# Lysophosphatidylcholine-induced cellular injury in cultured fibroblasts involves oxidative events

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**Abstract** **Lysophosphatidylcholine (lysoPC), formed during LDL oxidation and located within atherosclerotic plaques, induces numerous cellular responses, but via unknown mechanisms. Cellular events involved in sublethal lysoPC-induced injury were examined because these are relevant to mechanisms by which lysoPC alters cell behavior. LysoPC evoked transient membrane permeabilization in fibroblasts within 10 min. Cells underwent reversible rounding within 2 h, returning 3 h later to grossly normal appearance and a normal response to growth stimulation. We asked whether this sublethal permeabilization resulted from physical perturbation of the plasma membrane or if it required cellular events. LysoPC induced leakage of fluorescent dye from unilamellar phospholipid vesicles, suggesting physical membrane perturbation was a significant contributor. To characterize this further we increased the cholesterol content of cells and vesicles to stabilize membranes, and found decreased lysoPC-induced permeabilization in both cell and cell-free systems as cholesterol levels increased. Interestingly, vitamin E, a known antioxidant, blunted lysoPC-induced permeabilization and morphological changes in cells. Thus, lysoPC appeared to cause an unexpected oxidant stress-dependent enhancement of cell injury. To confirm this, several structurally distinct antioxidants, including *N,N′*-diphenyl-1,4-phenylenediamine, Desferal, Tiron, and 4-hydroxy TEMPO, were applied and these also were inhibitory. Oxidant stress was observed by a lysoPC-induced increase in fluorescence of 5- and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, an intracellular marker of reactive oxygen species. Lysophosphatidylethanolamine (lysoPE) caused qualitatively similar morphological changes to cells and induced permeabilization, but injury by lysoPE was not inhibited by antioxidants. These data suggest that generation of intracellular reactive oxygen species follows lysoPC-induced plasma membrane destabilization and that this lysoPC-specific oxidant stress enhances cell injury. This intracellular oxidant stress in response to lysoPC may be an integral part of the multiple influences lysoPC has on gene expression and cell function.**—Colles, S. M., and G. M. Chisolm. **Lysophosphatidylcholine-induced cellular injury in cultured fibroblasts involves oxidative events. *J. Lipid Res.* 2000.** 41: **1188–1198.**

**Supplementary key words** antioxidant • oxidant • permeabilization • cholesterol • vitamin E

Lysophosphatidylcholine (lysoPC) is one of numerous lipid products formed during the oxidation of low density lipoprotein (LDL) (1, 2). LysoPC formation during LDL oxidation has been attributed to a phospholipase $A_2$ activity associated with the LDL particle (3, 4). The presence of lysoPC has also been reported in the lipid-rich core of atherosclerotic plaques (5, 6). It has been proposed that lysoPC is responsible for many of the cellular effects identified in vitro for oxidized LDL (oxLDL) (7–9) and through these, lysoPC may contribute to the development of atherosclerotic plaques.

LysoPC has been reported to regulate a variety of cellular functions, some of which could be construed to promote atherosclerotic lesion development. For example, lysoPC enhances vascular smooth muscle cell proliferation (10–12), attracts monocytes (1), inhibits endothelium-dependent relaxation (13), and retards endothelial cell migration (14). LysoPC alters the expression of multiple genes, including increasing NO synthase in endothelial cells (15), upregulating growth factors such as heparin-binding epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) in various cells (16–18), suppressing endothelin 1 in endothelial cells (19), and enhancing the expression of adhesion molecules in endothelial cells (20). It is also well known that lysoPC injures a variety of cells (11, 21, 22).

LysoPC is an amphipathic molecule and possesses "detergent-like" properties that can alter cellular membranes. The membrane effects of lysoPC have been studied extensively in relation to lysis of red blood cells (22, 23). In addition, exposure to concentrations of lysoPC well above its critical micellar concentration has been re-

Abbreviations: carboxy-$H_2$DCFDA, 5- and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; Desferal, desferoxamine mesylate; DMEM, Dulbecco's modified Eagle's medium; DOPC, dioleoylphosphatidylcholine; DPPD, *N,N′*-diphenyl-1,4-phenylenediamine; EGF, epidermal growth factor; GSH, glutathione; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; 4-OH TEMPO, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl; PDGF, platelet-derived growth factor; TCA, trichloroacetic acid; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; TLC, thin-layer chromatography.

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ported to transiently increase permeability in plasma membranes (24, 25). As a result of the ability of lysoPC to affect multiple cellular systems it has been difficult to delineate the events through which lysoPC influences particular cellular responses.

The cellular injury caused by lysoPC may influence a number of the varied cell behaviors that follow exposure to lysoPC. We focused on sublethal cellular injury by lysoPC, at concentrations well below its reported critical micellar concentrations, to gain insight into its basic interaction with cells. Our interest in this stems from our unexpected observation that vitamin E was able to blunt lysoPC-induced injury to human fibroblasts (21), and that vitamin E diminished the lysoPC-induced proliferation of smooth muscle cells (10, 26). These observations suggested that lysoPC may induce an oxidant stress in cells. If so, common pathways may be involved between cellular injury by lysoPC and the influence of lysoPC on important cell functions.

Thus, the focus of our study was to examine the nature of lysoPC-induced injury and determine specifically whether lysoPC induced the generation of intracellular reactive oxygen species leading to oxidative stress, which could contribute to cell injury. We were also interested in elucidating the relationship between membrane destabilization by lysoPC and the putative oxidant effects.

## MATERIALS AND METHODS

Lysophosphatidylcholine (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). 5-Carboxyfluorescein and 6-carboxyfluorescein (mixed isomers) and 5- and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA) were purchased from Molecular Probes (Eugene, OR). Vitamin E, *N,N′*-diphenyl-1,4-phenylenediamine (DPPD), [$^{14}$C]adenine, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl (4-OH TEMPO), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), desferoxamine mesylate (Desferal), and glutathione were purchased from Sigma-Aldrich (St. Louis, MO).

### Cell culture

Human fibroblasts were isolated from neonatal foreskins as previously described (27). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)–F12 medium containing 7.5% bovine fetal bovine serum (FBS). Medium was changed every 2–3 days and cells were passaged at confluence.

### Measurement of cellular injury

The extent of membrane permeabilization was measured by the percent specific release of $^{14}$C from cells previously loaded with [$^{14}$C]adenine as previously described (21, 28, 29). Briefly, cells were loaded overnight with [$^{14}$C]adenine (0.2 μCi/mL) in the presence of 7.5% FBS. At the end of the loading period cells were washed at least two times with DMEM–F12 to remove excess [$^{14}$C]adenine and FBS. Cells were approximately 75% confluent at the onset of treatment with lysoPC. Cells were incubated with lysoPC at various concentrations and times as indicated. The percent specific release of $^{14}$C was defined as $^{14}$C release in response to lysoPC less the basal $^{14}$C release (from untreated cells) divided by the total $^{14}$C release possible (obtained with 0.5% Triton X-100) less the basal release. Pretreatments with antioxidants were initiated 24 h before the addition of lysoPC, and then removed. The cells were washed with DMEM–F12 to minimize noncellular interactions between the antioxidants and lysoPC. No effect on either the basal release of $^{14}$C or total uptake of $^{14}$C was observed in response to treatment with either antioxidants or sterols. Vitamin E and DPPD were added from stock solutions of ethanol and dimethyl sulfoxide (DMSO), respectively. The total concentration of organic solvent was maintained below 0.5%. Cells were loaded with cholesterol using either cholesterol–albumin coacervates (65 μm cholesterol) or free cholesterol from an ethanol stock solution (65 μm cholesterol in 0.5% ethanol in medium), resulting in a 2-fold increase in total cellular cholesterol.

### Time-lapse photography for morphological visualization

Human fibroblasts were seeded in 35-mm dishes in DMEM–F12 medium. Cells were maintained in medium containing 5% FBS without or with either 65 μM cholesterol or 40 μM vitamin E. After washing to remove excess cholesterol or vitamin E cells were treated with lysoPC in serum-free medium. The effect on cell morphology was monitored with a charge-couple device (CCD) video camera module mounted on an Olympus (Norwood, MA) CK2 microscope. Images were captured at 3-min intervals over a 10-h period, using NIH Image (NIH, Bethesda, MD).

### Synthesis of DOPC–cholesterol vesicles

Vesicles were generated by coating DOPC, cholesterol, and/or vitamin E from stock solutions in chloroform onto the wall of a 13 × 100 mm test tube under a stream of argon, followed by overnight incubation under vacuum. Lipids were resuspended in 20 mM TRIS buffer (pH 7.5) containing the fluorescent dye 5- and 6-carboxyfluorescein at 100 mM, which undergoes self-quenching at this concentration (30). Vesicles were formed by sonication on ice until optical clarity was achieved. Dye not incorporated into vesicles was removed on a 5-mL exocellulose desalting column (Pierce, Rockford, IL). Vesicles were quantitated on the basis of their phospholipid content (31), stored under argon in the dark, and used within 2 days.

### Disruption of DOPC–cholesterol vesicles by lysoPC

Vesicles were prepared as described above, diluted, and resuspended in 20 mM TRIS buffer. The concentration of total lipid was kept constant. Incubation of vesicles with lysoPC was performed for 3 h at room temperature in the dark. After incubation an aliquot of the sample was diluted in buffer and the fluorescence measured (excitation, 490 nm; emission, 520 nm). An increase in vesicle disruption released the 5- and 6-carboxyfluorescein dye into the buffer, resulting in decreased self-quenching and an increase in fluorescence. Maximal fluorescence for each vesicle preparation was determined after total disruption by Triton X-100. LysoPC disruption of vesicles was reported as a percentage of the total release.

### Detection of intracellular reactive oxygen species

Generation of cellular reactive oxygen species by lysoPC was measured with carboxy-$H_2$DCFDA. This dye is freely permeable to membranes, but after entering cells, cleavage of the diacetate by esterases renders it impermeable. The resulting dye fluoresces on interaction with reactive oxygen species, including hydrogen peroxide and hydroxyl radical but not superoxide anion (32). Human dermal fibroblasts were seeded on coverslips in 6-well plates. The next day cells were exposed to 6 μM lysoPC for increasing periods of time. Carboxy-$H_2$DCFDA (25 μM) was incubated with the cells for the final 2 h of incubation with lysoPC. In some experiments the fluorescent marker carboxy-$H_2$DCFDA

was incubated with cells at 25 μM as a 2-h pretreatment and removed from medium before the addition of lysoPC. Reactive oxygen species generation in cells was detected with an Oncor (Gaithersburg, MD) video imaging system with a Nikon (Garden City, NY) fluorescent microscope with excitation at 490 nm and emission at 515 nm. Generation of reactive oxygen species was readily determined by a marked increase in intracellular fluorescence.

### Measurement of cellular proliferation

To ascertain the effect of cholesterol on cellular proliferation cells were incubated overnight with 65 μM cholesterol. Proliferation was then measured with [$^3$H]thymidine as previously described (10). Briefly, cells were washed three times with DMEM–F12 to remove excess cholesterol. [$^3$H]thymidine (1 μCi/mL) was added for 10 or 24 h. Cells were washed once with ice-cold 10% trichloroacetic acid (TCA). Fresh 10% TCA was added and the cells were placed on ice for 15 min. The TCA was then removed and cells solubilized with 0.25 N NaOH for 15 min with agitation. An aliquot was removed and [$^3$H]thymidine incorporation quantified.

### Uptake and metabolism of lysoPC

Cellular uptake and metabolism of lysoPC were monitored with radiolabeled lysoPC. Briefly, human dermal fibroblasts were seeded into 48 wells. The next day cells were washed and then exposed to 3 μM lysoPC that was spiked with [$^{14}$C]lysoPC. At designated intervals the medium was removed and cells were digested with 0.1% sodium dodecyl sulfate (SDS). Total uptake of lysoPC was quantified by measuring the total label present in the lysate, using a scintillation counter. To monitor the metabolism of lysoPC the lipids were extracted and subjected to thin-layer chromatography (TLC) as previously described (1). The TLC plate was exposed to film at −70°C for 3 weeks.

### Determination of plasma membrane cholesterol content

Cholesterol content of the plasma membrane was determined as previously described (33). Briefly, cells were seeded in 6-well plates and incubated overnight with antioxidants or solvent control. Cells were washed with phosphate-buffered saline (PBS) to remove excess antioxidants and extracellular cholesterol. Cells were fixed with glutaraldehyde (2.5%) for 15 min on ice, washed with PBS to remove glutaraldehyde, and incubated with 0.25 unit of cholesterol oxidase for 60 min to convert plasma membrane cholesterol to cholestenone. Cells were washed with PBS to remove cholesterol oxidase and scraped in the presence of 0.1% SDS, and the entire lysate extracted with acetonitrile. Cholestenone present in the lysate was determined by reversed-phase high-performance liquid chromatography (HPLC) with detection at 235 nm. Total cellular free cholesterol was determined by lysing the cells with 0.1% SDS and then incubating the lysate with cholesterol oxidase to convert cholesterol to cholestenone.

## RESULTS

The nature of lysoPC-induced cellular injury was examined by multiple approaches. Initially, cell permeabilization by lysoPC was quantified by monitoring the specific release of $^{14}$C from human fibroblasts that had been previously loaded with [$^{14}$C]adenine (28). A time course of $^{14}$C release revealed that initiation of the lysoPC-induced permeabilization began within 10 min and concluded approximately 5 h after lysoPC addition (**Fig. 1**). As Fig. 1 illustrates, the release of $^{14}$C was separable into two distinct phases: an initial, rapid phase that occurred in the first 30 min and a more prolonged and slower release that lasted several hours. Typically, $^{14}$C release measured in this assay has been associated with cytotoxicity and has been shown to correlate with other indicators of cell death (28). Somewhat unexpectedly, our other data (see below) revealed that the specific $^{14}$C release by lysoPC under the conditions of our experiments reflected permeabilization during a transient, nonlethal injury.

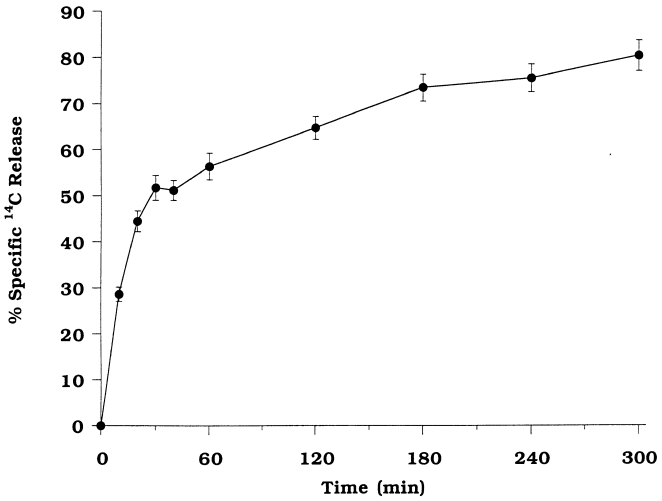


**Fig. 1.** Time course of lysoPC-induced permeabilization. Human dermal fibroblasts, loaded with [$^{14}$C]adenine and washed, were treated with 7 μM lysoPC in serum-free medium for up to 5 h. Specific release of $^{14}$C was measured starting at 10 min. Data are means of three replicates ± SD.

Another characteristic of lysoPC-induced injury was a significant morphological change when cells were exposed to concentrations of lysoPC comparable to those used above. Approximately 20–30 min after addition of 6 μM lysoPC, cells could first be observed by light microscopy to constrict and round. This was well after the start of the rapid $^{14}$C release, which was significant at 10 min (Fig. 1). Time-lapse photography revealed that the morphological changes were maximal at 1.5–2 h and by approximately 5 h cells returned to a typical fibroblast morphology that resembled their appearance before lysoPC addition (**Fig. 2**). Monitoring individual cells in a filmed population of cells, we observed that while cells underwent significant changes in morphology and were delayed in dividing, there was no significant decrease in the total number of viable cells in response to the lysoPC concentrations used (**Table 1**). Even stronger evidence for the viability of lysoPC-injured cells was provided by our finding that when serum was provided to cells 5 h after exposure to lysoPC, cells were able to proliferate readily (data not shown). The recovery of cells after exposure to lysoPC may be dependent on the metabolism of the lysophospholipid. With radiolabeled lysoPC with the tag on the fatty acid, we tracked the uptake and metabolism of lysoPC, using lipid extracts from cells. Uptake was evident as early as 10 min, as was the conversion of lysoPC to PC. Both processes were evident over the 5 h of exposure to lysoPC (data not shown). We

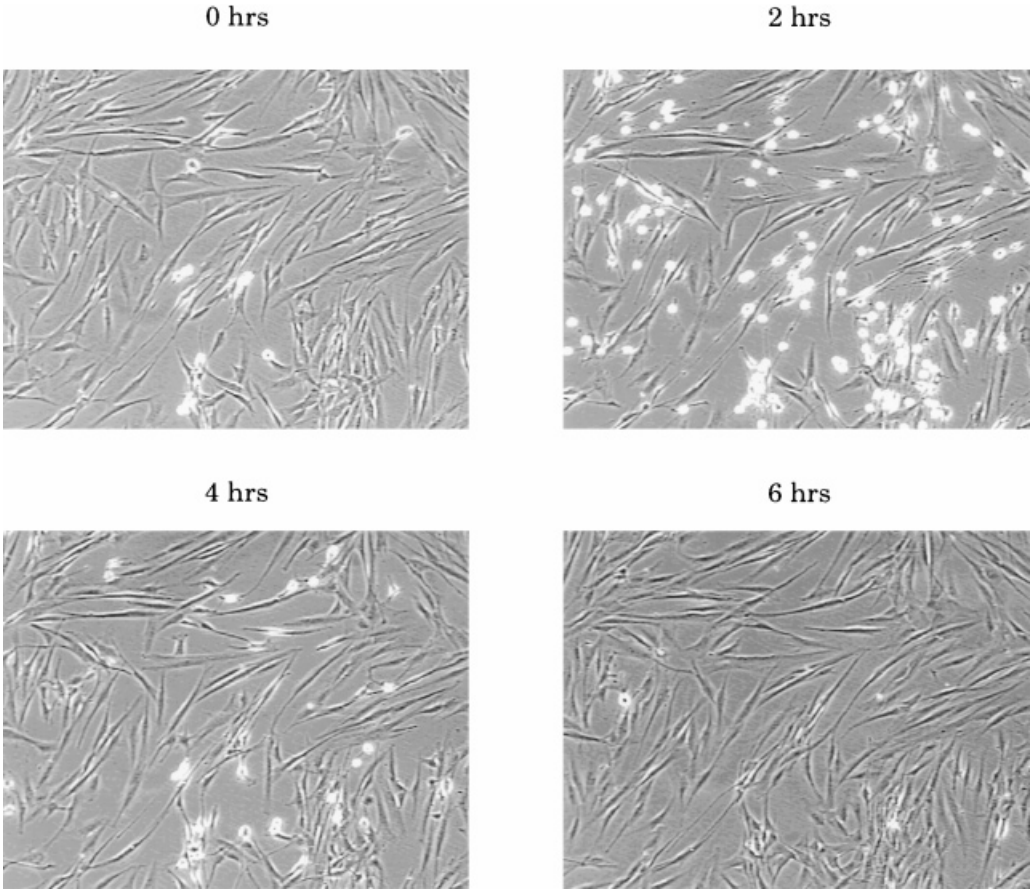


**Fig. 2.** LysoPC-induced changes in cellular morphology. Human dermal fibroblasts were treated with 6 μM lysoPC for increasing periods of time. Changes in cellular morphology were monitored by time-lapse photography, with images captured at 3-min intervals. Pictured are images from 2-h intervals.

hypothesize that the recovery of cells occurs as the metabolism of lysoPC exceeds the uptake of lysoPC at about 5 h.

Two approaches, with and without cells, were used to determine whether the changes observed were attributable to membrane destabilization by lysoPC. In the first, cellular levels of cholesterol were increased to enhance membrane stability. The overnight treatment of cells with cholesterol was previously reported to double the level of total cellular cholesterol (21). We used the oxidation of cholesterol by cholesterol oxidase in intact cells as a marker of plasma membrane cholesterol (33) and observed a significant increase in the level of cholesterol in the plasma membrane after overnight cholesterol treatment (data not shown). Cells loaded with cholesterol exhibited a marked reduction in specific $^{14}C$ release in response to lysoPC (**Fig. 3**). Even the initial rise in specific $^{14}C$ release could be overcome by incubating cells with cholesterol (65 μM) before treatment with lysoPC. The effect of cholesterol on the uptake and metabolism of lysoPC was examined with radiolabeled lysoPC. Cells were loaded with cholesterol overnight and then exposed to radiolabeled lysoPC for up to 5 h. Lipid extracts from cells were collected and subjected to TLC analysis. The only deviation in lysoPC metabolism in cholesterol-loaded cells was a slight increase in the level of PC formed at 120 min. However, this was significantly later than when the protective effects of cholesterol were observed. The data suggest that altered metabolism of lysoPC in response to cholesterol was not responsible for the decreased specific $^{14}C$ release observed after loading cells with cholesterol. An increase in cellular cholesterol also significantly reduced the number of cells that underwent morphological changes in response to lysoPC (Table 1). In a separate experiment we

TABLE 1. Inhibition by vitamin E and cholesterol of lysoPC-induced changes in cell morphology and proliferation

| | % of Cells Rounding During 10 h[a] | Fold Change in Total Cell Number after 10 h |
|---|---|---|
| Lysophosphatidylcholine | 72 | 1.05 |
| LysoPC + vitamin E | 60 | 1.17 |
| LysoPC + cholesterol | 44 | 1.41 |

Cells were treated overnight without or with 40 μM vitamin E or 65 μM cholesterol. Cells were washed extensively and then exposed to 5 μM lysoPC for 10 h. Seventy to 90 individual cells were monitored at 3-min intervals for 10 h and the fate of each described in terms of rounding morphology, cell division, and recovery to grossly normal morphology.

[a] Cells rounding due to lysoPC only. Cells that rounded and then divided were excluded from the numerator (cells rounding). The denominator is the number of cells present at the onset of lysoPC exposure.

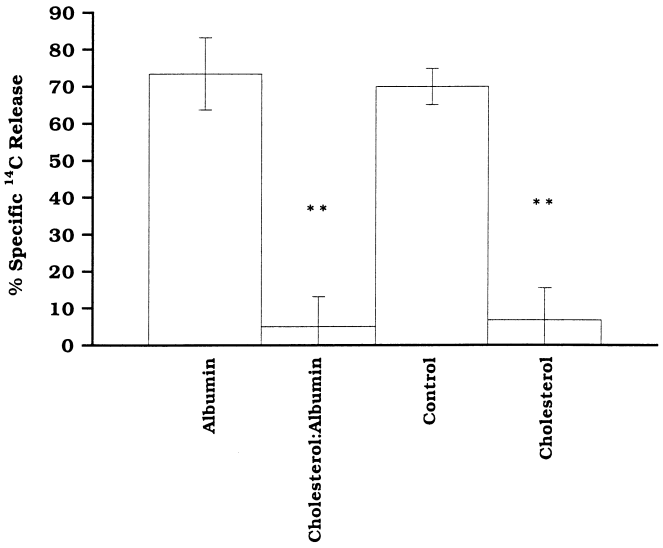


**Fig. 3.** Effect of cholesterol on lysoPC-induced specific release of $^{14}$C. Human dermal fibroblasts were pretreated overnight with cholesterol–albumin coacervates (65 μM cholesterol) or cholesterol (65 μM). Cells were washed extensively to remove excess cholesterol. Cells were then treated with 6 μM lysoPC for 24 h and the specific release of $^{14}$C quantified. Data are means of three replicates ± SD. ** $P < 0.01$ versus control.

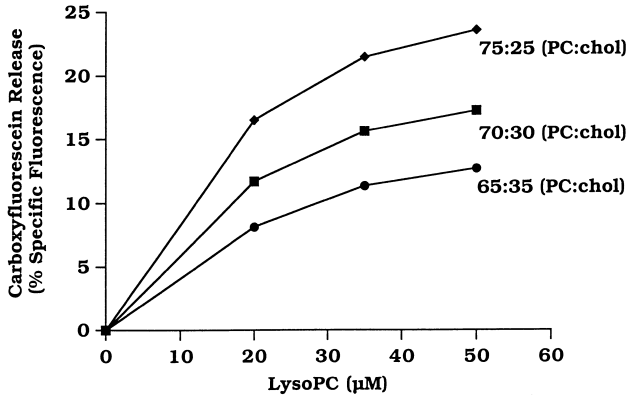


**Fig. 4.** Effect of cholesterol on lysoPC-induced disruption of DOPC–cholesterol vesicles. Vesicles were prepared with differing cholesterol composition in the presence of 5,6-carboxyfluorescein and then exposed to lysoPC for 3 h. Circles, DOPC–cholesterol vesicles (65:35, mol%); squares, DOPC–cholesterol vesicles (70:30, mol%); diamonds, DOPC–cholesterol vesicles, (75:25, mol%).

examined whether loading cells overnight with cholesterol induced cellular proliferation as measured by [$^3$H]thymidine incorporation. There was no increase in [$^3$H]thymidine incorporation either 10 or 24 h after removal of the cholesterol (data not shown). Our data suggest that cholesterol protection against lysoPC-induced injury allowed cells to proceed through the cell cycle, as observed by an increase in total cell number compared with cells treated with lysoPC (Table 1). Our results are consistent with the interpretation that decreased membrane stability by lysoPC resulted in increased release of $^{14}$C, followed by changes in cell morphology.

Numerous cellular events could have influenced the preceding results. For example, permeabilization could require lysoPC-induced cell signaling events or metabolism of lysoPC. Therefore, in our second approach we examined the capacity of lysoPC to induce permeabilization in a cell-free system. LysoPC caused release of the fluorescent dye 5,6-carboxyfluorescein from DOPC–cholesterol unilamellar vesicles. Vesicles were also prepared in the presence of the dye with differing DOPC–cholesterol ratios. As shown in **Fig. 4**, lysoPC induced dye leakage from the vesicles. Furthermore, as the proportion of cholesterol present in the vesicle membrane was raised, the vesicles became progressively less sensitive to dye release by lysoPC (Fig. 4), demonstrating the ability of cholesterol to blunt membrane destabilization. Thus, membrane permeabilization by lysoPC and stabilization by cholesterol could be demonstrated in both cell and cell-free systems.

To examine whether this protective effect was unique to cholesterol we examined the ability of two cholesterol analogs to modulate lysoPC-induced permeabilization of fibroblasts. Both β-sitosterol (whose 3-position hydroxyl is like that of cholesterol, but which has an ethyl group at the 24-position) and stigmasterol (with the 3-position hydroxyl group plus an ethyl group at the 24-position and a double bond at position 22) were taken up by cells to similar extents. Treatment with cholesterol or either of these cholesterol analogs resulted in a significant increase in the respective sterol content in the plasma membranes. As shown in **Fig. 5**, both analogs also protected against lysoPC-induced injury. In separate experiments β-sitosterol and stigmasterol afforded protection quantitatitively comparable to that by cholesterol (data not shown).

We had previously observed that vitamin E was able to blunt certain effects attributed to lysoPC (10, 21, 26). We

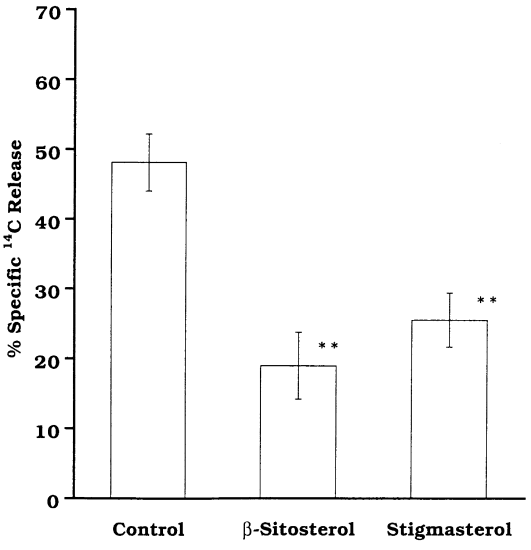


**Fig. 5.** Effect of sterol analogs on lysoPC-induced release of $^{14}$C. Human dermal fibroblasts were pretreated overnight with a 65 μM concentration of the two sterol analogs indicated. Cells were washed extensively to remove excess sterol before treating with 6 μM lysoPC for 24 h. The specific release of $^{14}$C was quantified. Data are means of three replicates ± SD. ** $P < 0.01$ versus control.

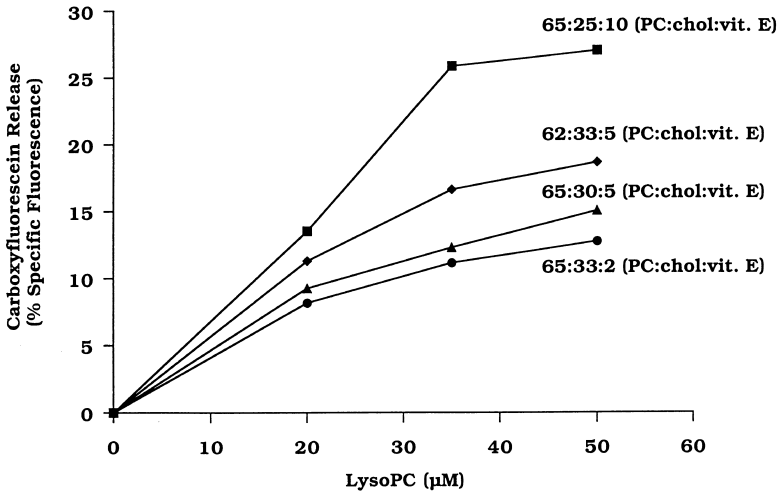


**Fig. 6.** Effect of vitamin E on lysoPC-induced disruption of DOPC–cholesterol vesicles. Vesicles were prepared with differing vitamin E composition in the presence of 5,6-carboxyfluorescein and were then treated with lysoPC. Circles, DOPC–cholesterol–vitamin E vesicles (65:33:2, mol%), squares, DOPC–cholesterol–vitamin E (65:25:10, mol%); diamonds, DOPC–cholesterol–vitamin E (62:33:5, mol%); triangles, DOPC–cholesterol–vitamin E (65:30:5, mol%).

examined the protective nature of vitamin E more closely, using time-lapse photography to determine if vitamin E protection from lysoPC-induced injury could be observed morphologically as was seen with cholesterol. Pretreatment overnight with 40 μM vitamin E reduced the number of cells undergoing rounding in response to lysoPC (Table 1). In addition, vitamin E partially protected the ability of cells to continue proliferation in the presence of lysoPC (Table 1). Vitamin E is a known antioxidant, suggesting that one component of lysoPC-induced injury may involve oxidant stress. However, vitamin E also has multiple effects unrelated to its antioxidant capability (34–36).

Multiple approaches were used to determine whether lysoPC was eliciting an oxidative response. Initially, to study whether protection by vitamin E may have been through its ability to stabilize membranes (37), DOPC–cholesterol vesicles were formed without or with increasing molar fractions of vitamin E. However, rather than offering increased membrane stability, the presence of vitamin E resulted in an increase in lysoPC-induced leakage from vesicles (**Fig. 6**). This suggested that the reduction in $^{14}C$ release attributed to vitamin E (**Fig. 7B**) may be occurring through a mechanism other than membrane stabilization.

To ascertain whether the antioxidant properties of vitamin E contributed to the reduction in lysoPC-induced injury, the effectiveness of other antioxidants was examined. DPPD was selected because it is a general antioxidant that is capable of scavenging lipid and peroxyl radicals and would therefore likely be able to interfere with the progression of cellular lipid peroxidation (21, 38, 39). As Fig. 7A illustrates, overnight pretreatment with DPPD was able to significantly blunt injury by lysoPC. For comparison, the decrease in the lysoPC-induced specific release of $^{14}C$ as a result of overnight pretreatment with vitamin E can be observed in Fig. 7B. The inhibition of lysoPC-induced permeabilization afforded by DPPD in multiple experiments averaged approximately 27% (n = 36), while that by vitamin E averaged approximately 76% (n = 5). Protection by DPPD was observed when DPPD was administered either as an overnight pretreatment or as a concurrent treatment with lysoPC. DPPD provided protection to both asynchronous, proliferating cells and quiescent cells (data not shown), suggesting its effect was not a result of altering cell cycle progression. The presence of 1 mM reduced glutathione (GSH) was able to enhance the protective nature of DPPD to about 45% inhibition of $^{14}C$ release; however, GSH alone was unable to protect cells from injury by lysoPC (data not shown).

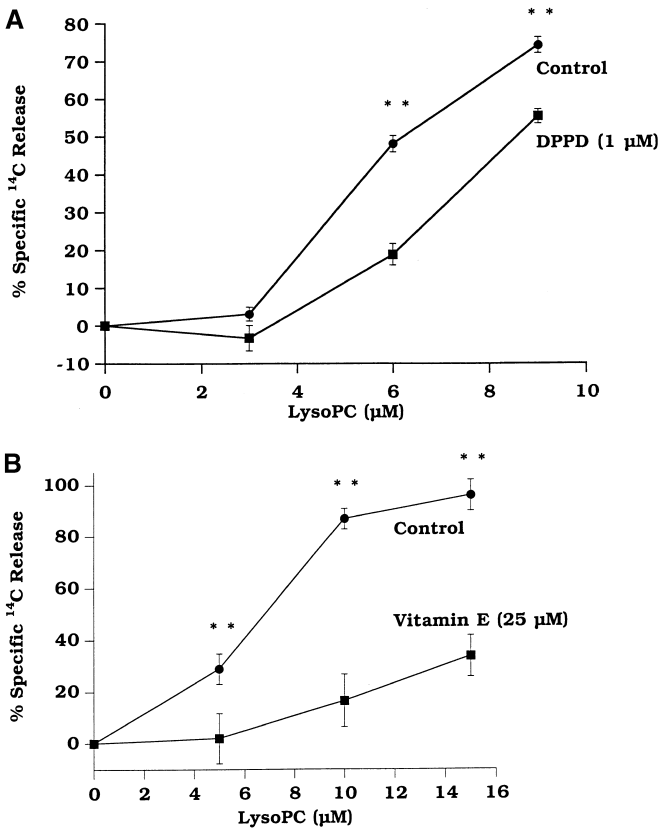


**Fig. 7.** Effect of antioxidants on lysoPC injury. (A) Human fibroblasts were treated overnight without (circles) or with (squares) 1 μM DPPD and then exposed to lysoPC for 24 h. (B) Human fibroblasts that were pretreated overnight without (circles) or with (squares) 25 μM vitamin E. Data are means of three replicates ± SD. ** $P < 0.01$ versus control at equivalent concentrations of lysoPC.

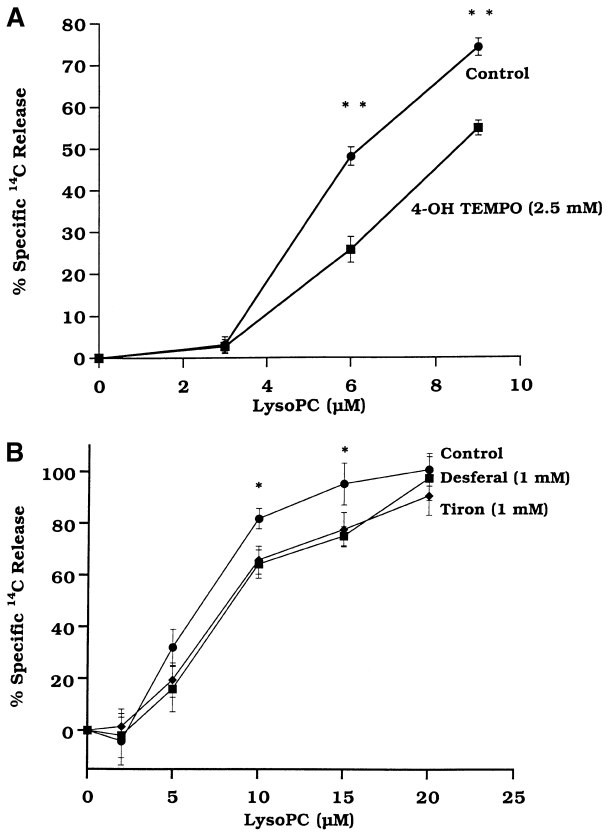


**Fig. 8.** Inhibition of lysoPC-induced injury by 4-OH TEMPO and metal chelators. (A) Human dermal fibroblasts were treated without (circles) or with (squares) 2.5 mM 4-OH TEMPO and then exposed to lysoPC for 24 h. Injury by lysoPC was blunted by this scavenger of free radicals. (B) Inhibition of lysoPC-induced injury by metal ion chelators. Cells were incubated without (circles) or with either 1 mM Desferal (squares) or 1 mM Tiron (diamonds). These agents provided a modest but consistent inhibition of lysoPC injury. Data are means of three replicates ± SD. * $P < 0.05$ and ** $P < 0.01$ versus control at equivalent concentrations of lysoPC.

The concept was further tested using additional, structurally distinct antioxidants known to act by different mechanisms. 4-OH TEMPO, a spin label, can act as an antioxidant via its ability to form stable complexes with free radicals and, like DPPD, it moderately but significantly inhibited lysoPC induced injury (average inhibition, up to 36% [n = 3]; **Fig. 8A**). Desferal and Tiron, which act as cellular metal ion chelators, also provided a modest but significant degree of protection against lysoPC injury (average inhibition for Desferal and Tiron was up to 28% [n = 6] and 31% [n = 4], respectively; Fig. 8B). The preceding findings support the concept that lysoPC injury toward cells had an oxidative component.

To examine whether these responses to lysoPC were general effects induced by lysolipids, cells were treated with lysoPE. LysoPE induced $^{14}$C release and transient changes in cellular morphology as observed for lysoPC. The release of $^{14}$C by lysoPE could be blunted by cholesterol, as observed with lysoPC. However, when cells were pretreated with antioxidants, vitamin E, DPPD, or Tiron, no decrease in the lysoPE response was observed. These results suggest that the induction of reactive oxygen species may be unique to lysoPC and not solely a response to membrane permeabilization.

On the basis of the observations that cholesterol was protective against lysoPC injury we examined whether antioxidant protection was secondary to antioxidant-associated increases in the levels of cholesterol in cells or more specifically in the plasma membrane. As determined by the conversion of free cholesterol to cholestenone in total cell lysates by cholesterol oxidase, the total level of free (nonesterified) cholesterol was unchanged in response to overnight treatment with the antioxidants DPPD and Desferal (data not shown). More importantly, when the cholesterol levels in the plasma membrane were examined after treatment with antioxidants, no significant changes were observed. The effect of antioxidants on the uptake and metabolism of lysoPC was also examined with [$^{14}$C]lysoPC. Uptake of lysoPC was not modified in response to the antioxidants. No alteration in the metabolism of lysoPC was detected by TLC analysis of cell extracts. To determine further whether antioxidants may be working through membrane stabilization we examined whether antioxidants could prevent lysoPC-induced disruption of vesicles. We examined lysoPC-induced vesicle leakage after incubations with both metal chelators (Desferal and Tiron) and DPPD under various conditions. As was observed with vitamin E, these compounds did not prevent lysoPC-induced lysis of the vesicles (data not shown). These observations suggest that these antioxidants were not protecting cells through increasing membrane stability.

The data collected with the preceding inhibitors of lipid peroxidation suggested that oxidative stress was a contributor to lysoPC-induced injury. As an additional and distinct approach to confirm this finding, the fluorescent indicator carboxy-$H_2$DCFDA was used to monitor more directly the presence of intracellular reactive oxygen species. Cells exposed to lysoPC exhibited a marked increase in fluorescence, indicating the generation of reactive oxygen species (**Fig. 9**). The same qualitative results were obtained when the fluorescent indicator was administered to cells as a pretreatment only, with removal of the dye from the medium before exposure of cells to lysoPC (data not shown).

## DISCUSSION

Lysophosphatidylcholine, an amphipathic molecule, is believed to be of significant biological importance. It induces a wide range of important cellular effects, but the mechanisms through which it evokes such diverse cellular responses are not clearly defined. LysoPC has been reported to stimulate various signaling pathways. Depending perhaps on the cell system, it can activate protein kinase C (PKC) (40–42), increase cAMP through a G protein-dependent pathway (43), stimulate phospholipase D (40), alter membrane $Na^+$,$K^+$-ATPase (44), increase intracellular $Ca^{2+}$ (11), and induce NF-κB-binding activity (45). Because the preceding signaling phenomena are

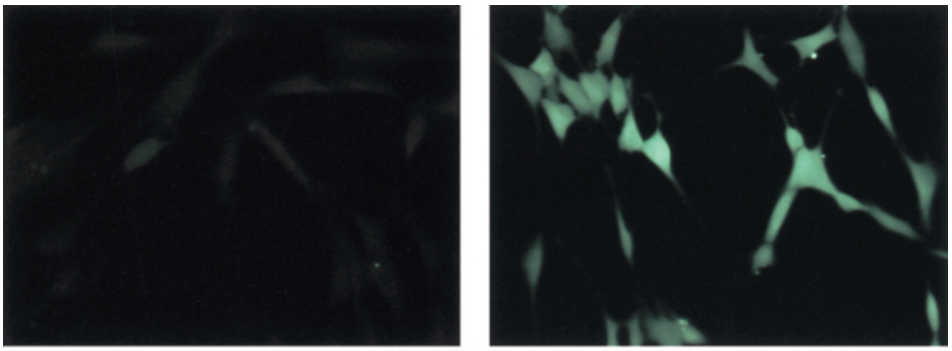


**Fig. 9.** Time course of lysoPC-induced generation of reactive oxygen species. Human dermal fibroblasts treated without (A) or with (B) 6 μM lysoPC for 2 h. During this period cells were loaded with carboxy-$H_2$DCFDA, which detects reactive oxygen species. Cells were visualized with the Oncor video imaging system and a Nikon fluorescence microscope (excitation, 490 nm; emission, 515 nm).

likely influenced by the sublethal injury induced by lysoPC, we focused on the effects of lysoPC on membrane permeabilization and a hypothesized induction of oxidant stress.

LysoPC is a naturally occurring product resulting from phospholipase $A_2$ activity to remove fatty acids from membrane and lipoprotein phospholipids. LysoPC is rapidly taken up by cells, and can undergo either further deacylation to form diacylglycerol or reacylation to form phosphatidylcholine (46). Delivery of exogenous lysoPC to cells may disrupt membranes after exceeding the ability of these pathways to metabolize lysoPC. For example, higher concentrations of lysoPC [164 μM, which is above the reported critical micellar concentration in aqueous medium of ~50 μM (47)] increased plasma membrane permeability sufficiently to enhance the delivery of anticancer agents to cells (24, 25). In the present study, however, the concentration of lysoPC was maintained well below the reported critical micellar concentration. This would presumably minimize effects that could be attributed to micellar disruption of the plasma membrane, such as removal of membrane proteins (23). We found that lysoPC, below its critical micellar concentration, was able to induce a rapid release of $^{14}$C from [$^{14}$C]adenine-loaded human foreskin fibroblasts. This rapid release was followed by a more prolonged and gradual release of $^{14}$C (Fig. 1). The initial rapid release of $^{14}$C may have resulted from the rapid uptake and possible localization of lysoPC within membrane domains, resulting in a loss of barrier function. While exposure of cells to high concentrations of lysoPC can result in cell lysis, our results show clearly that, within a specific concentration range, membrane permeabilization can occur that does not lead to cell death. This was based on multiple lines of evidence. By tracking morphological changes in individual cells, we observed rounding followed by reversion to initial, pre-lysoPC morphology for all cells. Further evidence of this nonlethal reversibility is in part revealed in Table 1. A 10-h exposure to lysoPC apparently delayed cell cycle progression, but cell number was not decreased. Most importantly, cells exposed to lysoPC competently proliferated on subsequent stimulation with serum. These observations are consistent with the theory that in our system lysoPC induced a transient decrease in membrane stability, leading to increased membrane permeability that did not ultimately lead to cell lysis.

Our experiments demonstrating the marked protection offered both to cells and unilamellar vesicles by cholesterol support the hypothesis that lysoPC destabilizes membranes. Previous studies have demonstrated a stoichiometric interaction between cholesterol and lysoPC (48) and suggested that these two "cone-shaped" molecules combine to form a more cylindrically shaped complex (48, 49). It has been proposed that interaction of the choline group of the phospholipid with the hydroxyl group at the 3-position of cholesterol was responsible for helping to stabilize this interaction (49). In indirect support for the interpretation that cholesterol acted via such a stabilizing effect our results showed that two cholesterol analogs that also have the hydroxyl group at the 3-position, β-sitosterol and stigmasterol, also partly inhibited lysoPC-induced $^{14}$C release. These findings suggest that cholesterol may interact directly with lysoPC, perhaps within cholesterol-enriched membrane domains, to prevent disruption of the plasma membrane. When the uptake of lysoPC was examined no significant effect of increased cholesterol on lysoPC uptake was observed. When TLC analysis for lysoPC metabolites was performed on cell lipid extracts, no deviations in cellular metabolism of lysoPC were induced by the sterols.

LysoPC also induced remarkable, yet transient, changes in cell morphology. These changes were not observed by light microscopy until 20–30 min after the addition of lysoPC, well after the beginning of the marked permeabilization, which was readily detectable 10 min after addition of lysoPC. Such reversible changes in cell morphology have been attributed to disturbances in the balance between the phosphatases and kinases responsible for maintaining cell shape. LysoPE, another amphipathic molecule, also induced these transient changes in cellular morphology. This suggests that in our system the lysophospholipids may be effecting such an imbalance, possibly as

a result of alteration in calcium ion (11, 50, 51) or a result of an effect of lysoPC on phosphatases and/or kinases (40–42, 45, 52, 53).

The inhibition by vitamin E of lysoPC-induced release of $^{14}C$ from [$^{14}C$]adenine-loaded fibroblasts (21) could have resulted from one or more of the known activities of vitamin E. Vitamin E could have stabilized membranes or interacted directly with lysoPC, as has been reported to occur in certain systems (34, 35). Alternatively, lysoPC could injure cells through a mechanism involving induction of oxidant stress. We examined these possibilities by multiple approaches. Although vitamin E has been reported to interact with lysoPC (34, 35), our measurements of dye leakage from vesicles made with increasing amounts of vitamin E suggested that vitamin E did not stabilize membranes of unilamellar vesicles against lysoPC. The hypothesis that lysoPC was causing oxidative stress was supported by our findings that several antioxidants, including those that are structurally distinct from vitamin E, namely, DPPD, 4-OH TEMPO, Desferal, and Tiron, diminished $^{14}C$ release moderately but significantly. It is interesting to note that these antioxidant compounds inhibited the lysoPC-induced injury by up to only 25–35%; the combination of DPPD and GSH inhibited up to approximately 45% and vitamin E inhibited up to approximately 75%. The explanation for the variable degree of protection is not known.

Two lines of indirect evidence suggested the transient permeabilization of the plasma membrane was required for the oxidant response to lysoPC. First, if membrane permeabilization is a prerequisite for the lysoPC induction of oxidant stress or the change in morphology, then cholesterol, which stabilizes membranes, would be anticipated to blunt all these effects, but antioxidants would not be expected to blunt membrane permeabilization as successfully as cholesterol. Indeed, cholesterol markedly reduced permeabilization, changes in morphology and the putative oxidant effects induced by lysoPC; antioxidants inhibited permeabilization significantly, but less effectively than cholesterol. Antioxidants, which did not alter the cholesterol content of the plasma membrane, did not inhibit the permeabilization by lysoPE, suggesting specificity of the oxidant effect of lysoPC.

Second, it appeared from our measurements that membrane permeabilization preceded both the detection of reactive oxygen and the changes in morphology. The ability of lysoPC to induce oxidant stress was examined with the fluorescent dye carboxy-$H_2$DCFDA, which has been used previously as an intracellular marker of reactive oxygen species (54–56). LysoPC induced the generation of reactive oxygen species that was maximal approximately 1 to 2 h after exposure to lysoPC (Fig. 9 and data not shown), a time course similar to that observed for the morphological changes (Fig. 2).

Taken together, our data demonstrate clearly that lysoPC can evoke an intracellular oxidant effect in cells in addition to its membrane-destabilizing effects. This interpretation is indirectly supported by our previous reports of vitamin E inhibition of lysoPC-induced cellular effects (10, 21, 26) and reports showing that lysoPC led to increased extracellular $O_2^{\cdot -}$ via a PKC-dependent pathway in rabbit thoracic aorta (42) and a brief burst of $O_2^{\cdot -}$ production in human endothelial cells (57). The generation of reactive oxygen species that we measure in our system with carboxy-DCFDA does not appear to be dependent on PKC activation, based on the lack of effectiveness of any of multiple PKC inhibitors and phorbol ester downregulation to reduce lysoPC-induced fluorescence (S. M. Colles and G. M. Chisolm, unpublished observations). In addition, we observed a more prolonged production of reactive oxygen species than was observed in the human endothelial cells. In the endothelial cell system $O_2^{\cdot -}$ production was maximal in less than 5 min (57). We observed peak production approximately 2 h after addition of lysoPC, suggesting distinct mechanisms may be involved.

How the generation of reactive oxygen species contributes to the cellular injury is unclear; the specific cellular signals evoked by lysoPC that lead to oxidant stress are unknown. Certainly, disruption of the plasma membrane by lysoPC appears to be one factor involved in the initiation process. Permeabilization is readily detectable before our detection of reactive oxygen species (Figs. 1 and 9). The ability of lysoPC to induce oxidant stress may be a factor important in the ability of lysoPC to alter gene expression, possibly through altering redox-sensitive transcription factors (45, 58).

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