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# Quantitative Analysis of Phospholipid Peroxidation and Antioxidant Protection in Live Human Epidermal Keratinocytes

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To characterize oxidative stress in phospholipids of normal human epidermal keratinocytes we metabolically labeled their membrane phospholipids with a natural oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid, and exposed the cells to two different sources of oxidants—a lipid-soluble azo-initiator of peroxyl radicals, 2,2'-azobis(2,4-dimethyl-valeronitrile), AMVN, and a superoxide generator, xanthine oxidase/xanthine. We demonstrated that both oxidants induced pronounced oxidation of four major classes of *cis*-parinaric acid-labeled phospholipids—phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol—in normal human epidermal keratinocytes that was not detectable as any significant change of their phospholipid composition. Vitamin E was effective in protecting the cells against phospholipid peroxidation. Since viability of normal human epidermal keratinocytes was not changed either by labeling or exposure to oxidants the labeling protocol and oxidative stress employed are compatible with the quantitative analysis of phospholipid peroxidation in viable cells.

**KEY WORDS:** Keratinocytes; phospholipid peroxidation; oxidative stress; xanthine/xanthine oxidase; AMVN, $\alpha$-tocopherol.

## INTRODUCTION

Constant exposure to air, chemicals and light is said to make skin cells particularly vulnerable to oxidative stress. Lipid peroxidation induced through either enzymatic or non-enzymatic pathways has been hypothesized to cause a number of adverse effects in skin such as inflammatory response, apoptosis (Pugliese, 1995; Lee *et al.*, 2000, Maziere *et al.*, 2000), genetic instability, tumor promotion and carcinogenesis (Nair *et al.*, 2000). These hypotheses are mainly supported by indirect evidence that

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supplementation with antioxidants provides protection against these effects (Youn *et al*., 1992; Wells *et al*., 1997; Jones *et al*., 1999; Zhao *et al*., 2000).

Surprisingly, no reliable quantitative data on lipid peroxidation in live skin cells has so far been reported. This is mainly due to functioning of effective repair systems that remodel oxidatively modified phospholipids (Rashba-Step *et al*., 1997). This is achieved through the phospholipase $A_2$-catalyzed cleavage of peroxidized phospholipids to peroxy-fatty acid residues and lysophospholipids and subsequent reacylation of lysophospholipids (Van der Vliet and Bast, 1992; McLean *et al*., 1993). As a result, accumulation of peroxy-fatty acid containing phospholipids cannot be readily detected in live skin cells if the oxidative challenge does not overwhelm the capacity of the repair systems.

We have recently developed a novel approach to quantitatively assess oxidative stress in live cells (Kagan *et al*., 1998). For this, we engineered cells in which we metabolically integrated an oxidation-sensitive fluorescent fatty acid containing four conjugated double bonds, *cis*-parinaric acid (PnA) into different classes of membrane phospholipids. Oxidation of PnA results in disruption of the conjugated double bond system that cannot be resynthesized in mammalian cells. Therefore, oxidation of PnA residues in different phospholipids is not affected by their remodeling and its loss can be readily assayed by normal-phase HPLC separation of phospholipids with a fluorescence detector (Kagan *et al*., 1998). Thus, quantitative measurements of site-specific oxidative stress in different classes of phospholipids in membranes of live cells are feasible.

In this study, we applied this methodology to characterize oxidative stress in phospholipids of normal human epidermal keratinocytes (NHEK) exposed to two different sources of oxidants—a lipid-soluble azo-initiator of peroxyl radicals, 2.2′-azobis(2,4-dimethyl-valeronitrile), AMVN, and a superoxide generator, xanthine oxidase/xanthine. We demonstrated that both oxidants induced pronounced oxidation of different classes of PnA-labeled phospholipids in keratinocytes that was not detectable as any significant change of their phospholipid composition. Vitamin E was effective in protecting the cells against phospholipid peroxidation.

## METHODS

### Reagents

*cis*-Parinaric acid [(9Z, 11E, 13E, 15Z)-octadecatetraenoic acid] was obtained from Molecular Probes (Eugene, OR). Chloroform, methanol, hexane, 2-propanol (HPLC grade), Tween 20, butylated hydroxytoluene (BHT), xanthine, xanthine oxidase, malachite base green were purchased from Sigma (St. Louis, MO). $\alpha$-Tocopherol acetate was purchased from Aldrich Chemical (Milwaukee, WI). KGM-2 medium was purchased from Clonetics (San Diego, CA). The azo-initiator, 2.2'-azobis (2,4-dimethyl-valeronitrile) (AMVN) was purchased from Wako Chemicals USA (Richmond, VA).

### Normal Human Epidermal Keratinocytes (NHEK) Cell Culture

NHEK from adults were obtained from Clonetics (San Diego, CA). Cells were plated at a density of $6.25 \times 10^4$ cells per 75 ml tissue culture flask (Greiner Laboratories, GmbH, Germany) and grown in KGM-2 medium at 37°C in tissue culture

incubator (5% $CO_2$) until confluent monolayers were obtained. Repeated counts of NHEK using a hemacytometer revealed a concentration of $1–2 \times 10^6$ cells per flask at the time of harvest. To supplement NHEK with $\alpha$-tocopherol the cells ($\approx$100% confluence) were cultured in the presence of $\alpha$-tocopherol acetate (2.5 $\mu$M or 50 $\mu$M) for another 24 hr at 37°C in KGM-2 medium. Cell viability was determined by Trypan Blue exclusion.

### Treatment with AMVN and Xanthine/Xanthine Oxidase

To induce oxidative stress NHEK were washed twice with KGM-2 medium and loaded with PnA as described earlier (Kagan *et al.*, 1998; Shvedova *et al.*, 2000). PnA-loaded NHEK were incubated in the presence or in the absence of 500 $\mu$M 2,2′-azobis(2,4-dimethyl-valeronitrile) (AMVN), or xanthine (250 $\mu$M)/xanthine oxidase (X/Xo) (0.075U per flask (15 ml medium)) for 2 hr at 37°C. X/Xo were added every 30 min during 2 hr incubation. After incubation, cells were scraped washed twice with PBS and total lipids were extracted according to Folch *et al.* (1959) in the presence of BHT (100 $\mu$M) to retard subsequent oxidation. In all cases, the levels of PnA-labeled phospholipids after 2 hr incubation of NHEK without oxidants were used as controls for comparisons. During 2 hr incubation in the absence of oxidants, the content of PnA labeled phospholipids decreased only insignificantly (within 10% of the initial levels).

### HPTLC Analysis of NHEK Lipids

Lipid extracts were separated by HPTLC on silica G plates ($5 \times 5$ cm) as described earlier (Kagan *et al.*, 1998). The phospholipid spots were identified by iodine staining, scraped and lipid phosphorus was determined as described by Bottcher *et al.* (1961).

### HPLC Analysis of NHEK Lipids

The lipid extracts were dried under $N_2$, dissolved in 0.2 ml of 2-propanol:hexane:water (4:3:0.16, by vol.) and separated by normal phase HPLC using a 5-$\mu$M Microsorb-MV$^{TM}$ Si column ($4.6 \times 250$ mm) and an ammonium acetate gradient as described earlier (Kagan *et al.*, 1998). The separations were performed using a Shimadzu HPLC LC-600 system (Kyoto, Japan) equipped with an in-line RF-551 fluorescence detector. Fluorescence of PnA was measured at 420 nm after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined using a micro method (Chalavardjian and Rubincki, 1970).

### Statistical Evaluation

The results are presented as mean $\pm$ SE values of five experiments and statistical analysis was performed by Student's *t*-test or one-way analysis of variance (ANOVA). The statistical significance of differences was set at $p < 0.05$.

## RESULTS

### HPTLC Analysis of NHEK Phospholipids

In our initial experiments aimed at detection of oxidative stress in phospholipids, we attempted to determine whether changes in phospholipid composition occurred in NHEK exposed to AMVN or X/Xo. Seven different phospholipid spots were detected by HPTLC of lipids extracted from either control NHEK or oxidant-challenged NHEK. Phosphatidylcholine (PC) represented $42.8 \pm 1.2\%$ of the total phospholipids in NHEK, phosphatidylethanolamine (PE) being the next most prominent phospholipid ($29.8 \pm 1.1\%$). The other phospholipids detected in the order of their abundance were sphingomyelin (SPH) > phosphatidylinositol (PI) > phosphatidylserine (PS) $\gg$ diphosphatidylglycerol (DPG) $\gg$ lysophosphatidylcholine (LPC) (Table 1). No significant difference in phospholipid distribution was detected in NHEK following exposure to AMVN and X/Xo (Table 1).

Oxidatively modified phospholipids are known to undergo rapid and effective repair and remodeling (Kagan *et al.*, 1998; Rashba-Step *et al.*, 1997). Hence, the lack of HPTLC-detectable changes in the phospholipid composition of NHEK exposed to either AMVN or X/Xo might be due to effective repair of phospholipids via deacylation/reacylation pathways (Van der Vliet and Bast, 1992; McLean *et al.*, 1993). Therefore, we applied a PnA-based assay that permits sensitive detection of specific oxidative stress in selected membrane phospholipids of cells without interference from repair mechanisms.

### HPLC Analysis of PnA-Labeled Phospholipid Peroxidation in NHEK

Values for specific incorporation of PnA into membrane phospholipids of NHEK suggest that incorporation of PnA in the various phospholipids is different, and amounts of PnA incorporated can be ranked in the following order: PC = PE > PS > Pl (Table 2). We found that AMVN (500 $\mu$M) and X/Xo caused substantial time-dependent oxidation of PnA-labeled phospholipids (Table 3). The acyl chains of three classes of phospholipids in NHEK, namely PE, PC, and Pl, were the major targets for AMVN-induced peroxidation (2 hr) whereas the acid phospholipid,

**Table 1.** Phospholipid Composition of Normal Human Epidermal Keratinocytes

| | Phospholipid content, % of total phospholipids | | |
|---|---|---|---|
| Phospholipid | Control | AMVN | X/Xo |
| Phosphatidylcholine | $46.0 \pm 1.2$ | $44.7 \pm 1.2$ | $44.8 \pm 1.2$ |
| Phosphatidylethanolamine | $28.0 \pm 1.4$ | $28.0 \pm 1.1$ | $27.4 \pm 1.1$ |
| Phosphatidylserine | $6.8 \pm 0.4$ | $7.0 \pm 0.4$ | $7.2 \pm 0.3$ |
| Sphingomyelin | $10.5 \pm 1.4$ | $9.8 \pm 0.8$ | $9.4 \pm 1.6$ |
| Phosphatidylinositol | $6.6 \pm 0.6$ | $7.4 \pm 0.6$ | $7.6 \pm 1.1$ |
| Diphosphatidylglycerol | $2.1 \pm 0.6$ | $2.2 \pm 0.5$ | $2.9 \pm 0.5$ |
| Lysophosphatidylcholine | trace | $0.9 \pm 0.3$ | $0.7 \pm 0.2$ |

All values are means $\pm$ SD ($n = 3$).
Trace—less than 0.2%.

**Table 2.** Specific Incorporation of PnA into Phospholipids of Normal Human Epidermal Keratinocytes

| Phospholipid | Incorporation of PnA | |
|---|---|---|
| | ng PnA/$\mu$g of Pi in phospholipid fraction | mol PnA:mol phospholipid |
| Phosphatidylinositol | $25.9 \pm 1.2$ | 1:350 |
| Phosphatidylethanolamine | $91.3 \pm 4.0$ | 1:95 |
| Phosphatidylserine | $40.8 \pm 2.8$ | 1:210 |
| Phosphatidylcholine | $118 \pm 4.2$ | 1:73 |

NHEK were incubated in the presence of PnA (5 $\mu$g/$10^6$ cells) for 2 hr at 37°C. After incubation cells were washed in the presence and absence of fatty acid free human serum albumin then lipids were extracted and resolved by HPLC.

PS was marginally affected. Exposure of NHEK to X/Xo for 2 hr resulted in significant peroxidation in all phospholipid classes (Table 3).

To analyze positional distribution of PnA integrated into membrane phospholipids of NHEK we treated homogenates of PnA-prelabeled NHEK with phospholipase $A_2$ in the presence of mellitin to determine the positional distribution of PnA in phospholipids. Since phospholipidase $A_2$ specifically hydrolyzes phospholipids in *sn-2*-position fluorescence-HPLC as well as HPTLC analysis of the products permits determination of the relative amounts of PnA esterified in the *sn-2*-position of membrane phospholipids. The results of this analysis are shown in Table 4. HPTLC results demonstrated that >95% phospholipids underwent hydrolysis under the conditions used. Our HPLC data showed that >80% of PnA was confined to *sn-2*-position in all major classes of phospholipids (81.5%, 82.4%, 72.4%, and 90.5% for PC, PE, PS, and Pl, respectively). Thus PnA containing four conjugated double-bonds was predominantly integrated into *sn-2*-position of NHEK phospholipids in line with the positional distribution of polyunsaturated fatty acid residues in mammalian phospholipids (Lehninger *et al.*, 1993). This suggests that oxidative attack

**Table 3**. Effect of AMVN and Xanthine–Xanthine Oxidase on Oxidation of PnA–labeled Phospholipids in Normal Human Epidermal Keratinocytes

| Treatment | PnA-labeled phospholipids, ng PnA/$\mu$g total lipid Pi | | | |
|---|---|---|---|---|
| | Pl | PE | PS | PC |
| Control[a] | $2.1 \pm 0.1$ | $27.2 \pm 1.2$ | $2.9 \pm 0.2$ | $50.9 \pm 1.8$ |
| AMVN | | | | |
| 1 hr[b] | $1.5 \pm 0.2$ | $22.2 \pm 0.4$** | $2.9 \pm 0.1$ | $38.6 \pm 0.7$** |
| 2 hr[c] | $0.4 \pm 0.1$** | $12.5 \pm 1.9$** | $2.6 \pm 0.3$ | $30.4 \pm 3.6$** |
| X/Xo | | | | |
| 1 hr[b] | $1.0 \pm 0.3$* | $24.7 \pm 0.3$ | $2.4 \pm 0.2$ | $34.9 \pm 0.7$** |
| 2 hr[c] | $0.4 \pm 0.1$** | $13.3 \pm 1.1$** | $1.9 \pm 0.2$** | $21.4 \pm 1.6$** |

NHEK loaded with PnA were exposed to AMVN (500 $\mu$M) or xanthine (250 $\mu$M)–xanthine oxidase (0.075U/1–2 $\times$ $10^6$ cells) (X/Xo) at 37°C. At the end of incubation, NHEK were washed with PBS, lipids were extracted and resolved by HPLC. All data are mean $\pm$ SE; [a] $n = 11$; [b] $n = 3$; [c] $n = 6$; *$p < 0.05$, **$p < 0.005$ vs. control.

**Table 4.** Effect of Phospholipase $A_2$ on Phospholipid Composition and Content of PnA-labeled Phospholipids in Normal Human Epidermal Keratinocytes

| | Content of phospholipids % of total phospholipids | | PnA-labeled phospholipids ng PnA/$\mu$g total lipid Pi | |
|---|---|---|---|---|
| Phospholipid | Control | Phospholipase $A_2$ | Control | Phospholipase $A_2$ |
| Phosphatidylcholine | $46.0 \pm 1.2$ | $1.0 \pm 0.2$ | $50.9 \pm 1.8$ | $0.38 \pm 0.07$ |
| Phosphatidylethanolamine | $28.0 \pm 1.4$ | $1.8 \pm 1.2$ | $27.2 \pm 1.2$ | $0.18 \pm 0.04$ |
| Phosphatidylserine | $6.8 \pm 0.4$ | $0.6 \pm 0.5$ | $2.9 \pm 0.2$ | $0.01 \pm 0.01$ |
| Sphingomyelin | $10.5 \pm 1.4$ | $12.4 \pm 1.3$ | ND | ND |
| Phosphatidylinositol | $6.6 \pm 0.6$ | $0.6 \pm 0.5$ | $2.1 \pm 0.1$ | $0.01 \pm 0.01$ |
| Diphosphatidylglycerol | $2.1 \pm 0.4$ | $2.8 \pm 0.2$ | ND | ND |
| Lysophospholipids | ND | $80.8 \pm 4.8$ | ND | $15.2 \pm 2.2$ |
| Free *cis*-parinaric acid | ND | ND | $2.8 \pm 0.4$ | $68.8 \pm 1.9$ |

All values are means $\pm$ SE ($n = 3$). ND—not detectable.
Lysophospholipids: Lysophosphatidylcholine; Lysophosphatidylethanolamine; Lysophosphatidylserine; Lysophosphatidylinositol.

on PnA-labelled cells should be also happening in *sn-2*-position. In fact, our fluorescence-HPLC measurements of NHEK phospholipids after exposure to AMVN and X/Xo revealed no peaks corresponding to fluorescently-labeled lysophospholipids. This confirms that PnA esterified in *sn-2*-position was by far the major substrate for peroxidation in NHEK.

## Vitamin E Protection against Phospholipid Peroxidation

To determine the effects of vitamin E on lipid peroxidation induced by AMVN or X/Xo, NHEK ($\approx$100% confluence) vitamin E ($\alpha$-tocopheryl acetate) was added to the growth medium 24 hr prior to PnA-labeling and exposure to oxidants. Excess of vitamin E that has not been integrated into cells was removed by washing NHEK with the KGM2 medium. Analysis of $\alpha$-tocopherol content demonstrated that NHEK contained $3.4 \pm 0.3$ and $28.4 \pm 1.2$ nmoles $\alpha$-tocopherol/mg proteins after loading with 2.5 and 50 $\mu$M vitamin E, respectively. The effect of $\alpha$-tocopherol on the AMVN-induced oxidation of PnA-labeled phospholipids is presented in Fig. 1. Supplementation with 2.5 $\mu$M $\alpha$-tocopherol did not afford any significant protection. Essentially complete protection of all phospholipids (Pl, PE, and PC) was achieved with 50 $\mu$M $\alpha$-tocopherol.

Figure 2 shows the effect of $\alpha$-tocopherol on the oxidation of PnA-labeled phospholipids induced by X/Xo. In this case, even at the low concentration of 2.5 $\mu$M, $\alpha$-tocopherol significantly reduced the loss of PnA-labeled phospholipid from oxidation induced by X/Xo, except for PS. Increasing the $\alpha$-tocopherol concentration up to 50 $\mu$M resulted in a complete inhibition of peroxidation in all classes of phospholipids including PS.

## Cell Viability

Neither PnA labeling nor exposure to oxidants (AMVN, X/Xo) in the absence or presence of antioxidants caused any significant changes in viability of NHEK as assessed by Trypan Blue exclusion (*data not shown*).

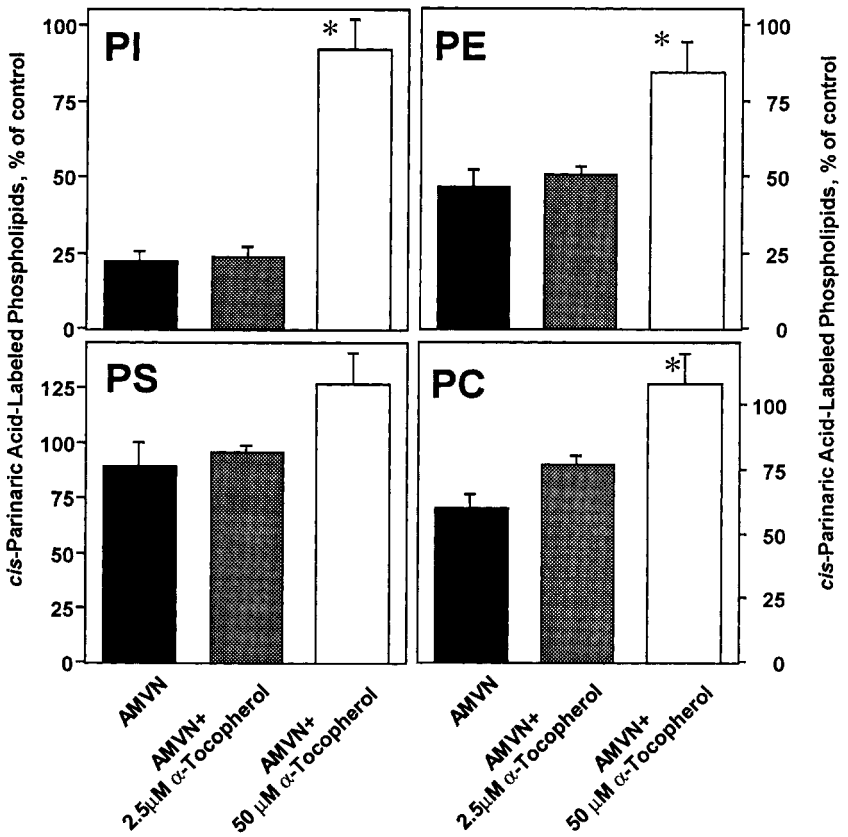


**Fig. 1.** Effect of $\alpha$-tocopherol on oxidation of PnA-labeled phospholipids induced by AMVN in normal human epidermal keratinocytes. NHEK were cultured in the presence or in the absence of $\alpha$-tocopherol (2.5 $\mu$M or 50 M) for 24 hr at 37°C in KGM-2 medium. Unloaded NHEK and NHEK loaded with $\alpha$-tocopherol were exposed to AMVN (500 $\mu$M). At the end of the incubation, cells were scraped and lipids were extracted and resolved by HPLC. Pl, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. *$p < 0.05$ vs. AMVN, $n = 6$, $n = 7$, and $n = 3$ for AMVN, 2.5 $\mu$M $\alpha$-tocopherol plus AMVN and 50 $\mu$M $\alpha$-tocopherol plus AMVN, respectively.

## DISCUSSION

Phospholipid peroxidation has been implicated in a number of pathological conditions in skin induced by UV-irradiation as well as by different toxic chemicals (Pugliese, 1995; Lee *et al.*, 2000; Maziere *et al.*, 2000; Nair *et al.*, 2000). However, the exact quantitative measurements of oxidative stress in phospholipids of skin cells have not been reported. Conventional methods for phospholipid analysis fail to provide adequate information on phospholipid peroxidation in viable cells. In line with this, we were not able to detect any significant changes in the relative proportions of phospholipids after exposure to AMVN or X/Xo in viable NHEK. Since both AMVN and X/Xo undoubtedly generated radicals that attacked intracellular

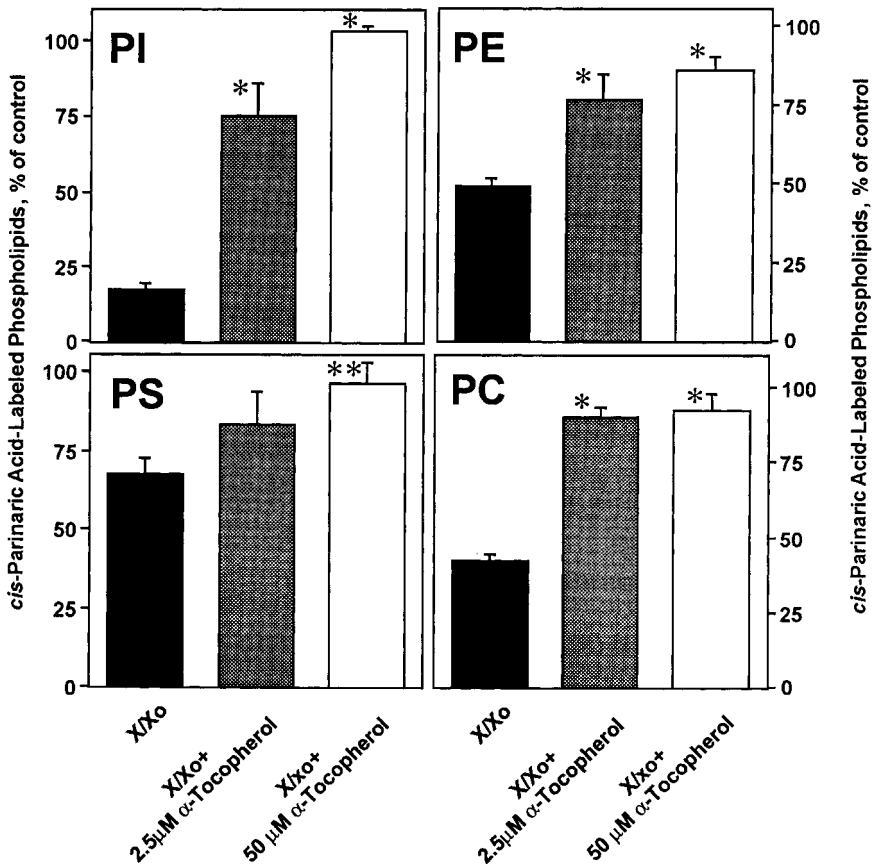


**Fig. 2.** Effect of $\alpha$-tocopherol on oxidation of PnA-labeled phospholipids induced by xanthine plus xanthine oxidase in normal human epidermal keratinocytes. NHEK were cultured in the presence or in the absence of $\alpha$-tocopherol (2.5 $\mu$M or 50 M) for 24 hr at 37°C in KGM-2 medium. Unloaded NHEK and NHEK loaded with $\alpha$-tocopherol were exposed to xanthine (250 $\mu$M) plus xanthine oxidase (0.075U/1–2 $\times$ $10^6$ cells) (X/Xo) for 2 hr at 37°C. X/Xo were added every 30 min during incubation. At the end of the incubation, cells were scraped and lipids were extracted and resolved by HPLC. Pl, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. *$p < 0.03$, **$p < 0.05$ vs. X/Xo, $n = 6$ for X/Xo and 2.5 $\mu$M $\alpha$-tocopherol plus X/Xo, $n = 6$ and $n =$ 3 for 50 $\mu$M $\alpha$-tocopherol plus X/Xo.

constituents, the lack of detectable changes in phospholipid composition may reflect the presence of effective mechanisms of repair for peroxidized phospholipids in viable cells (Van der Vliet and Bast, 1992; McLean *et al.*, 1993).

One may assume that oxidation of phospholipids is accompanied by changes in their relative abundance. In mammalian cells, phospholipids usually contain polyunsaturated fatty acid residues in their *sn-2*-position. Since polyunsaturated phospholipids are mostly prone to oxidative attack (Rashba-Step *et al.*, 1997) the phospholipid oxidation products should contain hydroperoxy-fatty acid residues in

the *sn-2*-position at the initial stages of free radical oxidation (Kagan, 1988). Subsequent decomposition of hydroperoxides and $\beta$-scission may give rise to esterified short-chain fatty acid fragments likely containing a carbonyl function (Kagan, 1988). These oxidatively modified phospholipids have a different hydrophobic/hydrophilic balance (Kagan, 1988) and, hence, behave differently during HPTLC. Therefore, HPTLC analysis of cell phospholipids after oxidative challenge may reveal changes in relative distribution of different phospholipid classes. This, however, will be only true if the repair systems in live cells do not remodel oxidized phospholipids such that no changes in relative abundance of phospholipids occur. The repair systems include hydrolysis of oxidized fatty acid residues by phospholipases $A_2$ and subsequent enzymatic reacylation of lysophospholipids formed (Rashba-Step *et al.*, 1997; Van der Vliet and Blast, 1992; McLean *et al.*, 1993).

To overcome this problem, we attempted to detect phospholipid peroxidation by labeling phospholipids with a natural, fluorescent fatty acid, PnA, that fulfills three requirements: (i) it is extremely sensitive to oxidative stress due to the presence of four conjugated double bonds in its structure, (ii) its fluorescence is lost as a result of oxidation, and (iii) the unique conjugated double bond system cannot be resynthesized in mammalian cells. NHEK readily integrated PnA into their phospholipids in such a way that only a small proportion of each phospholipid class is labeled with PnA. The amounts of PnA-labeled phospholipid do not exceed 2% in two major phospholipids, PC and PE, and they are significantly less for PS and Pl ($\approx$0.5% and 0.3%, respectively). These amounts of PnA-labeled phospholipids are sufficient for reliable detection of their peroxidation in NHEK. Even in the least labeled phospholipid, Pl, exposure to either type of oxidant (AMVN or X/Xo) did not consume all the PnA–Pl suggesting that the amount of label was not a limiting factor for the assay. In other phospholipids, oxidant-induced loss of PnA represented only a small fraction of the PnA-labeled phospholipids.

Importantly, we were able to detect that different classes of phospholipids are differently affected by the peroxidation process induced by either AMVN or X/Xo. PS turned out to be surprisingly resistant to AMVN-induced oxidative stress under the conditions used. Given that AMVN is a lipid-soluble azo-initiator that readily partitions into lipid domains of membranes this may suggest that some specific, as yet unidentified, mechanisms were involved in PS protection against its peroxidation in NHEK. While all four major phospholipid classes were oxidized during incubation of NHEK with X/Xo PS was again relatively less susceptible to peroxidation than the other phospholipids.

To further prove that PnA-labeled NHEK represent a good model for quantitative assay of oxidative stress and the protective effects of antioxidants we investigated protective effects of vitamin E on peroxidation of PnA-labeled phospholipids. We found the NHEK cultured in the presence of $\alpha$-tocopherol acetate were, indeed, protected against phospholipid peroxidation. This protective effect of vitamin E was concentration-dependent (with AMVN) and the effectiveness of vitamin E protection was significantly higher in the case of X/Xo as compared to AMVN.

Since the amount of Trypan Blue-negative NKEK was not changed by either labeling or exposure to oxidants the labeling protocol and oxidative stress employed were compatible with the quantitative analysis of phospholipid peroxidation in

viable cells. In conclusion, our results clearly indicate that PnA-labeled NHEK offer a unique model cellular system for quantitative studies of oxidative stress in different classes of phospholipids in skin cells. Based on this model, we are currently studying specific signaling pathways through which phospholipid oxidation is involved in execution of apoptotic program in NHEK.

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