Exogenous Oxidants Initiate Hydrolysis of Endothelial Cell Inositol Phospholipids

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Oxidants released from inflammatory cells contribute to the pathogenesis of acute inflammatory edema in many models. Chemically produced oxidants can reversibly alter the barrier properties of cultured endothelial and epithelial monolayers. This report examines the effects of nonlytic doses of H2O2 on endothelial cell lipids. H2O2 oxidized ω-6 fatty acids in the endothelial cells and initiated hydrolysis of endothelial cell phospholipids. When endothelial cells were exposed to peroxidized linoleic acid, it caused lysis of the cells at doses 1,000-fold lower than effective doses of H2O2. The phospholipase A activity resulted in increases in free fatty acids

Measuring albumin transfer. Micropore filters (Nucleopore, Pleasanton, CA) were gelatin impregnated and glued to polycarbonate cylinders. Polycarbonate cylinders were placed into the wells of 24-well plates mounted on filters. Experiments with phorbol esters, phorbol-12,13-dibutyrate (PDBU), and 4a-phorbol-12,13-didecanoate were dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL), aliquoted, and stored at -20°C. iO

| MATERIALS AND METHODS |

Cell culture and monolayer preparation. Porcine pulmonary artery endothelial cells were cultured as previously described. Cells from passages 5 to 12 were used in all experiments. Cells were identified as endothelial by uniform fluorescent staining after uptake of fluorescently labeled acetylated low-density lipoprotein. Microscope filters (Nucleopore, Pleasanton, CA) were gelatin impregnated and glued to polycarbonate cylinders. Polycarbonate cylinders were placed into the wells of 24-well plates mounted on filters. Experiments with phorbol esters, phorbol-12,13-dibutyrate (PDBU), and 4a-phorbol-12,13-didecanoate were dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL), aliquoted, and stored at -20°C. iO
Measuring hydrocarbon release. Release of hydrocarbons from endothelial cells exposed to H$_2$O$_2$ was used as an index of lipid peroxidation in the cells. Endothelial cells were removed from tissue culture flasks with brief (15 second) exposure to trypsin (0.25%) and EDTA (0.1%) and immediately added to 50 mL M199 with 10% fetal bovine serum (FBS) and the cells pelleted by centrifugation (100 g for ten minutes). Pelleted cells were washed three times in Hanks' balanced salt solution (HBSS) with 15 mmol/L HEPES, pH 7.4 (Hanks-HEPES), and finally suspended in Hanks-HEPES at 1.8 ± 0.1 x 10$^5$ cells/mL. The cells (1 mL) were aspirated into a 10-mL blood gas syringe previously purged with nitrogen, leaving 6-mL head space. The syringe was closed with a three-way stopcock fitted with a rubber gasket. A Hamilton syringe was used to inject 100 μL of H$_2$O$_2$ in HBSS through the gasket and stopcock into the cell suspension. Control syringes received 100 μL HBSS alone. A control syringe from the same passage of cells used in the H$_2$O$_2$ exposure was included for every experimental syringe. In additional experiments, the same protocol was followed except that 5 μmol/L A23187 was injected instead of H$_2$O$_2$. This dose of A23187 has consistently caused prostacyclin release from porcine pulmonary endothelial cells cultured as mentioned earlier (data not shown).

Hydrocarbons in the gas phase were detected on a Varian gas-liquid chromatograph (Walnut Creek, CA) equipped with a flame ionization detector linked to a Spectra Physics Integrator (Mountain View, CA) to quantitate the peaks. The sample was injected onto a Porasil (Supelco Inc, Bellefonte, PA) C column (80/100 mesh, 1/8 in diameter, 5 m long). After injection, the column was heated to 28°C for eight minutes, after which the temperature was increased to 50°C at a rate of 2°C/min and maintained at 50°C for 20 minutes. Standards of ethane and pentane were analyzed prior to test injections, and the sensitivity of the assay was consistently 1 pmol. The chromatograph is the property of Dr Robert Roberts who graciously made it available for this part of the study.

Cell lipid analyses. Endothelial cells in 60-mm-diameter tissue culture plates were incubated with 1.8 ± 0.2 x 10$^8$ cpm of 3H-inositol for 16 hours, washed three times, and then exposed to H$_2$O$_2$ in Hanks-HEPES with 10 mmol/L LiCl for ten minutes. After the incubation the media was removed and the cells immersed and scraped into 2 mL ice-cold methanol. The lipids were extracted by the subsequent addition of 2 mL methanol, 2 mL chloroform, and 1.4 mL 2.4 mol/L HCl followed by 2.0 mL chloroform and 2.0 mL 2.4 mol/L HCl. Approximately 80% of counts were recovered in the single phase. The phases were separated by centrifugation at 2,000 g for ten minutes. The lower chloroform phase was washed with methanol:HCl, dried under nitrogen, and redissolved in chloroform methanol (2:1) for application to silica gel plates. The inositol lipids were separated on oxalate-impregnated plates by using a mixture of chloroform, acetone, ethanol, acetic acid, and water (40:15:13:12:8). To measure inositol polyphosphates, the upper phase was washed with chloroform, dried under nitrogen, and redissolved in water with the pH adjusted to 7.6 with 0.2 mol/L Tris. Inositol polyphosphates were then separated on Dowex 1 columns (Bio-Rad, Richmond, CA) as described. Fractions were collected and counted on a liquid scintillation counter.

For determination of lipid phosphate, endothelial cells in 35-mm-diameter tissue culture plates were preincubated with 30 μCi$^{32}$P (New England Nuclear, Cambridge, MA) for 16 hours in M199 with 10% FBS. The cells were washed five times with Hanks-HEPES and then exposed to 1 mmol/L H$_2$O$_2$ in Hanks-HEPES for ten minutes. The incubation medium was then removed and the cells scraped into 4 mL methanol. Lipids were extracted by using the same procedure described earlier for inositol phospholipids. Aliquots of the chloroform phase were then taken for analysis of $^{32}$P activity in the individual phospholipids by using CHCl$_3$, MeOH, and methylamine (60:35:5) to separate the phospholipids. Another aliquot of the chloroform phase was dried under nitrogen and then analyzed for phosphate as described. Total protein in sister monolayers on the same plates was determined according to Lowry et al.

Lactate dehydrogenase assay. To quantitate lytic injury to the cells, lactate dehydrogenase (LDH) activity released to the bathing medium after exposure to H$_2$O$_2$ or oxidized fatty acid was determined and compared with the amount of LDH activity released from the cells by Triton X-100 (0.1%). In each protocol, cells were twice exposed to H$_2$O$_2$ or oxidized fatty acid in M199 for a specified time, crystallized catalase (Sigma, C3155) added when H$_2$O$_2$ was used, and the medium removed and replaced with M199. The original medium and the M199 were combined and the LDH released from the cells measured as described. Equivalent effects of catalase were observed when catalase was exposed to polymyxin B sepharose before the experiment or when catalase without polymyxin B exposure was used.

Oxidation of fatty acid. Fatty acids were oxidized by reacting the fatty acid (Nu-Check Prep, Inc, Elsian MN; 1 mmol/L, stabilized in Tween 80) with soybean lipoxynase (Sigma) for 30 minutes. The fatty acids were extracted with 20 vol chloroform:
methanol (2:1) that was then acidified to pH 4.0 with 4 vol acid saline and the phases split by gravity. The chloroform phase was dried under nitrogen and the fatty acids redissolved in chloroform: methanol (2:1). Oxidized fatty acids were quantitated by measuring the change in absorbance at 290 nm following the reaction under nitrogen of oxidized fatty acid in the sample with recrystallized KI in methanol. An extinction coefficient of $4.41 \times 10^4$ mol$^{-1}$·cm$^{-1}$ at 290 nm was used. When 1-14C fatty acid was added with unlabeled fatty acid to the lipoxygenase system, recovery was greater than 90%, and greater than 85% of the acid was oxidized. For experimental use aliquots of oxidized fatty acid were dried under nitrogen and then complexed as the Na salt to 1 mmol/L fatty acid–free albumin in the appropriate aqueous solution.

**Statistical analysis.** All data are reported as means ± SE. Comparisons between two paired groups were made by using a paired t test. Differences between more than two groups were tested by using one-way analysis of variance with differences between individual groups tested by using a studentized range test. Differences were considered significant at a level of $P < 0.05$.

**RESULTS**

**Effects of H$_2$O$_2$ on fatty acid oxidation.** When H$_2$O$_2$ was added to endothelial cells suspended in Hanks-HEPES in blood gas syringes, pentane was released to the head space (Fig 1). Doses of H$_2$O$_2$ as low as 7.8 $\mu$mol/L caused detectable pentane release, and more pentane was released with higher doses. In contrast, the addition of 5 $\mu$mol/L A23187 did not result in detectable pentane release.

When endothelial cells were preloaded with 1-14C linoleic acid and then exposed to increasing doses of H$_2$O$_2$, increased counts were released to the incubation medium at a dose of H$_2$O$_2$ of 1 mmol/L (data not shown). In subsequent experiments monolayers from the same passage of cells were labeled with 1-14C linoleic or arachidonic acid and exposed to 1 mmol/L H$_2$O$_2$ for 30 minutes. Increased counts were released to the incubation medium after exposure to H$_2$O$_2$, and more arachidonate than linoleate was released (Fig 2). Based on the mass estimates these experiments suggest that approximately 1.9 nmol of linoleate and 5.7 nmol of arachi-

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**Fig 1.** Pentane released from 1.8 ± 0.1 x 10$^7$ porcine pulmonary artery endothelial cells suspended in Hanks-HEPES in a blood-gas syringe and exposed to increasing concentrations of H$_2$O$_2$ for 30 minutes. Bars represent means ± SE of at least four samples.

**Fig 2.** Radioactivity released from 9.4-cm$^2$ monolayers of confluent porcine pulmonary artery endothelial cells prelabeled with 1-14C linoleic acid or arachidonic acid for 12 hours and then exposed to 1 mmol/L H$_2$O$_2$ in Hanks-HEPES for 30 minutes. Monolayers incorporated an average of 1.8 ± 0.2 x 10$^7$ cpm linoleate and 1.4 ± 0.2 x 10$^7$ cpm arachidonate. Significant release of both fatty acids occurred. Bars represent means ± SE of seven monolayers.

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donate were released from the cells. Additional cells were labeled with 1-14C linolenate and then exposed to 1 mmol/L H$_2$O$_2$. H$_2$O$_2$ increased the release of counts to the incubation medium when linolenate was used as the labeled fatty acid as well (control, 0.59% ± 0.04%; H$_2$O$_2$ exposed, 0.80% ± 0.05%; n = 5).

The lipids released to the incubation medium were extracted and separated by using thin-layer chromatography (Fig 3A). In the medium from control monolayers labeled with 1-14C linoleic acid, 75% ± 3% of the counts comigrated with phospholipid standards, and 15% ± 1% of the counts comigrated with free fatty acids. Of the 15% comigrating with the fatty acids, 9% ± 1% migrated as native fatty acid and 6% ± 1% as oxidized fatty acid. In contrast, in the medium from 1-14C linoleate–labeled monolayers exposed to H$_2$O$_2$ (1 mmol/L, 30 minutes) 65% ± 1% of the label comigrated with the phospholipids, and 27% ± 2% comigrated with free fatty acids. Of the 27% comigrating with the fatty acids, 5% ± 0.5% migrated as native fatty acid and 21% ± 3% as oxidized fatty acid. Similar changes occurred when cells prelabeled with 1-14C arachidonic acid was exposed to H$_2$O$_2$ (1 mmol/L, 30 minutes, Fig 3B) and when cells prelabeled with 1-14C linolenate were exposed to H$_2$O$_2$ (1 mmol/L, 30 minutes, data not shown). Hence, H$_2$O$_2$ caused fatty acid to be released from the cells, thus suggesting activation of phospholipase A activity. In addition, after exposure to H$_2$O$_2$ a larger fraction of the increased amount of fatty acid released comigrated as oxidized free fatty acid.

**Effects of H$_2$O$_2$ on cellular lipids.** The distribution of labeled fatty acid among the major cell lipids from control and H$_2$O$_2$-exposed cells was analyzed to determine from which cellular lipids label was released to the incubation medium (Fig 4). In control cells 90% ± 0.5% of 1-14C radioactivity was found in the phospholipids, and this decreased to 83% ± 0.3% in cells exposed to H$_2$O$_2$ (1
exposure to H\textsubscript{2}O\textsubscript{2}. Hence, H\textsubscript{2}O\textsubscript{2} increased the fraction of the label in control cells, and this did not change with ±0.2% with H\textsubscript{2}O\textsubscript{2}. In control cells 1% ± radioactivity was in diglycerides, and this increased to 5% ± mmol/L, 30 minutes). In control cells 2% ± 0.1% of the radioactivity was in diglycerides, and this increased to 5% ± 0.2% with H\textsubscript{2}O\textsubscript{2}. In control cells 1% ± 0.1% of the label was in free fatty acids, and this increased to 7% ± 0.1% in cells exposed to H\textsubscript{2}O\textsubscript{2}. Triglycerides contained 4% ± 0.2% of the label in control cells, and this decreased to 3% ± 0.1% after H\textsubscript{2}O\textsubscript{2} exposure. Cholesterol esters contained 2% ± 0.1% of the label in control cells, and this did not change with exposure to H\textsubscript{2}O\textsubscript{2}. Hence, H\textsubscript{2}O\textsubscript{2} increased the fraction of label that was present as free fatty acid within the cell lipids. Based on the mass measurements these data suggest that 6.4 nmol linoleate/mg cell protein was present in the cells as free fatty acid. Similar qualitative changes occurred when cells were prelabeled with 1-\textsuperscript{14}C linolenate. Free fatty acid levels increased from 1.2% ± 0.2% of the total counts in control cells to 4.2% ± 0.4% in H\textsubscript{2}O\textsubscript{2}-exposed cells.

Since it appeared that most of the label was released from cell phospholipids, the distribution of 1-\textsuperscript{14}C linoleate and arachidonate among the different phospholipids in control and H\textsubscript{2}O\textsubscript{2}-exposed cells was determined. In cells prelabeled with 1-\textsuperscript{14}C linoleate, H\textsubscript{2}O\textsubscript{2} (1 mmol/L, 30 minutes) caused a decrease in the fraction of 1-\textsuperscript{14}C label that comigrated with phosphatidylserine (7% ± 0.5% in control, 4% ± 0.1% in exposed), but there was not a significant change in the fractional distribution of label among any of the other phospholipids (Fig 5A). Similar changes occurred when the cells were preincubated with 1-\textsuperscript{14}C arachidonate before exposure to H\textsubscript{2}O\textsubscript{2} (1 mmol/L, 30 minutes). The fraction of label that comigrated with phosphatidylinositol decreased from 17% ± 0.3% to 11% ± 0.3%, while the fractional distribution among the other phospholipids did not change (Fig 5B).
When $^1$H linolenate was the labeled fatty acid, a similar pattern evolved, with the fraction of label comigrating with phosphatidylinositol decreasing from $2.2\% \pm 0.03\%$ to $1.8\% \pm 0.01\%$ after exposure to H$_2$O$_2$ (1 mmol/L, 30 minutes).

The aforementioned data described phospholipase A-type hydrolysis of fatty acid from the inositol phospholipids. However, the neutral lipid chromatogram also demonstrated an increase in the amount of fatty acid in the diglyceride (Fig 4), a change consistent with activation of a phospholipase C. For this reason endothelial cells were prelabeled with $^{14}$C or $^3$H inositol and the inositol phospholipids separated after H$_2$O$_2$ exposure. H$_2$O$_2$ (1 mmol/L, ten minutes) increased the fraction of activity that comigrated with lysophosphatidylinositol ($1.478 \pm 175$ cpm, $3.9\% \pm 0.1\%$ for control; $2.673 \pm 185$ cpm, $7.4\% \pm 0.4\%$ for exposed, $n = 5$) and increased the amount of activity that comigrated with phosphatidylinositol biphosphate ($1.071 \pm 117$ cpm, $2.8\% \pm 0.2\%$ for control; $1.708 \pm 97$ cpm, $4.7\% \pm 0.2\%$ for exposed). Exposure to H$_2$O$_2$ for ten minutes also increased the inositol polyphosphates recovered from the cells (Fig 6).

Total lipid phosphate values were not different between control cells ($133.4 \pm 12.3$ nmol lipid phosphate/mg cell protein) and cells exposed to 1 mmol/L H$_2$O$_2$ for 30 minutes ($124.7 \pm 11.3$ nmol lipid phosphate/mg cell protein). This was true when lipid phosphate was expressed per milligram cell protein or per square centimeter of confluent monolayer. When the phospholipids were separated with CHCl$_3$, MeOH, and methylamine ($60:35:5$), the distribution of $^{32}$P radioactivity among the major phospholipid classes was not different between control and H$_2$O$_2$-exposed cells. When the organic phase from these cells was separated with CHCl$_3$, acetone, ethanol, acetic acid, and H$_2$O (40:15:13:12:8), it could be appreciated that phosphatidic acid levels increased from $8.94 \pm 0.36$ nmol/mg cell protein in control cells to $11.0 \pm 0.58$ nmol/mg cell protein in exposed cells ($n = 12$). This increase in phosphatidic acid levels combined with the increases in diglyceride and inositol polyphosphate concentrations suggested activation of a phospholipase C as well as a phospholipase A.

Effects of oxidized fatty acids on endothelial cell lysis. The intent of this investigation was to determine some of the mechanisms causing the reversible effects of oxidants on the endothelium. Hence, it was important to determine whether the oxidized fatty acids had reversible or primarily lytic effects on endothelial cells. Endothelial monolayers were exposed to increasing concentrations of oxidized linoleate complexed to 15 $\mu$mol/L fatty acid–free albumin for five minutes, washed with M199 with 75 $\mu$mol/L fatty acid–free albumin, and then incubated for 235 minutes in M199. When 17.5 $\mu$mol/L oxidized linoleate was complexed to the albumin, LDH release was not increased above control. However, 37.5 $\mu$mol/L oxidized linoleate complexed to 15 $\mu$mol/L albumin caused the release of 44$\% \pm 4\%$ of cellular LDH activity (Fig 7). If 10% FBS was added along with the oxidized linoleate, the release of LDH was reduced to 13$\% \pm 1\%$ of the total cellular LDH. Similarly, if the cells were initially exposed to the oxidized linoleate for five minutes in the absence of serum, washed, and then incubated in M199 with 10% serum added, LDH activity released was only 10$\% \pm 1\%$ of the total cellular LDH. Thus, a five-minute exposure to oxidized fatty acid initiated a process causing lysis of the cells, and this process was partially reversed by incubation in medium with 10% serum. If the cells were exposed to oxidized linoleate (37 $\mu$mol/L complexed to 15 $\mu$mol/L albumin) for 15 minutes, washed, and incubated in M199 with 10% serum, the serum was no longer able to protect the cells, and LDH release was increased (49$\% \pm 3\%$ of total).

In contrast to the lytic effect of brief exposure of the cells to oxidized linoleate, when cells were exposed to H$_2$O$_2$ for 30 minutes followed by catalase treatment (10,000 U/35-mm-diameter plate) and then incubated in M199 with no serum, no increase in LDH activity released over four hours was seen, even with 5 mmol/L H$_2$O$_2$ (Fig 8). If the catalase was not added until 60 minutes after the addition of the H$_2$O$_2$,
Effects of $H_2O_2$ and phorbol esters on endothelial barrier function. The rapid cytolytic effect of the oxidized fatty acids on the endothelial cells did not make the fatty acids likely candidates for mediating the reversible effects of oxidants on endothelial barrier function. However, the initiation of inositol phospholipid hydrolysis by phospholipase C is an important transmembrane signaling event in many cells and might contribute to the reversible effects of oxidants on the cells. One of the consequences of inositol phospholipid hydrolysis by phospholipase C is activation of protein kinase C. Phorbol esters directly stimulate activation of protein kinase C, so we determined the effect of phorbol esters on albumin flux across monolayers of endothelial cells cultured on micropore filters. PDBU at $10^{-7}$ mol/L increased the albumin flux across the cultured endothelial monolayers (Fig 9). In contrast, 4a-phorbol didecanoate ($1 \times 10^{-7}$ mol/L), which does not activate protein kinase C, was not effective (less than 5% increase, $P < 0.05$ for each).

When endothelial cells cultured on tissue culture plates were exposed to $10^{-7}$ mol/L PDBU, we did not see the marked cell retraction that we had seen when similar cells were exposed to exogenous oxidants or A23187. However, there did appear to be an increased frequency of small separations between adjacent endothelial cells that was noticeable after 30 to 45 minutes' exposure to PDBU (Fig 10A and B).

To relate the functional effects of the phorbol esters to initiation of inositol phospholipid hydrolysis by $H_2O_2$, endothelial monolayers cultured on micropore filters were exposed to $H_2O_2$ for 15 minutes. After the 15 minutes, catalase (10,000 U/mL) was added to the monolayers, and then the amount of albumin that crossed the monolayer in the next 60 minutes was determined and compared with the albumin flux recorded prior to $H_2O_2$ exposure. As demonstrated, 2.5 mmol/L $H_2O_2$ for 15 minutes increased the albumin flux across the monolayers (Fig 11).

Fig 8. Release of LDH from monolayers of endothelial cells (9.6 cm$^2$) exposed to $H_2O_2$ in M199 for 30 minutes. At the end of the 30 minutes catalase (10,000 units) was added and the monolayers washed, and the monolayers were incubated for 3.5 hours in M199 without serum. LDH activity in the supernatants from the initial exposure and the 3.5 hours were combined. Percent release was calculated as described in Methods. None of the doses increased LDH release above control values.

Fig 9. The control period (Con) albumin flux across monolayers of endothelial cells cultured on micropore filters was determined. Monolayers were then exposed to M199 alone (Con) or M199 with $10^{-7}$ or $10^{-8}$ mol/L PDBU in M199 for 15 minutes. After 15 minutes, M199 with PDBU and 150 pmol/L albumin was placed on the luminal surface, and the flux of albumin over the next 60 minutes was measured (n = 7 for each group). $P < 0.05$ for $10^{-7}$ mol/L PDBU (PDBU $-7$) v control (Con) and $10^{-8}$ mol/L PDBU (PDBU $-8$). Solid bars represent control period flux and crosshatched bars represent test period flux.

DISCUSSION

Oxidants can have both reversible functional and irreversible lytic effects on cells. Our previous studies demonstrated that exposure of cultured endothelial and epithelial monolayers to oxidants for short periods of time caused reversible changes in the barrier properties of the monolayers. In this report we found that exposure of cultured endothelial monolayers to these doses of oxidants for short periods of time caused oxidation of fatty acids in the cell lipids and hydrolysis of inositol phospholipids by both phospholipase A and phospholipase C pathways. Exposure of endothelium to very low concentrations of oxidized linoleic acid for short periods caused lysis of the cells and suggested that the oxidized fatty acids were not likely to account for reversible effects. Phospholipase C hydrolysis of inositol phospholipids results in activation of protein kinase C in most cells. An activator of protein kinase C, PDBU, increased endothelial monolayer permeability to albumin. We believe these observations are relevant to both the reversible and the lytic effects of oxidants on endothelial cells.

Our observation that a brief exposure of the endothelial cells to $H_2O_2$ was not cytolytic but that a more prolonged exposure was cytolytic is similar to the data of Harlan et al. While the brief $H_2O_2$ exposure was not cytolytic, it did alter the barrier function of the cultured endothelium, similar to our earlier reports. By monitoring the cellular events that occurred during this initial brief exposure, we hoped to begin to understand some of the reversible effects of oxidants on endothelial cells.

The release of pentane from endothelial cells exposed to $H_2O_2$ provided evidence that an $\omega-6$ fatty acid was being oxidized. Linoleate and arachidonate are important $\omega-6$ fatty acids of the cells, and metabolism of arachidonate by cyclooxygenase can cause pentane release. However, A23187 also causes cyclooxygenase metabolism of arachidonate by these porcine endothelial cells, and the assay did not detect...
pentane release from cells exposed to A23187. This suggested that the pentane detected by the assay reflected an effect of H₂O₂ on the fatty acids and not simply activation of cyclooxygenase metabolism. It was of interest that H₂O₂ caused detectable pentane release at concentrations well below those having functional effects on the cells. This suggests that oxidation of fatty acids is a sensitive and early effect of oxidants on endothelial cells.

In addition to oxidation of fatty acid residues, H₂O₂ also initiated hydrolysis of endothelial cell phospholipids. The release of labeled fatty acids from the cells and the increase in free fatty acid and lysophospholipid levels within the cell lipids are consistent with activation of phospholipase A activity. Oxidant activation of phospholipase A activity in endothelial and other cells has been reported by others as well.²¹-²² While H₂O₂ caused a small increase in the release of free fatty acids from the endothelial cells, there was an even more remarkable increase in free fatty acid levels that remained in the cell lipids. Even modest concentrations of free fatty acids are known to cause dysfunction of both cell and organelle membranes, whether the fatty acids are oxidized or not.²⁴

Oxidized linoleic acid has been observed to increase the calcium conductance of liposomes and sarcolemmal membranes.²⁵,²⁶ This was the basis for our original hypothesis, that oxidized linoleate, acting as a calcium ionophore, might account for the reversible oxidant-induced changes in endothelial and epithelial cell shape and actin cytoskeletons.¹,² However, the cytolytic effects of very brief exposures of the endothelial cells to low concentrations of oxidized linoleate

Fig 10. (A) Phase-contrast photomicrograph of endothelial cell monolayer prior to exposure to 10⁻⁷ mol/L PDBU. (B) Phase-contrast photomicrograph of endothelial cell monolayer after exposure to 10⁻⁷ mol/L PDBU for 40 minutes. Note the spaces between adjacent cells. (Original magnification ×630; current magnification ×440).
washed. M199 with 150 I

period when comparing change from control

Catalase (10,000 units) was added. and the monolayer.

hydrolysis of phosphatidylinositol biphosphate by phospholi-

monolayer were then exposed to M199 alone (C) or

P measured (n = 6 for each group). P < .05 for all doses v control (C) when comparing change from control period to test period by using analysis of variance.

reduced our enthusiasm for this hypothesis. Based on the
data of Spector et al., the effective free oxidized fatty acid
concentration for lysing the cells was between 1 x 10^{-7}
mol/L (17.25 \mu mol/L fatty acid complexed to 15 \mu mol/L
albumin) and 5.6 x 10^{-7} M (37 \mu mol/L fatty acid com-
plexed to 15 \mu mol/L albumin). This contrasts dramatically
with effective H_{2}O_{2} concentrations in the millimolar range.

Because of the important role of inositol phospholipids in
transmembrane signal processing we were especially inter-
ested in the fact that the inositol phospholipids were most
affected by the hydrolysis. The increases in inositol polyphos-
phate, phosphatidic acid, and diglyceride levels are consist-
tent with activation of phospholipase C in addition to the
activation of phospholipase A discussed earlier. While the
observed increase in phosphatidylinositol bisphosphate is not
necessarily expected from activation of phospholipase C, it is
consistent with the recent report of Lacal et al.

Phospholipase C hydrolysis of phosphatidylinositol biphos-
phate, with resultant increases in inositol trisphosphate and
diacylglycerol levels, causes an increase in intracellular Ca^{2+}
activity and activation of protein kinase C. The increased Ca^{2+}
activity would be expected to alter the actin structure
through its interaction with proteins such as gel
solin. However, it is also possible that phosphorylation of
cytoskeletal-associated proteins by protein kinase C contrib-
utes to the observed changes in endothelial barrier function.

The experiments with the phorbol esters are consistent with
this hypothesis. Mullin and O'Brien found that phorbol
esters increased the paracellular conductance of a cultured
epithelium, similar to our findings with the endothelium.
Neither Mullin and O'Brien nor we have demonstrated
phorbol ester-induced phosphorylation of cytoskeletal-asso-
ciated proteins, and other effects of the phorbol esters remain
a plausible explanation.

In an earlier publication we did not observe an effect of
phorbol myristate acetate alone on albumin flux across the
endothelium. While the effective doses in this manuscript
were greater than those used previously, we are not entirely
certain of the explanation for this discrepancy.

Lambert et al. found that bradykinin stimulated the
hydrolysis of phosphatidylinositol biphosphate by phospholi-
pase C in cultured porcine aortic endothelial cells. In our
earlier report we observed an important role for extracellular
calcium in the oxidant effects on the cells. A recent report
from Morgan-Boyd et al suggests that the effects of bradyk
inin on intracellular calcium in endothelial cells is partly
dependent on extracellular calcium even though bradykinin
acts through the inositol pathway. Bradykinin is a proin-
flammatory molecule that is able to cause an acute edema-
tous response and changes in endothelial cell shape. The
results of Lambert et al in conjunction with our own suggest
that inositol phospholipid hydrolysis may be an important
pathway involved in the alteration of endothelial permeabili-
ty.

Oxidants can alter cell function in many ways, and there
are undoubtedly multiple pathways that contribute to cellu-
lar dysfunction and injury. We have tried to focus on doses of
oxidants that cause reversible effects on endothelial cells
because we believe that these are likely to be important
primary effects of oxidants on endothelium. The experiments
reported in this manuscript suggest that effects of oxidants
on cell lipids are a sensitive and early effect of oxidants on
endothelial cells. The oxidant-initiated hydrolysis of inositol
phospholipids may help explain some of the reversible effects
of oxidants on the endothelial barrier. The oxidized fatty
acids were especially toxic to the cells and lead us to believe
they are not good candidates for reversible effects. However,
agents such as these, which alter membrane ionic conduc-
tances, would be likely to be especially injurious to cells
depleted of energy stores, an effect of oxidants recently
described by Spragg et al.

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Fig 11. The control period (C) albumin flux across monolayers
of endothelial cells cultured on micropore filters was determined.
Monolayers were then exposed to M199 alone (C) or H_{2}O_{2} in M199
at increasing concentrations for 15 minutes. After 15 minutes
catalase (10,000 units) was added, and the monolayers were
washed. M199 with 150 \mu mol/L albumin was then added to the
luminal surface, and the albumin flux over the next 60 minutes was
measured (n = 6 for each group). P < .05 for all doses v control (C) when comparing change from control period to test period by
using analysis of variance.
Exogenous oxidants initiate hydrolysis of endothelial cell inositol phospholipids

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