36	0.11	0.71	0.95
128	0.03	0.34	0.11	0.65	0.92

[a] CHO cells growing in dishes were incubated for 2 h at 37°C in PBS with *cis*-parinaric acid (4 μg/ml), scraped from the dishes, washed once with PBS containing 0.5 mg/ml human serum albumin and once with PBS, isolated by centrifugation, and suspended in 1 ml of PBS. The cells were exposed to gamma irradiation at room temperature, then extracted with chloroform-methanol (see Section 2). This experiment was repeated two times; the one shown is representative.

[b] CL, cardiolipin; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine.

unaffected, even at the highest dose of radiation-induced oxidative insult.

3.5. Oxidation of polyunsaturated fatty acids and PnA by high concentrations of t-BuOOH

To compare the susceptibility of polyenoic fatty acid residues with that of PnA in membrane phospholipids, we performed experiments in which two different concentrations of t-BuOOH were used to cause either a selective oxidation or random oxidation of PnA in membrane phospholipids, respectively, and compared them with the disappearance of polyenoic fatty acids after saponification of the sum of phospholipid fractions (measured by conventional reverse-phase C_{18}-HPLC [14]). From Fig. 9 it is evident that loss of highly unsaturated fatty acids such as 22:6 and 20:4 was significant when the cells were incubated with t-BuOOH at a high concentration (5.0 mM).

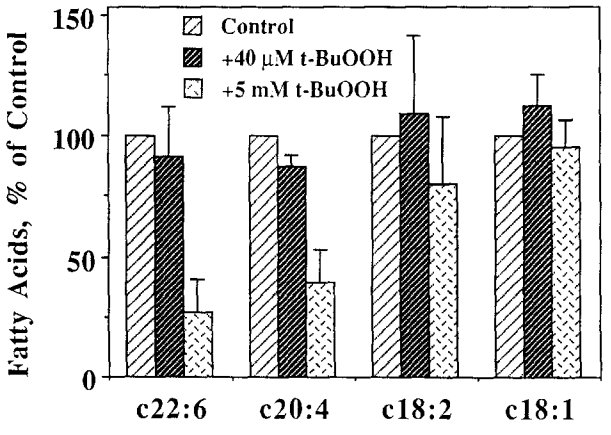

Fig. 9. Effects of t-BuOOH on unsaturated fatty acids in phospholipids of K562 cells. Cells ($1 \cdot 10^6$/ml) were incubated with t-BuOOH (5.0 mM) in L1210 medium for 60 min at 37°C. Cell lipids were extracted with chloroform-methanol and then separated by normal phase HPLC. Phospholipid fractions (CL, PC, PEA, PI, PS and SPH) were collected and combined. Assays of unsaturated fatty acids in phospholipids were performed by C_{18} HPLC as described in Section 2. This experiment was repeated three times; the one shown is representative.

Under these conditions, a pronounced and random oxidation of PnA was observed in all classes of phospholipids in K562 cells (see Fig. 4). No significant decrease of polyenoic fatty acid residues was detected at a lower concentration of t-BuOOH (40 μM). In contrast, a significant and selective oxidation of PnA-PS was detected in PnA-labelled cells incubated with 40 μM t-BuOOH (Fig. 8).

4. Discussion

Hydroperoxides are the primary molecular products of lipid peroxidation, a process that mainly involves polyunsaturated fatty acid residues of phospholipids in biomembranes [22]. A multitude and variety of secondary products formed from decomposition of the lipid hydroperoxides are known to cause not only damage to membranes, but also oxidative modification of critical cell proteins and DNA, causing cytotoxic and mutagenic effects [23,24].

While numerous techniques have been developed to quantitate lipid peroxidation in model systems [25], they have found only limited application for assays of lipid peroxidation in intact, living cells or *in vivo* [26,27]. Since PnA is a natural fatty acid, its structure is similar to that of other polyunsaturated fatty acid residues in phospholipids; hence, PnA causes minimum membrane-perturbing effects [28]. Oxidative destruction of any part of the conjugated double bond system of PnA causes the disappearance of its characteristic fluorescence [5]. Consequently, PnA has been successfully used as a marker of oxidative stress in model systems, ghost cell membranes and lipoproteins [5–9]. Recently, PnA has also been utilized for measurements of oxidative damage in cells [7,29,30]. Unfortunately, in previous studies on cells, only high and physiologically irrelevant concentrations of oxidants (usually in excess of 500 μM) were used to produce loss of PnA fluorescence. These high doses of oxidants also caused significant cell death [31]. Thus, to date it has not been clear as to whether or not oxidation of PnA in such systems occurred in intact, live cells (undergoing *oxidative stress*), or in damaged, dying or dead cells (as a result of *oxidative damage*).

We hypothesized that PnA, metabolically incorporated under physiological conditions into different classes of membrane phospholipids in cells in culture, could potentially be a sensitive and specific reporter molecule for measurement of oxidative stress in membranes in *live* cells. In the present work, we examined the metabolic incorporation of PnA into the membrane phospholipids of four different cell lines, human leukemia HL-60, K562 and K/VP.5 cells and Chinese hamster ovary (CHO) fibroblasts. Further, we examined the utility of PnA as a reporter for membrane phospholipid peroxidation induced by low levels of oxidative stress.

Incubation of cells with the PnA-hSA complex resulted in a time- and concentration-dependent incorporation of the fluorescent fatty acid into phospholipids (Figs. 6 and 7). PnA fluorescence was detected in all five major classes of membrane phospholipids, i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin, in all four cell lines studied. In K562, K/VP.5 and HL-60 cells, approximately 50% of PnA was recovered in these major phospholipid peaks. In CHO cells, the amount of PnA integrated into phospholipids did not exceed 15% of total PnA incorporation in cells. Significant differences were seen in PnA incorporation into specific phospholipids among the cell types used. These differences may be attributed to specific intracellular/plasma membrane ratios or differences in metabolic pathways for polyenoic phospholipids for each kind of cells (e.g., specificity of phospholipases A, acyl-CoA transferases).

Several significant characteristics of PnA as a biomarker of oxidative stress in membrane phospholipids were evident in these studies. The first was that PnA incorporated into membrane phospholipids without damaging cells. The lack of PnA toxicity in the range of PnA concentrations used in this study concurs with results previously reported by Spector et al. [32] although significant toxic effects do occur when cells are incubated with PnA for longer periods (24–48 h) [32].

The second was that metabolically integrated PnA constituted less than 1% of unsaturated fatty acid residues (22:6, 20:4, 18:2 and 18:1), (certainly, much less than 1% of total fatty acid residue pool) as estimated by HPLC analysis of fatty acids freed by saponification of total lipid extracts. Hence, it is unlikely that membrane structure and characteristics were changed by the presence of such small amounts of incorporated PnA.

Incubation of cells with 0.02–0.1 mM t-BuOOH did not change the overall fluorescence response from cell suspensions (Table 4) despite significant oxidation of PnA in individual membrane phospholipids (Fig. 8). One explanation for these results may be that neutral lipids, that were likely to be responsible for at least half of the total fluorescence intensity from cell suspensions, were not oxidized by t-BuOOH. In support of this hypothesis, phospholipids (not neutral lipids) are known to be the major targets for lipid peroxidation *in vitro* and *in vivo* [33].

An important feature of the method developed is its utility for monitoring oxidative stress in individual phospholipids of live cells, allowing observation of a very early responses to oxidative stress. For example, PnA-PS in K562 cells was selectively oxidized upon exposure to low concentrations of t-BuOOH. Yet, the fluorescence of the total lipid extract from the cells did not appreciably change under these conditions. This is because the PnA-PS fluorescence constituted only a few percent of the total fluorescence intensity in lipid extracts. The high sensitivity of PnA-PS in K562 or K/VP.5 cells to t-BuOOH-induced oxidation is intriguing. It is possible that the specific topography of PS in the cytoplasmic leaflet of plasma membrane bilayer may be responsible for its higher availability to free radicals. Interestingly, energy-dependent and protein-dependent aminophospholipid translocase activity present in plasma membranes, which specifically transports PEA and PS from the outer leaflet toward the inner leaflet of the membrane lipid bilayer [34,35], is sensitive to H_2O_2-induced oxidative stress [36]. While both aminophospholipids – PS and PEA – have nearly the same transbilayer distribution in plasma membranes [37], PEA was not significantly oxidized by low concentrations of t-BuOOH. Thus, specific transbilayer topography is not likely to be the sole cause for the preferential oxidation of PnA-PS. Since cells exposed to low concentrations of t-BuOOH retained their viability, specific oxidation of PnA-PS was not an artifact arising from dead cells – a problem with other, less sensitive peroxidation assays that require higher doses of oxidants. Not surprisingly, the specificity of PnA-PS response was lost at higher t-BuOOH concentrations, likely as a result of indiscriminate oxidative damage arising from cell death and lysis.

Our results demonstrate that the cell lines used have different susceptibility to oxidation by t-BuOOH. PnA-phospholipids of K562 and K/VP.5 cells were more prone to oxidation by low concentrations of t-BuOOH than in CHO or HL-60 cells. K562 and a subclone, K/VP.5, are erythroleukemic cells that express hemoglobin [16]. We and others have shown that hydrogen peroxide and organic hydroperoxides interact with hemoglobin to form a potent oxidant, ferrylhemoglobin [38], which is capable of oxidizing different biomolecules including unsaturated fatty acids [39]. Thus, ferrylhemoglobin may be responsible for the higher succeptibility of K562 and K/VP.5 cells to t-BuOOH-induced oxidation. In a separate study, we used K562 and K/VP.5 cells in which we manipulated endogenous levels of hemoglobin by incubating them with hemin [40]. We found that oxidation of PnA-PS by low concentrations of t-BuOOH (20–40 μM) is dependent on the concentrations of intracellular hemoglobin. Since this specific hemoglobin-mediated peroxidation mechanism is not likely to be present in the epithelial CHO or myeloleukemic HL-60 cells, they are more resistant to t-BuOOH-induced oxidation. HL-60 cells are known to have high activity of myeloperoxidase [41]. Hence, PnA-peroxidation induced by myeloperoxidase-catalyzed pathways is expected to be enhanced in HL-60 cells. Indeed, we found that H_2O_2/phenol, substrates of myeloperoxidase, caused massive oxidation of PnA-labelled phospholipids in HL-60 cells [42].

A lipid-soluble azo-compound, AMVN, which initiates peroxyl radical formation at a constant rate [21], caused nearly equal PnA peroxidation in all classes of phospholipids. Importantly, cell viability was not significantly decreased by this treatment. The differences between the patterns of t-BuOOH- and AMVN-induced oxidations can

be attributed to the different mechanisms by which these two chemicals generate free radicals in cells. As mentioned above, t-BuOOH causes ferrylhemoglobin-induced oxidation, dependent on the compartmentalization of endogenous hemoglobin in K562 cells [40], while thermally-induced decomposition of the lipophilic AMVN, which randomly partitions into membrane lipid bilayers [21], is independent of intracellular constitutents.

In gamma-irradiated CHO cells, the response of PnA was minimal and confined to PS and PI, suggesting that this form of oxidative stress did not significantly involve membrane phospholipids. Our results also indicate that, in the absence of exogenously added oxidants such as t-BuOOH or AAPH, endogenous antioxidant mechanisms were sufficient to protect PnA against oxidation during a 1 h incubation period.

One of the major problems in the study of oxidative stress is that efficient lipid repair mechanisms may be activated, e.g., deacylation of oxidatively modified fatty acid residues and subsequent reacylation of phospholipids with normal (non-oxidized) fatty acids [43,44], making accurate determination of lipid oxidation a difficult endeavor in otherwise unperturbed cells. We used experimental conditions (low concentrations of PnA, short incubation periods, removal of PnA adhering to the cell surface) under which the integration of PnA into major membrane phospholipids in cells was at or below saturation levels. Our results demonstrated that loss of PnA fluorescence occurred proportionally to the dose of the oxidant applied. Since loss of PnA fluorescence is a direct result of oxidative loss of its conjugated tetraene system [8], we conclude that no interference with lipid repair mechanisms occurred. Even if PnA that had been oxidized was replaced with an unoxidized fatty acid by such repair mechanisms, evidence for PnA oxidation remained (as the loss of PnA-specific fluorescence). Thus, the procedures we have introduced in this paper for the assay of PnA oxidation in membrane phospholipids represent a method insensitive to lipid repair mechanisms, that in turn contributes to its high sensitivity.

Our data suggest that measurements of PnA-labelled phospholipids were more sensitive than conventional HPLC assay of 22:6- and 20:4-residues in K562 cells exposed to t-BuOOH (Figs. 8 and 9 and Table 3). Since the low concentration of t-BuOOH (40 μM) did not affect cell viability (more than 95% viable cells after 1 h incubation), lipid repair mechanisms that minimized losses of polyunsaturated fatty acids in membrane phospholipids were retained by the cells [43,44]. On the other hand, a 1 h incubation of K562 cells in the presence of 5.0 mM t-BuOOH caused almost 100% cell death. Under these conditions, we observed a significant loss of polyunsaturated fatty acid residues in phospholipids as well as random oxidation of PnA in all classes of phospholipids.

In conclusion, we have developed a method for quantitative probing of peroxidation in different classes of membrane phospholipids that allows for selective and sensitive monitoring of oxidative stress in live cells without interference from cell repair mechanisms. Using this method, we found that oxidative stress induced by different oxidants is specific for different phospholipid classes. This selectivity was not observed at higher concentrations of oxidants, where all phospholipid classes were involved in oxidative damage. Higher concentrations of oxidants, however, caused cell death and lysis, and the phospholipid damage seen can only be ascribed to the resulting random oxidation reactions that then occurred.

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