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 phospholipid hydrolysis was directed primarily at the inositol phospholipids and consisted of both A and C type phospholipase activity. The phospholipase A hydrolysis resulted in increases in endothelial cell free fatty acids and lysophosphatidylinositol. The phospholipase C hydrolysis resulted in increases in diglycerides, phosphatidic acid, and inositol polyphosphate levels. The phospholipase C hydrolysis of phosphatidylinositol is known to activate protein kinase C in most cells. Stimulation of protein kinase C with phorbol-12,13-dibutyrate increased albumin flux across endothelial monolayers and altered endothelial cell shape, similar to effects of oxidants. These data are consistent with the hypothesis that oxidant-initiated hydrolysis of endothelial cell inositol phospholipids contributes to oxidant-mediated reversible changes in endothelial monolayer barrier function.

REPORTS from several laboratories including our own have demonstrated that reactive oxidants are some of the molecules that contribute to the pathogenesis of acute inflammatory edema. More recently we found that chemically generated oxidants caused reversible increases in albumin levels and ionic permeabilities of cultured endothelial and epithelial monolayers, respectively. Our own observations are consistent with those of Till et al who found that the intravascular activation of complement caused a rapidly reversible polymorphonuclear cell (PMN)-dependent lung edema that was inhibited by antioxidant enzymes.

Oxidants can alter cells in several ways including oxidation of cellular lipids, oxidation of enzymatic and structural proteins, and oxidation of nucleic acids. Our interest was especially in understanding the reversible effects of low doses of oxidants on endothelial and epithelial cells. The oxidants released from PMN are short-lived, rapidly reacting with molecules they meet. The cell membrane is the first part of the cell exposed to exogenously derived oxidants, and many of the lipids in the cell membrane rapidly react with oxidants. Hence, it was probable that a large fraction of the low doses of oxidants would react with the cell membrane. In this manuscript we report some of the effects of brief exposure to these doses of oxidants on endothelial cell lipids.

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 (Biomedical Technologies, Cambridge, MA; fourth-passage cells).

Micropore filters (Nucleopore, Pleasanton, CA) were gelatin impregnated and glued to polycarbonate cylinders (ADAPS, Dedham, MA). After sterilization in ethylene oxide, gelatin-impregnated filters were exposed to fibronectin and then seeded with 2 x 10⁶ endothelial cells/cm².

Measuring albumin transfer. Monolayers on filters mounted on the polycarbonate cylinders were placed into the wells of 24-well tissue culture plates (Costar, Inc, Cambridge, MA) that had previously been filled with 1.5 mL albumin-free M199. The lumen of the cylinder was then filled with 0.5 mL M199 containing 150 µmol/L fatty acid–free bovine serum albumin (A5711; Sigma Chemical Co., St. Louis). With these volumes the fluid levels within the cylinder and the well of the plate were balanced. After one hour the media from the well of the tissue culture plate and from within the polycarbonate cylinder were sampled and the albumin concentration measured following the reaction of albumin in the sample with bromcresol green at 630 nm.

Each monolayer was tested by following the aforementioned protocol and the fractional transfer of albumin calculated as the concentration of albumin in the well of the tissue culture plate after one hour divided by the concentration of albumin in the lumen of the cylinder. Monolayers with a control fractional transfer less than 0.02 (permeability less than 8 x 10⁻⁶ cm/s) were accepted for study. The monolayers were distributed among the experimental groups so that there was a similar distribution of low and high fractional transfers in each group. Each monolayer then served as its own control during exposure to H₂O₂.

For experiments with phorbol esters, phorbol-12,13-dibutyrate (PDBU) and 4α-phorbol-12,13-didecanoate were dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL), aliquoted, and stored at -20°C. Just prior to use the aliquots were thawed at 25°C and diluted in M199 to the appropriate concentration. Final DMSO concentrations were less than one part in 1,000. Phorbol esters in M199 were added to the monolayers for 15 minutes in the absence of albumin, and then 150 µmol/L albumin with the appropriate concentration of phorbol ester was added for 60 minutes to determine albumin flux.

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$^6$ 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. The cells were then incubated in the syringe at 37°C for 30 minutes. At the end of the 30 minutes the cells were expressed from the syringe and the remaining gas analyzed for hydrocarbons. 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$^6$ cpm of 1-14C linoleic or arachidonic acid in M199 with 10% FBS for 12 hours. Incorporation was 82% ± 5% for linoleate (18:2), 75% ± 5% for linolenate (18:3), and 76% for arachidonate (20:4). Prior to carrying out experiments the cells were washed five times with Hanks-HEPES to remove free fatty acid (counts in the fifth wash equaled background). Conversion of linoleate to arachidonate was less than 10% when labeled cell lipids were analyzed by gas chromatography as previously reported by Spector et al. For analysis of lipid phosphate, endothelial cells in 35-mm-diameter tissue culture plates were preincubated with 30 μCi32P (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 32P 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, Elysian MN; 1 mmol/L, stabilized in Tween 80) with soybean lipoxygenase (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 × 10^4 M⁻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 M HClO₄.

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 < .05.

RESULTS

Effects of H₂O₂ on fatty acid oxidation. When H₂O₂ was added to endothelial cells suspended in Hanks-HEPES in blood gas syringes, pentane was released to the head space (Fig 1). Doses of H₂O₂ as low as 7.8 μmol/L caused detectable pentane release, and more pentane was released with higher doses. In contrast, the addition of 5 μmol/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 H₂O₂, increased counts were released to the incubation medium at a dose of H₂O₂ of 1 mmol/L (data not shown). In subsequent experiments monolayers from the same passage of cells were labeled with 1-¹⁴C linoleic acid and then exposed to 1 mmol/L H₂O₂ for 30 minutes. Increased counts were released to the incubation medium after exposure to H₂O₂, 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 arachidonate were released from the cells. Additional cells were labeled with 1-¹⁴C linolenate and then exposed to 1 mmol/L H₂O₂. H₂O₂ 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%; H₂O₂ 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 H₂O₂ (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 preloaded with 1-¹⁴C arachidonic acid were exposed to H₂O₂ (1 mmol/L, 30 minutes, Fig 3B) and when cells preloaded with 1-¹⁴C linolenate were exposed to H₂O₂ (1 mmol/L, 30 minutes, data not shown). Hence, H₂O₂ caused fatty acid to be released from the cells, thus suggesting activation of phospholipase A activity. In addition, after exposure to H₂O₂ a larger fraction of the increased amount of fatty acid released comigrated as oxidized free fatty acid.

Effects of H₂O₂ on cellular lipids. The distribution of labeled fatty acid among the major cell lipids from control and H₂O₂-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 H₂O₂ (1
exposure to \( H_2O_2 \). Hence, \( H_2O_2 \) increased the fraction of the label in control cells, and this did not change with \( H_2O_2 \)-exposed cells. Triglycerides contained 4\% of the total counts in control cells, and this increased to 5\% in \( H_2O_2 \)-exposed cells. In control cells 1\% of the total counts was in diglycerides, and this increased to 5\% in \( H_2O_2 \)-exposed cells. Cholesterol esters contained 2\% of the total counts in control cells, and this decreased to 3\% in \( H_2O_2 \)-exposed cells. Since it appeared that most of the label was released from cell phospholipids, the distribution of \( 1^\text{H} \) linoleate and arachidonate among the different phospholipids in control and \( H_2O_2 \)-exposed cells was determined. In cells prelabeled with \( 1^\text{H} \) linoleate, \( H_2O_2 \) (1 mmol/L, 30 minutes) caused a decrease in the fraction of \( 1^\text{H} \)-label that comigrated with phosphatidylinositol (7\% ± 0.5\% in control, 4\% ± 0.1\% in \( H_2O_2 \)-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 \( 1^\text{H} \) arachidonate before exposure to \( H_2O_2 \) (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).
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When 1-14C linolenate was the labeled fatty acid, a similar pattern evolved, with the fraction of label comigrating with phosphatidylinositol decreasing from 2.2% ± 0.03% to 1.8% ± 0.01% after exposure to H2O2 (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 diglycerides (Fig 4), a change consistent with activation of a phospholipase C. For this reason endothelial cells were prelabeled with 14C or 3H inositol and the inositol phospholipids separated after H2O2 exposure. H2O2 (1 mmol/L, ten minutes) increased the fraction of activity that comigrated with lysophosphatidylinositol (1.478 ± 157 cpm, 3.9% ± 0.1% for control; 2,673 ± 185 cpm, 7.4% ± 0.4% for exposed, n = 5) and increased the amount of activity that comigrated with phosphatidylinositol biphosphate (1.071 ± 117 cpm, 2.8% ± 0.2% for control; 1,708 ± 97 cpm, 4.7% ± 0.2% for exposed). Exposure to H2O2 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 ± 12.3 nmol lipid phosphate/mg cell protein) and cells exposed to 1 mmol/L H2O2 for 30 minutes (124.7 ± 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 CHCl3, MeOH, and methylamine (60:35:5), the distribution of 32P radioactivity among the major phospholipid classes was not different between control and H2O2-exposed cells. However, the neutral lipid chromatogram also demonstrated an increase in the amount of fatty acid in the diglycerides (Fig 4), a change consistent with activation of a phospholipase C.

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 μmol/L fatty acid–free albumin for five minutes, washed with M199 with 75 μmol/L fatty acid–free albumin, and then incubated for 235 minutes in M199. When 17.5 μmol/L oxidized linoleate was complexed to the albumin, LDH release was not increased above control. However, 37.5 μmol/L oxidized linoleate complexed to 15 μmol/L albumin caused the release of 44% ± 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% ± 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% ± 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 μmol/L complexed to 15 μmol/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% ± 3% of total).

In contrast to the lytic effect of brief exposure of the cells to oxidized linoleate, when cells were exposed to H2O2 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 H2O2 (Fig 8). If the catalase was not added until 60 minutes after the addition of the H2O2,
Effects of H$_2$O$_2$ 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 × 10$^{-7}$ mol/L), which does not activate protein kinase C, was not effective (less than 5% increase, P > 0.20 v control, n = 5 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$_2$O$_2$, endothelial monolayers cultured on micropore filters were exposed to H$_2$O$_2$ 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$_2$O$_2$ exposure. As demonstrated, 2.5 mmol/L H$_2$O$_2$ for 15 minutes increased the albumin flux across the monolayers (Fig 11).

**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$_2$O$_2$ was not cytolytic but that a more prolonged exposure was cytolytic is similar to the data of Harlan et al. While the brief H$_2$O$_2$ 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$_2$O$_2$ provided evidence that an ω-6 fatty acid was being oxidized. Linoleate and arachidonate are important ω-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 H$_2$O$_2$ on the fatty acids and not simply activation of cyclooxygenase metabolism. It was of interest that H$_2$O$_2$ 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, H$_2$O$_2$ 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 H$_2$O$_2$ 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.$^{23}$

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
washed. M199 with 150 units catalase (10,000 units) was added, and the monolayer was placed in hydrolysis of phosphatidylinositol bisphosphate by phospholipase C at increasing concentrations for 15 minutes. After 15 minutes, monolayer were then exposed to M199 alone (C) or H2O2 in M199. Albumin flux over the next 60 minutes was measured (n = 6 for each group). P < .05 for all doses vs 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 × 10^{-7} mol/L (17.25 μmol/L fatty acid complexed to 15 μmol/L albumin) and 5.6 × 10^{-7} M (37 μmol/L fatty acid complexed to 15 μmol/L albumin). This contrasts dramatically with effective H2O2 concentrations in the millimolar range.

Because of the important role of inositol phospholipids in transmembrane signal processing, we were especially interested in the fact that the inositol phospholipids were most affected by the hydrolysis. The increases in inositol polyphosphate, phosphatidic acid, and diglyceride levels are consistent 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 bisphosphate, 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 gelonin. However, it is also possible that phosphorylation of cytoskeletal-associated proteins by protein kinase C contributes 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 us have demonstrated phorbol ester–induced phosphorylation of cytoskeletal-associated 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 bisphosphate by phospholipase 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 bradykinin on intracellular calcium in endothelial cells is partly dependent on extracellular calcium even though bradykinin acts through the inositol pathway. Bradykinin is a proinflammatory molecule that is able to cause an acute edematous 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 permeability.

Oxidants can alter cell function in many ways, and there are undoubtedly multiple pathways that contribute to cellular 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 conductances, 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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Exogenous oxidants initiate hydrolysis of endothelial cell inositol phospholipids

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