Protein targets of oxidized phospholipids in endothelial cells[S]

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Abstract **Oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine (Ox-PAPC) are found in atherosclerotic lesions, apoptotic cells, and oxidized LDL and stimulate human aortic endothelial cells (HAECs) to produce inflammatory cytokines, leukocyte chemoattractants, and coagulation factors. This regulation is thought to be a receptor-mediated process in which oxidized phospholipids activate specific receptors on HAECs to evoke an inflammatory response. To characterize the HAEC proteins with which oxidized phospholipids interact, a biotinylated PAPC analog, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (PAPE-*N*-biotin), was synthesized. Oxidation of PAPE-*N*-biotin in air generated a mixture of biotin-labeled oxidized lipids analogous to Ox-PAPC. Ox-PAPE-*N*-biotin, like Ox-PAPC, induced interleukin-8 (IL-8) protein synthesis and stimulated IL-8, low density lipoprotein receptor, heme oxygenase-1, and activating transcription factor-3 mRNA expression in HAECs. After treatment of HAECs with Ox-PAPE-*N*-biotin, the cellular proteins were isolated and separated by SDS-PAGE. Western analysis with streptavidin-HRP demonstrated at least 20 different biotinylated HAEC proteins to which the Ox-PAPE-*N*-biotin was associated, which were not detected with unoxidized PAPE-*N*-biotin treatment.[JLR] This work suggests that oxidized phospholipids, such as those found in oxidized LDL, apoptotic cells, and atherosclerotic lesions, form tight interactions with specific endothelial cell proteins, which may be responsible for the inflammatory response. Identification of these putative oxidized phospholipid targets may reveal therapeutic targets to modulate inflammation and atherosclerosis.**—Gugiu, B. G., K. Mouillesseaux, V. Duong, T. Herzog, A. Hekimian, L. Koroniak, T. M. Vondriska, and A. D. Watson. **Protein targets of oxidized phospholipids in endothelial cells.** ***J. Lipid Res.*** **2008.** 49: **510–520.**

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Atherosclerosis, the major cause of heart attack and stroke, kills more people in the Western world than cancer, chronic lung disease, and accidents combined (1). Atherosclerotic plaque development is initiated and propagated by lipid deposition and oxidation, infiltration of inflammatory cells, and accumulation of matrix and cellular debris within the blood vessel wall (2–4). A critical first step in this process is the recruitment of blood monocytes into the subendothelial space (5). It is these monocytes that differentiate into macrophages within the lesion and begin to take up oxidized lipids (mostly in the form of oxidized LDL) to become foam cells, the signature inflammatory cell of the atherosclerotic lesion (6). What stimulates this monocyte-endothelial interaction? One prime candidate is the oxidation of LDL directly underneath the endothelial cells.

Lipid peroxidation plays an important role in normal cellular physiology and disease (7–9). The fundamental chemical reaction that initiates this process is the abstraction of a hydrogen atom from a polyunsaturated fatty acid, leaving a carbon-centered radical (10). Enzymes like cyclooxygenase (11), lipoxygenase (12), and cytochrome P450 (13) can facilitate this reaction under certain physiological conditions to produce prostaglandins, leukotrienes, and other arachidonic acid metabolites that are potent mediators of inflammation. In the absence of en-

Abbreviations: BODIPY, dipyromethene boron difluoride; CKAP4, cytoskeleton-associated protein-4; HAEC, human aortic endothelial cell; IL-8, interleukin-8; Ox-X, oxidation products of X; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine; PAPE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylethanolamine; PAPE-*N*-biotin, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine); PEIPC, 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidylcholine; PEIPE-*N*-biotin, 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine); PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine; PVDF, polyvinylidene difluoride; sulfo-NHS-biotin, biotin ester of *N*-hydroxysuccinimide sulfonate.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of sixteen figures and one table.

zymes, lipid peroxidation occurs by free radicals produced under oxidative stress (10, 14). The physiological and pathophysiological importance of these nonenzymatically generated lipid peroxidation products has been increasingly recognized, particularly with respect to inflammation and immunity.

Mild oxidation of LDL results in mostly phospholipid oxidation, the products of which seem to be more proinflammatory than toxic (15–17). Extensive oxidation of LDL lipids forms large amounts of cytotoxic substances, such as oxysterols, malondialdehyde, and other reactive carbonyls, that can modify cellular proteins and/or DNA and that appear to play a role in the later stages of atherosclerosis (18, 19). In vitro studies using either minimally modified LDL or oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine (Ox-PAPC), a phospholipid present in LDL, showed that they stimulated cultured endothelial cells to express monocyte-specific adhesion molecules and secrete monocyte chemoattractants (20–22). Chromatographic separation of the many products formed by the oxidation of PAPC and testing of each for its ability to stimulate monocyte-endothelial interactions resulted in the identification of 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidylcholine (PGPC), and 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidylcholine (PEIPC) as potent lipid mediators of inflammation that evoked a specifically "atherogenic" response (17, 23). The activity of these lipids was dependent upon the oxidized arachidonic acid in the *sn*-2 position and was unaffected by substitutions made at the *sn*-1 or *sn*-3 position (24). We and others have reported that isolated components of Ox-PAPC, such as POVPC, PGPC, and PEIPC, have different inflammatory and anti-inflammatory effects on endothelial cells (15, 25). The mechanism for this differential activation may be explained by specific targeting of endothelial proteins by certain oxidized phospholipids. These lipids have been found in atherosclerotic plaques of animal models of atherosclerosis (17) as well as in human atherosclerotic lesions by immunostaining with E06, a monoclonal antibody that recognizes oxidized phosphatidylcholines (26, 27).

Despite the evidence linking phospholipid oxidation with endothelial inflammation, the mechanism by which oxidized phospholipids trigger this inflammatory response is not clear. Limited evidence suggests that oxidized phospholipids act through protein receptors on the surface of cells, similar to the cell activation by eicosanoids (28, 29), platelet-activating factor (30, 31), sphingolipids (32), and lysophosphatidic acid (33). In fact, the prostaglandin E_2 receptor appears to be partly responsible for the activation of human aortic endothelial cells (HAECs) in response to PEIPC (34). To gain more insight into the nature of the interaction between biologically active oxidized phospholipids and endothelial cells, we have investigated whether Ox-PAPC interacts with specific endothelial cell proteins at the cell surface and/or within the cell. Biotinylated analogs of Ox-PAPC were synthesized and used to treat cells, with subsequent detection using streptavidin-HRP for Western blots or avidin-FITC for fluorescence microscopy detection. These results suggest that oxidized, but not unoxidized, phospholipids modify endothelial cell proteins, which may contribute to vascular inflammation and atherosclerosis.

EXPERIMENTAL PROCEDURES

Reagents

Tissue culture media and reagents were obtained from Irvine Scientific, Inc. (Santa Ana, CA). FBS was obtained from Hyclone (Logan, UT). PAPC and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylethanolamine (PAPE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Biotin was purchased from Sigma-Aldrich (St. Louis, MO). TBS-T was produced by adding 1 ml of Tween-20 (Bio-Rad, Hercules, CA) to 1 liter of Tris-buffered saline (pH 7.2). EZ-link-sulfo-NHS-biotin (for biotin ester of *N*-hydroxysuccinimide sulfonate) was purchased from Pierce (Rockford, IL).

Lipid oxidation

Phospholipids were oxidized by transferring 1 mg, in chloroform, to a 13 × 100 mm glass test tube, evaporating the solvent under argon, and leaving the lipid residue to autoxidize in air. The extent of oxidation was monitored by ESI-MS daily. After adequate oxidation (usually 24–72 h), the lipid residue was resuspended in chloroform and stored at −80°C. The biotin-labeled analogs of POVPC, PGPC, and PEIPC were purified by sequential semipreparative reverse-phase LC-ESI-MS.

PAPE-*N*-biotin synthesis

A solution of PAPE (67.6 nmol, 50 mg) in 5 ml of dry dichloromethane was added drop-wise to a magnetically stirred solution of biotin (69.6 nmol, 17 mg), dicyclohexylcarbodiimide (140 nmol, 29 mg), and dimethylaminopyridine (140 nmol, 17 mg) in 3 ml of dry dichloromethane under argon at room temperature. The solution was mixed with a magnetic stirrer under argon for 12 h at room temperature. The solvent was evaporated in a rotary evaporator under vacuum, and the residue was purified by reverse-phase HPLC with ESI-MS detection to produce 45.6 mg (47.3 nmol) 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (PAPE-*N*-biotin) in 70% yield.

Liquid chromatography and mass spectrometry

PAPE-*N*-biotin and Ox-PAPE-*N*-biotin were analyzed by ESI-MS as phosphate anions in negative ion mode in 50% methanol with 1 mM ammonium acetate on a Thermo LCQ Advantage Max mass spectrometer (Thermo Electron Corp., West Palm Beach, FL). PAPE-*N*-biotin was purified after synthesis by LC-ESI-MS using a semipreparative Thermo Biosil C_8 10 × 250 mm column (Thermo Electron Corp.) at a flow rate of 2 ml/min and a gradient of 65% methanol to 100% methanol over 18 min and held at 100% methanol for 22 min. A postcolumn splitter with a flow ratio of ~40:1 was used to split the flow between the ESI-MS source and the fraction collector, which collected 1 min fractions. Individual components of Ox-PAPE-*N*-biotin were separated on the same semipreparative column using a linear gradient of 65% methanol to 100% methanol over 60 min at 2 ml/min. The fractions collected were enriched in the following compounds: *1*) 1-palmitoyl-2-(5-oxovaleroyl)/glutaroyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (POV/GPE-

N-biotin); *2*) 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (PEIPE-*N*-biotin); and *3*) unoxidized PAPE-*N*-biotin. ESI-MS and ESI-MS/MS were performed using a Thermo LCQ Advantage Max ion-trap mass spectrometer equipped with MS^n capability. The mass spectrometer was configured to scan in the negative ion mode from *m/z* 150 to 1,000.

Cell culture

HAECs were obtained from discarded donor aortic tissue during heart transplantation and cultured in M-199 containing 10% FBS and growth supplements as described previously (35).

Interleukin-8 assays

To determine interleukin-8 (IL-8) induction, confluent monolayers of HAECs were treated with test lipids in M-199 containing 1% FBS for 4 h at 37°C. The conditioned medium was collected, and its IL-8 content was measured by a commercially available ELISA (R&D Systems, Minneapolis, MN). Cells treated with M-199 with 1% FBS alone were used as a negative control.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described (36) on a Bio-Rad iCyclerIQ thermal cycler with the following primers designed using the Integrated DNA Technologies Primer Quest online design tool: hIL-8: 5′-ACCACACTGCGCCAACACAGAAAT-3′ (174F) and 5′-TCCAGACAGAGCTCTCTTCCATCAGA-3′ (236R); hLDL-R (for low density lipoprotein receptor): 5′-CGTGCTTGTCTGTCACCTGCAAAT-3′ (185F) and 5′-AGAACTGAGGAATGCAGCGGTTGA-3′ (259R); hHO-1 (for heme oxygenase-1): 5′-GGCAGAGAATGCTGAGTTCATGAGGA-3′ (81F) and 5′-ATAGATGTGGTACAGGGAGGCCATCA-3′ (174R); and hATF-3 (for activating transcription factor-3): 5′-ATGGTTCTCTGCTGCTGGGATTCT-3′ (R) and 5′-TTGCAGAGCTAAGCAGTCGTGGTA-3′ (F).

Affinity purification of HAEC protein targets of oxidized phospholipids

Tetrameric avidin beads (500 μl; Pierce) were added to 1 mg of isolated endothelial cell protein and incubated at room temperature with periodic mixing for 2 h. Nonbiotinylated proteins were removed by washing with 0.2% Nonidet-P40 in PBS. Biotinylated lipid-protein adducts retained on the avidin beads were eluted by boiling the beads for 5 min in reducing Laemmli sample buffer. The protein content of each fraction (cell lysate, supernatant, and affinity-purified lysate) was measured using a bicinchoninic acid protein assay kit (Pierce).

Gel electrophoresis and Western blotting

For analysis of lipid-protein interactions by Western blotting, cells were treated with test compounds in PBS for 30 min at 37°C. After incubation, the cells were washed and scraped into PBS containing 1 mM PMSF and protease inhibitor cocktail. Proteins were extracted from the cell pellet using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, and 0.1% SDS). Proteins extracted from cells (100 μg) were added to each well of an 8% polyacrylamide precast mini-gel (ICS Bioscience, Kaysville, UT). After electrophoresis, gels were either fixed with a solution of 7% acetic acid and 15% methanol for 30 min and stained with SYPRO® Ruby followed by visualization in a Gel Doc EQ System (Bio-Rad) or transferred onto a polyvinylidene difluoride (PVDF) membrane for Western analysis. The PVDF membrane was blocked with 5% nonfat milk at room temperature for 1 h, washed with TBS-T, and then incubated with streptavidin linked to horseradish peroxidase at a 1:200 dilution in 5% BSA for 1 h. Ox-PAPE-*N*-biotin-streptavidin-HRP-labeled proteins were visualized with the ECL-Plus chemiluminescent detection kit (Amersham Biosciences, Piscataway, NJ) in a Bio-Rad Versadoc™ model 4000 imaging system or a Kodak Image Station 2000MM.

Fluorescence microscopy

HAECs plated on an eight-chamber slide were rinsed with 500 μl of PBS, treated with 250 μl of 50 μg/ml Ox-PAPE-*N*-biotin, PAPE-*N*-biotin, or PBS at 37°C for 30 min, and then rinsed with PBS twice (500 μl each). Then, 250 μl of 5 μM dipyromethene boron difluoride (BODIPY) C_6-ceramide BSA complex (5 μM BODIPY C_6-ceramide in 5 μM BSA in PBS) was added to the chambers and left at 4°C for 30 min, then washed twice with 500 μl of ice-cold PBS. To each well was added 400 μl of FITC-avidin conjugate (Zymed, San Francisco, CA) at 6.25 μg/ml in PBS, and the slides were incubated for 30 min at room temperature. The cells were then washed twice with PBS and fixed for 20 min at room temperature with 2% formaldehyde in PBS. The wells were rinsed twice with PBS and covered with a cover slip after removal of the chambers. Fluorescence images were captured with a SPOT charge-coupled device camera and SPOT imaging software, version 4.0.4 (Diagnostic Instruments, Inc.), attached to an Olympus AX70 microscope (Olympus Imaging America, Inc., Center Valley, PA).

Proteomic analysis

Bands were excised from SYPRO® Ruby-stained one-dimensional SDS-PAGE gels, in situ-digested with trypsin, followed by reduction and alkylation. Reverse-phase LC-MS/MS was performed on a Thermo Electron LTQ ion-trap mass spectrometer with a dedicated online Surveryor HPLC system using a reverse-phase column (75 μm × 100 mm, BioBasic C_{18} 5 μm particle size; New Objective, Woburn, MA). The flow rate was 5 μl/min for sample loading and 250 nl/min for separation. Mobile phase A was 0.1% formic acid and 2% acetonitrile in water, and mobile phase B was 0.1% formic acid and 20% water in acetonitrile. A shallow gradient was used for analyses: linear gradient from 5% B to 40% B over 75 min, then to 100% B over 19 min, and finally keeping constant 100% B for 10 min. The ion transfer tube of the linear ion trap was held at 200°C, the normalized collision energy was 35% for MS/MS and MS^3, and the spray voltage was set at 1.9 kV. Spectra were acquired in data-dependent mode and searched against the international protein index human database using SEQUEST. Criteria for positive identification included the following: Xcorr (best score; evaluates how good is the best match) values of >2.0 (+1), >2.5 (+2), and >3.8 (+3); more than two sequenced peptides; and deltaCN > 0.1.

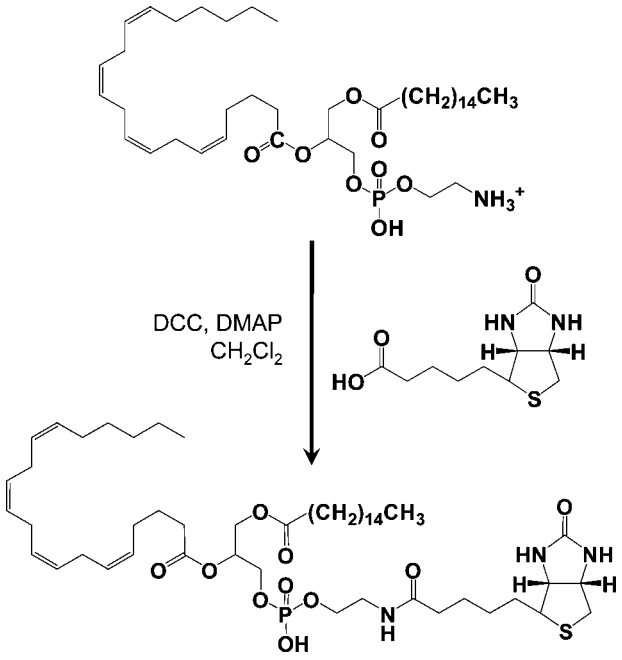
Statistics

The data are presented as means ± SD. Data were compared using ANOVA and were considered significantly different if the probability that the observed difference occurred by chance alone was <5% ($P < 0.05$).

RESULTS

Synthesis of oxidized biotinylated analogs of Ox-PAPC

Biotin was coupled to the primary amine of PAPE to produce PAPE-*N*-biotin (**Scheme 1**). Negative ion ESI-MS



Scheme 1. Synthesis of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (PAPE-*N*-biotin). DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine.

of the reaction mixture showed the presence of a negatively charged ion with *m/z* 964.6, which corresponded to the theoretical molecular weight of the phosphate anion of PAPE-*N*-biotin, *m/z* 964.59 (**Fig. 1**, inset). ESI-MS/MS studies confirmed the presence of palmitic acid in the *sn*-1 position and arachidonic acid in the *sn*-2 position (see supplementary material). After purification of the synthetic product by reverse-phase LC-ESI-MS, PAPE-*N*-biotin was oxidized by exposure to air as a lipid residue (17). To verify the presence and extent of oxidation, Ox-PAPE-*N*-biotin was resuspended in 50% methanol with 1 mM ammonium acetate and analyzed by negative ion ESI-MS. Multiple oxidation products were seen that produced a pattern similar to that of Ox-PAPC, except for a shift in mass by 182 Da caused by the presence of biotin at the *sn*-3 position (Fig. 1). We also observed "shadow ions" 16 Da larger than each oxidation product caused by oxidation of the sulfur atom on biotin to a sulfoxide. This was confirmed by ESI-MS/MS analysis (see supplementary material).

Biological activity of Ox-PAPE-*N*-biotin

We have previously shown that substituting choline for ethanolamine at the *sn*-3 position of PAPC did not affect the inflammatory properties of the molecule when oxidized (24). Ox-PAPE was just as active as Ox-PAPC at inducing IL-8 production in HAECs (24). Importantly, we now show that Ox-PAPE-*N*-biotin stimulated protein and mRNA expression of the inflammatory response genes known to be regulated by Ox-PAPC (37, 38). Ox-PAPE-*N*-biotin stimulated, in a dose-dependent manner, an increase in relative mRNA expression of IL-8, hemoxygenase-1, and the unfolded protein response gene, activating transcription factor-3 (**Fig. 2**). It also induced IL-8 and monocyte chemoattractant protein-1 protein production in HAECs. In general, Ox-PAPE-*N*-biotin was slightly less effective at inducing inflammatory responses than Ox-PAPC at the same concentration, which may have been attributable to the presence of the biotin group at the *sn*-3 position. However, the common inflammatory response of HAECs to Ox-PAPC and Ox-PAPE-*N*-biotin suggested that the mechanism by which they stimulated HAECs was similar, if not identical. This allowed us to use these biotin-labeled phospholipid analogs of Ox-PAPC to identify their specific targets in HAECs.

Endothelial cell protein targets of Ox-PAPE-*N*-biotin

Oxidized arachidonoyl phospholipids stimulate an inflammatory response by vascular wall cells, whereas unoxidized phospholipids do not. Based on previous studies suggesting that oxidized phospholipids form covalent interactions with proteins (39), we used Ox-PAPE-*N*-biotin as "bait" to identify its protein targets in HAECs. HAECs were treated with PBS, unoxidized PAPE-*N*-biotin, or Ox-PAPE-*N*-biotin in duplicate for 30 min. The test solution was removed, and the cells were washed twice with PBS. The cells were collected, and the protein was isolated (three conditions in duplicate). SDS-PAGE was performed using two identical gels. One gel was fixed and stained with SYPRO® Ruby to visualize total endothelial cell proteins. The protein patterns were nearly identical among the various treatments, suggesting that the treatments had no discernible effect on HAEC proteins (**Fig. 3A**). The proteins on the other gel were transferred to a PVDF membrane and probed with streptavidin-HRP to detect protein-associated biotin. A single band (Fig. 3B, open arrow; ~80 kDa) was detected in all lanes irrespective of treatment condition. This was identified by mass spectrometry as an intrinsic 80 kDa biotin binding protein, propionyl-CoA carboxylase, with the accession number IPI00465032.

In contrast, many proteins were identified from cells treated with Ox-PAPE-*N*-biotin in addition to the biotin binding protein (Fig. 3B, lanes 5, 6). In the most intense protein band (Fig. 3B, closed arrow; ~65 kDa), three proteins were identified by mass spectrometry, among which was the isoform 1 of cytoskeleton-associated protein-4 (CKAP4; accession number IPI00141318) (40), a type II reversibly palmitoylated transmembrane protein recently reported to regulate tissue plasminogen activator on the surface of smooth muscle cells (41).

Figure 3B, lanes 3, 4, which contained protein from the cells treated with unoxidized PAPE-*N*-biotin, showed two bands, the biotin binding protein and CKAP4 (level of the closed arrow; ~65 kDa), which was the strongest band in the Ox-PAPE-*N*-biotin treatment. We attributed the appearance of this band in the unoxidized PAPE-*N*-biotin treatment to the minimal oxidation of PAPE-*N*-biotin during cell treatment. The relative intensity pattern of proteins on the Western blot was clearly different from that on the SYPRO® Ruby-stained gel, suggesting that the

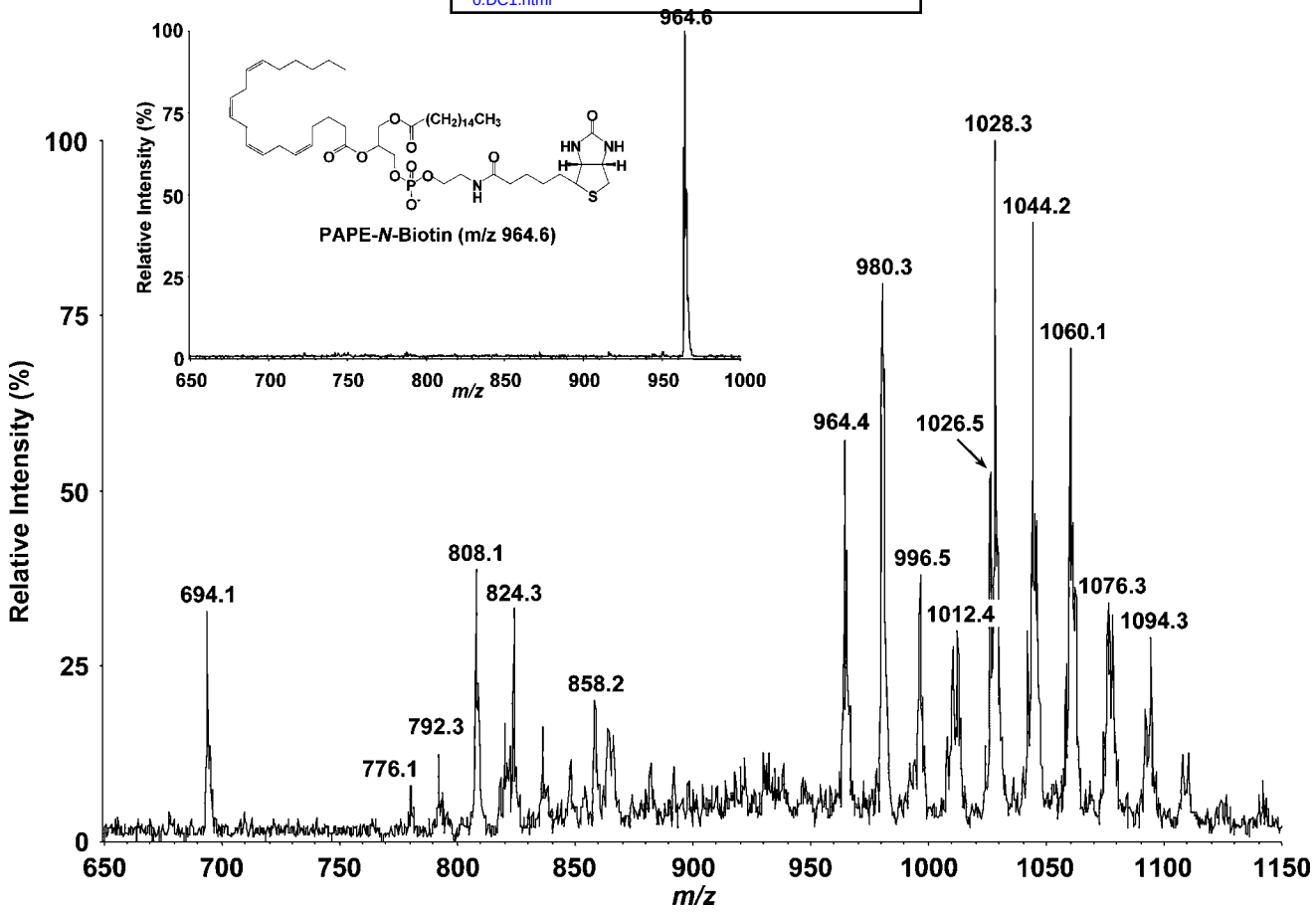


Fig. 1. ESI-MS of oxidized (Ox-)PAPE-*N*-biotin. PAPE-*N*-biotin was synthesized, purified by LC-ESI-MS, and oxidized in air. Ox-PAPE-*N*-biotin was resuspended in 50% acetonitrile with 1 mM ammonium acetate and analyzed by negative ion ESI-MS. ESI-MS analysis of unoxidized PAPE-*N*-biotin is shown for comparison (inset). The components of the mixture are as follows: *m/z* 808 (GPE-*N*-biotin); *m/z* 792 (OVPE-*N*-biotin); *m/z* 824 (hydroxy-methoxy-VPE-*N*-biotin, the hemiacetal of OVPE-*N*-biotin); *m/z* 694 [1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine)]; and *m/z* 980 (PAPE-*N*-biotin). See the supplementary material for detailed MS analysis and molecular structure confirmation.

biotin-labeled oxidized phospholipids were not interacting randomly with HAEC proteins based on abundance but rather were interacting with specific proteins.

Specificity of Ox-PAPE-*N*-biotin lipids for endothelial cell target proteins

To address the question whether oxidized lipids specifically modify proteins in endothelial cells, we treated HAECs in monolayer with either Ox-PAPE-*N*-biotin or sulfo-NHS-biotin according to the manufacturer's specifications (Pierce). Figure 3C shows a streptavidin Western blot of this experiment. Because of intense labeling with the NHS-biotin kit, the loading on the gel was 130 ng of the NHS-biotin-labeled proteins compared with 30 μg of the control or Ox-PAPE-*N*-biotin-labeled proteins. This experiment showed a different pattern for the biotinylation of surface proteins with sulfo-NHS-biotin than the pattern obtained by treating HAECs with Ox-PAPE-*N*-biotin.

Many oxidized species were produced by the oxidation of PAPE-*N*-biotin (**Scheme 2**, Fig. 1). To determine whether certain oxidation products targeted specific endothelial cell proteins, we separated Ox-PAPE-*N*-biotin on a semi-preparative reverse-phase column with online ESI-MS to collect fractions enriched in various Ox-PAPE-*N*-biotin species (see supplementary material). Selected fractions were then used to treat HAECs followed by Western analysis with streptavidin-HRP. These fractions were enriched in the following compounds: *1*) 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) and POVPE-*N*-biotin; *2*) GPE-*N*-biotin; *3*) PEIPE-*N*-biotin; and *4*) unoxidized PAPE-*N*-biotin. All compounds also contained the corresponding biotin sulfoxide (M+16) (data not shown). Reconstructed selected ion chromatograms of the ions of interest show the regions where these molecules were collected (see supplementary material). Each compound was well separated from one another. Streptavidin-HRP Western blot of proteins from HAECs treated with these compounds showed an apparently different staining pattern produced by the different fractions. Although we invariably observed a different pattern of protein staining with different oxidized lipids, there also appeared to be common HAEC proteins that were

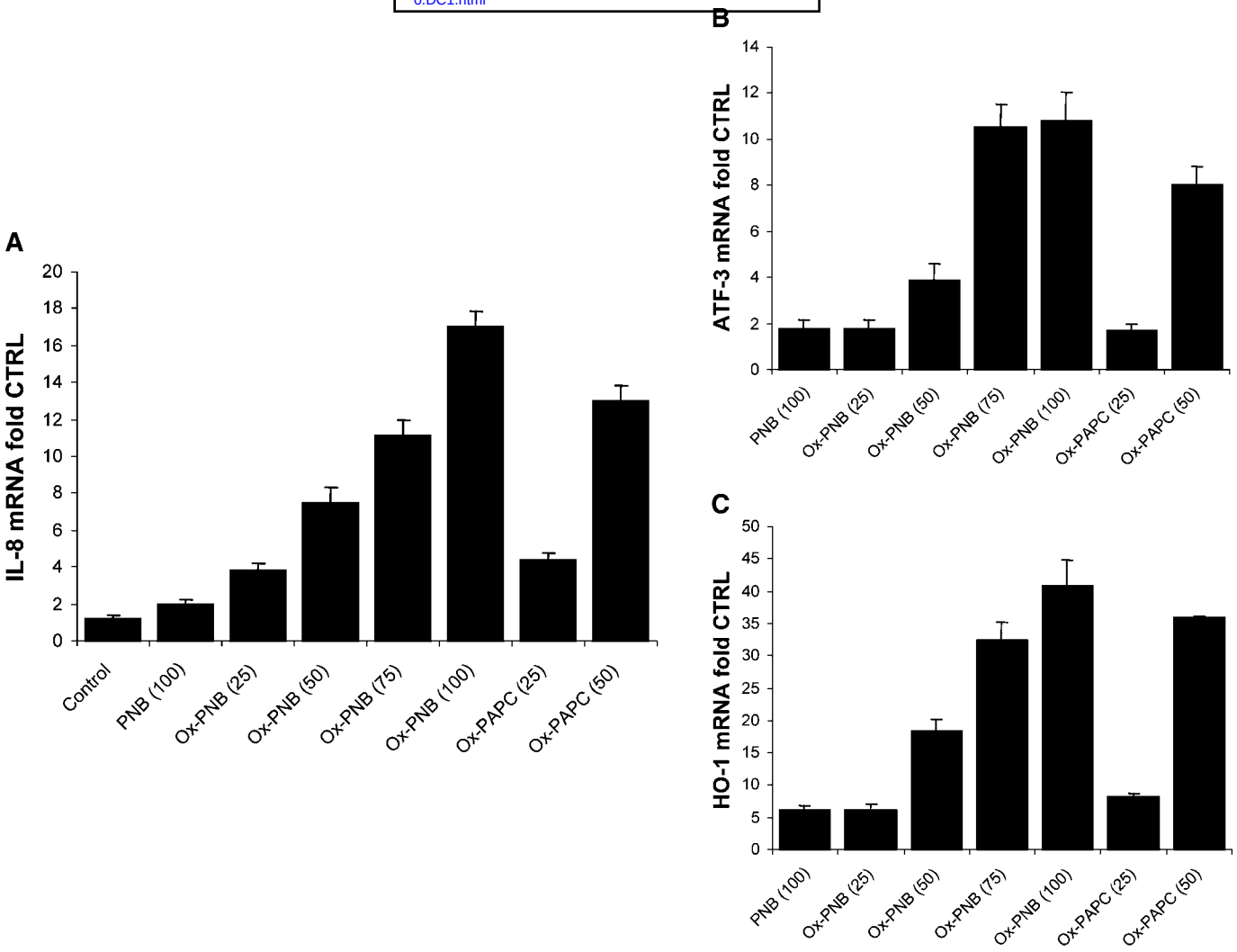


Fig. 2. Effect of Ox-PAPE-*N*-biotin on human aortic endothelial cell (HAEC) inflammatory response genes. Confluent monolayers of HAECs were treated with medium alone (control), unoxidized PAPE-*N*-biotin (PNB), Ox-PAPE-*N*-biotin (Ox-PNB), or oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine (Ox-PAPC) at the concentrations shown for 4 h at 37°C. Interleukin-8 (IL-8; A), activating transcription factor-3 (ATF-3; B), and heme oxygenase-1 (HO-1; C) relative mRNA were measured in the conditioned medium using quantitative real-time PCR with appropriate primers. Data shown are means ± SD.

modified (see supplementary material). More investigations with pure synthetic lipids rather than HPLC-isolated lipids will be necessary to completely elucidate this important observation.

Affinity purification of Ox-PAPE-*N*-biotin-labeled proteins in HAECs

Proteins extracted from HAECs treated with Ox-PAPE-*N*-biotin were affinity-purified using tetrameric avidin beads. After mixing the extracted protein with beads for 2 h at room temperature, nonbiotinylated proteins were removed by washing the beads with 0.2% Nonidet-P40 in PBS [supernatant (SP)]. Biotinylated lipid-protein adducts retained on the avidin beads were eluted [affinity-purified (AP)] and separated by SDS-PAGE. As before, protein electrophoresis was performed on two identical gels. One of the gels was stained with SYPRO® Ruby, and the other was transferred to a PVDF membrane for Western analysis. The SYPRO® Ruby-stained gel showed a significant decrease in the amount of protein after affinity purification (**Fig. 4A**, SP vs. AP); there was no visible difference between the whole cell lysate (WCL) and the fraction that did not bind to avidin beads, termed the supernatant (Fig. 4A, WCL vs. SP). In contrast, there was a noticeable enrichment of Ox-PAPE-*N*-biotin-labeled proteins achieved by affinity purification compared with the supernatant on the streptavidin-HRP Western blot (Fig. 4B, AP vs. SP). The avidin affinity-purified proteins that corresponded to those identified as biotinylated by Western analysis were cut from the SDS-PAGE gel and subjected to in-gel trypsin digestion followed by mass spectrometric identification (see supplementary Table I).

Localization of Ox-PAPE-*N*-biotin protein targets in HAECs

Endothelial cells were grown on poly-*D*-lysine- or gelatin-coated cover slips to 50% confluence and treated with

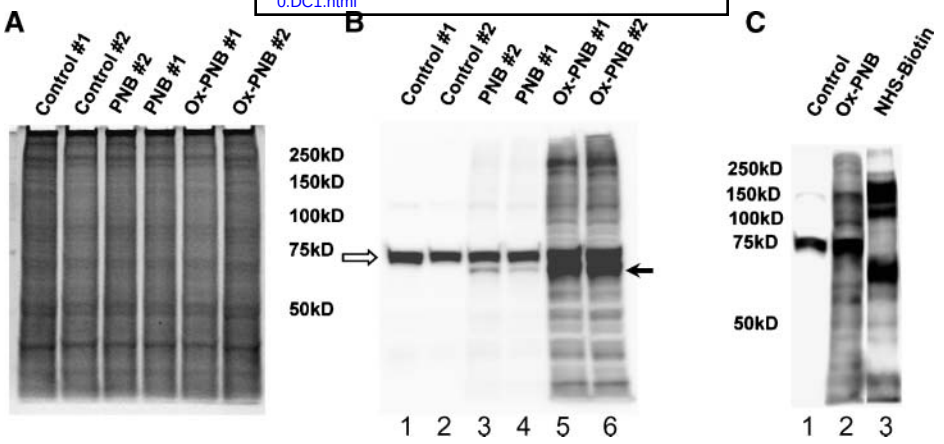


Fig. 3. HAEC oxidized phospholipid binding proteins. Confluent monolayers of HAECs were treated with PBS (control), 50 μg/ml unoxidized PAPE-*N*-biotin (PNB), or 50 μg/ml Ox-PAPE-*N*-biotin (Ox-PNB) for 30 min at 37°C in duplicate (#1 and #2). Proteins isolated from the treated cells were run on two identical 8% gels: one was fixed and stained with SYPRO® Ruby (A), and the other was transferred to a polyvinylidene difluoride (PVDF) membrane for Western analysis. Open arrow shows intrinsic biotin binding protein (non-specific). Closed arrow shows band specific to Ox-PAPE-*N*-Biotin treatment (B). In a separate experiment, HAECs were treated with PBS alone, Ox-PAPE-*N*-biotin, or biotin-hydroxysuccinimide sulfonate (NHS-biotin) at 4°C, according to the manufacturer's specifications. Proteins were separated on a 4–20% gradient gel and transferred onto a PVDF membrane. HAEC proteins labeled with biotin were visualized with streptavidin-HRP followed by ECL-Plus chemiluminescence (C).

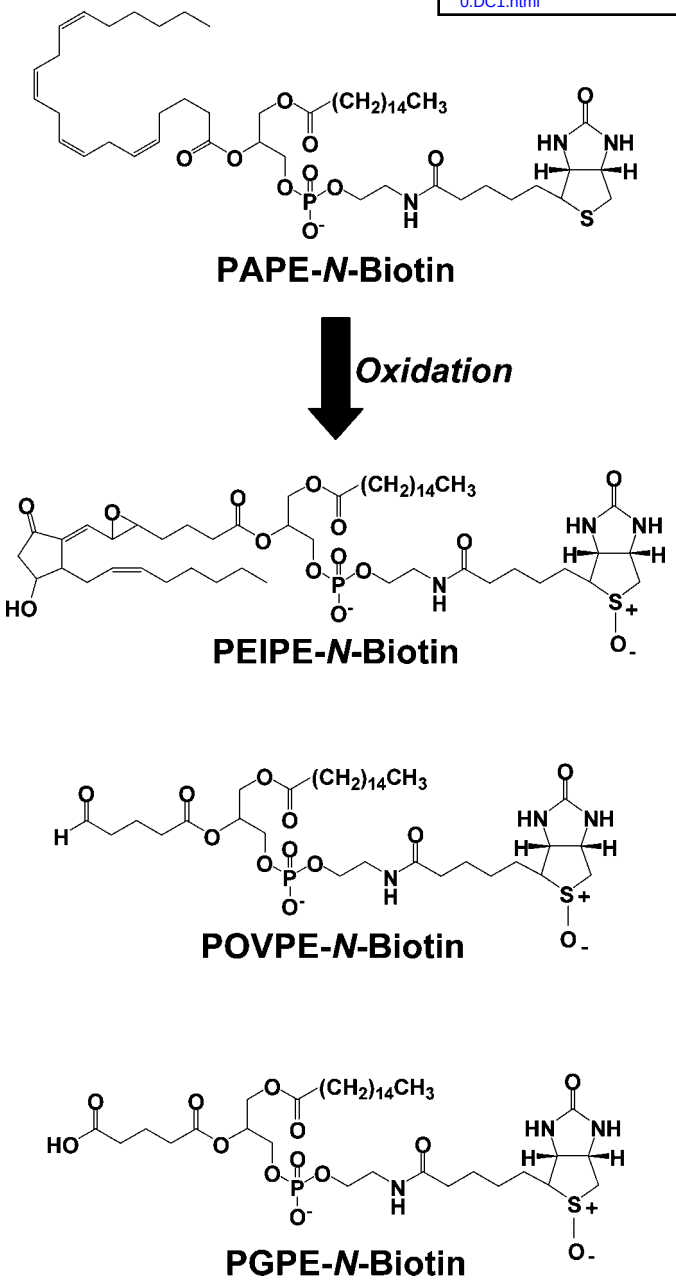
Ox-PAPE-*N*-biotin or unoxidized PAPE-*N*-biotin for 30 min in PBS. FITC-conjugated avidin (Zymed) and a Texas Red BODIPY C_6-ceramide/BSA complex were added, and the cells were fixed, quenched, and visualized by fluorescence microscopy. No significant FITC-avidin staining was detected in cells treated with unoxidized PAPE-*N*-biotin (**Fig. 5A**); however, there was abundant perinuclear staining in the Ox-PAPE-*N*-biotin-treated cells (Fig. 5D). A similar staining pattern was obtained with Golgi BODIPY staining (Fig. 5B, E). This showed that oxidized phospholipids enter the cells under the conditions of the experiment and that protein targets of oxidized phospholipids appear to be located in organelles surrounding the nucleus, such as the endoplasmic reticulum and/or the Golgi (Fig. 5F). This finding does not preclude the possibility of the direct interaction of oxidized lipids with proteins on the surface of endothelial cells.

DISCUSSION

Monocyte-endothelial interactions are thought to play a central role in the pathogenesis of atherosclerosis. This interaction is regulated, in large part, by the inflammatory state of the vessel wall, which can be stimulated by oxidized phospholipids and other mediators of inflammation. Specific oxidized products of PAPC have been shown to stimulate these inflammatory responses, but whether these lipids interact with specific proteins in endothelial cells is not known. The purpose of this study was to synthesize labeled oxidized phospholipids that could be used to detect specific protein-lipid interactions.

A biotin-labeled arachidonic acid-containing phospholipid was synthesized (Scheme 1) and oxidized. Not unexpectedly, oxidation of the arachidonic acid at the *sn*-2 position of PAPE-*N*-biotin occurred similar to that described previously for PAPC (42). Based on the expected molecular weight and relative HPLC retention time, analogs of all oxidation products previously found to be biologically active in Ox-PAPC were present in Ox-PAPE-*N*-biotin (see supplementary material). Unexpectedly, the sulfur atom in biotin was readily oxidized to a sulfoxide group, increasing the mass of the ions by 16 Da (Scheme 2). To confirm the biological activity of these biotin sulfoxide-containing lipids, we synthesized PAPE-*N*-biotin sulfoxide (43) and oxidized the compound in the air to produce oxidized phospholipids containing only biotin sulfoxide. These compounds were tested for biological activity in the same way as Ox-PAPE-*N*-biotin and showed similar induction of inflammatory gene expression (data not shown). The presence of the sulfoxide group did not affect the affinity of Ox-PAPE-*N*-biotin for HAEC proteins or the affinity of the biotin-containing head group for streptavidin. Certainly, the sulfoxide formation did not interfere with the ability to detect biotin-labeled proteins with streptavidin-HRP. Other investigators have described biotin sulfoxide formation under similar oxidation conditions (43).

Treatment of HAECs with Ox-PAPE-*N*-biotin resulted in the definitive detection of ~20 protein bands by streptavidin-HRP. Because these proteins were not detected with unoxidized PAPE-*N*-biotin, it seemed reasonable to conclude that the oxidation of arachidonic acid was responsible for the lipid-protein interaction. Furthermore, competition with excess Ox-PAPC during Ox-PAPE-*N*-biotin treatment of HAECs decreased the intensity of all of the proteins recognized by Ox-PAPE-*N*-biotin except propionyl-CoA carboxylase, suggesting that Ox-PAPC and



Scheme 2. Biologically active oxidation products generated by the oxidation of PAPE-*N*-biotin. PEIPE-*N*-biotin, 1-palmitoyl-2-(5, 6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine); PGPE-*N*-biotin, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidylcholine-(*N*-biotinylethanolamine); POVPE-*N*-biotin, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylethanolamine-(*N*-biotinylethanolamine).

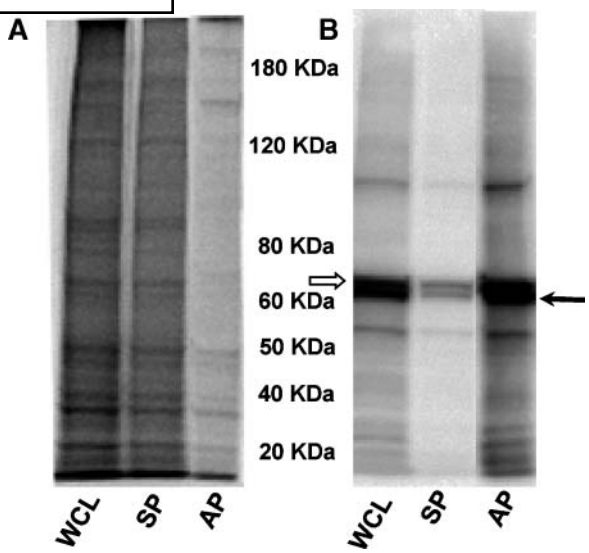


Fig. 4. Avidin affinity purification of HAEC proteins. HAECs were treated with Ox-PAPE-*N*-biotin (50 μg/ml) for 30 min in PBS at 37°C. Cellular proteins were isolated, added to beads coated with tetrameric avidin, and mixed for 2 h at room temperature. The beads were washed, and unbound proteins were collected in the supernatant. Bound proteins were recovered by boiling the beads in reducing Laemmli buffer. The initial cell lysate (WCL), the supernatant wash (SP), and the affinity purified proteins (AP) were analyzed by SDS-PAGE in duplicate. One gel was stained with SYPRO® Ruby (A), and the other gel was transferred to a PVDF membrane and probed with streptavidin-HRP to visualize proteins to which Ox-PAPE-*N*-biotin was bound. Open arrow shows intrinsic biotin binding protein (non-specific). Closed arrow shows band specific to Ox-PAPE-*N*-Biotin treatment (B).

Ox-PAPE-*N*-biotin were interacting with the same endothelial cell proteins (data not shown). The lipid-protein interaction was most likely covalent, because the endothelial cell proteins remained biotinylated throughout the denaturing conditions of SDS-PAGE; however, we cannot exclude the possibility that the lipids formed high-affinity, noncovalent interactions such as those described for platelet-activating factor, eicosanoids, and other lipid mediators. From the structures of oxidized lipids shown in Scheme 2, we would expect Schiff base formation for 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) (44) and Michael (39) and/or epoxide additions (45, 46) for PEIPE-*N*-biotin. 1-Palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidyl-(*N*-biotinylethanolamine) would most likely not participate in covalent interactions.

Serum was omitted from the medium during treatment of cells with lipids to avoid the hydrolysis of oxidized phospholipids by phospholipases and nonspecific interaction with serum proteins. The pattern of protein staining on the Western blot was clearly different from the pattern of protein staining with SYPRO® Ruby (Fig. 3), suggesting that certain proteins were recognized by oxidized phospholipids but other, more abundant, proteins were not. This may be related to the chemical nature of the proteins or their localization within the cell, because fluorescence microscopy showed the localization of oxidized phospholipids near the Golgi (Fig. 5). The 65 kDa protein detected on the Western blot (Fig. 3B, closed arrow) and purified by avidin affinity chromatography (Fig. 5B, closed arrow) was particularly susceptible to oxidized phospholipid modification. In fact, this protein was also slightly labeled by streptavidin-HRP in the unoxidized PAPE-*N*-biotin-treated cells. Later analysis of the unoxidized PAPE-*N*-biotin preparation by negative ion ESI-MS showed a trace amount of

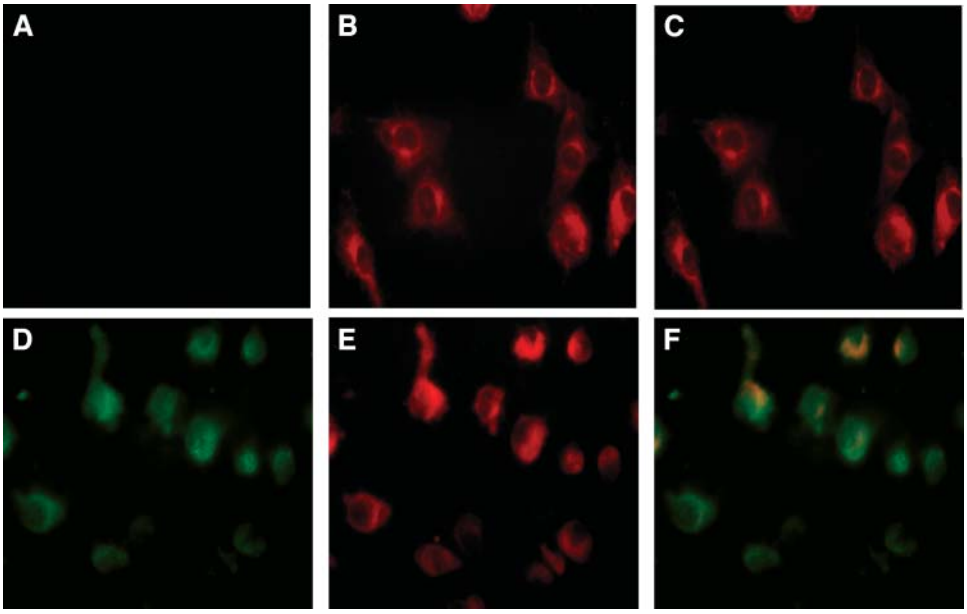


Fig. 5. Cellular localization of Ox-PAPE-*N*-biotin by fluorescence microscopy. HAECs were cultured on chamber slides and treated with either unoxidized PAPE-*N*-biotin (A–C) or Ox-PAPE-*N*-biotin (D–F). FITC-conjugated avidin was used to detect biotin (A, D), and Texas Red fluorescent dipyromethene boron difluoride (BODIPY) C6-ceramide/BSA complex was used to detect the Golgi (B, E). C shows merged panels A and B; F shows merged panels D and E.

oxidation products, which was likely responsible for the biotinylation of this protein. This observation emphasized the selectivity of these oxidized phospholipids for certain endothelial cell proteins and the possibility that, in vivo, small amounts of oxidized phospholipids may have specific and profound cellular effects.

We and others have reported that isolated components of Ox-PAPC, such as POVPC, PGPC, and PEIPC, have slightly different inflammatory and anti-inflammatory effects on endothelial cells (15, 25). The mechanism for this differential activation may be explained by specific targeting of endothelial proteins by certain oxidized phospholipids. This mechanism was supported by the observation that components of Ox-PAPE-*N*-biotin separated by LC-ESI-MS seemed to recognize different proteins (see supplementary material). More investigations are required using synthetic oxidized phospholipids to fully confirm this hypothesis.

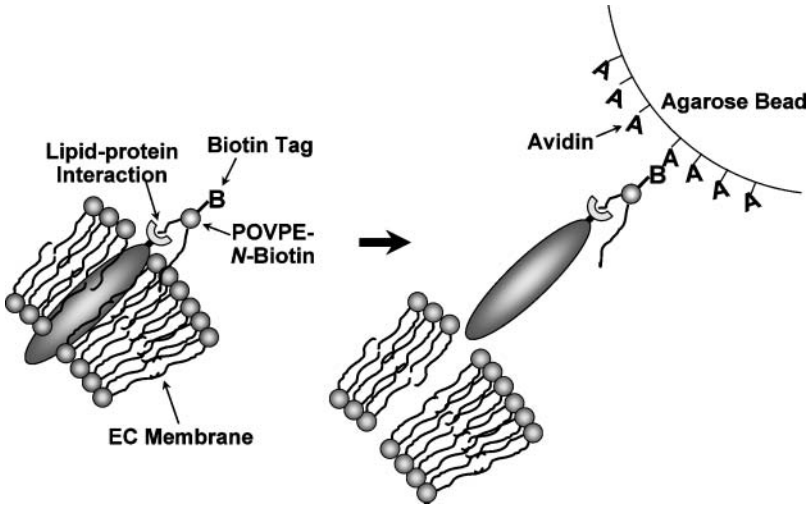


Fig. 6. Theoretical method of capturing oxidized phospholipid targets using avidin affinity chromatography. Oxidized phospholipids, such as POVPE-*N*-biotin, form a lipid-protein interaction with endothelial cell (EC) membrane proteins. The proteins can then be isolated by binding to avidin immobilized on a bead support and purified.

Avidin beads were used to affinity-purify HAEC lysates from cells treated with Ox-PAPE-*N*-biotin. The rationale behind this approach was that proteins in HAECs that are targeted by oxidized biotinylated phospholipids will acquire a biotin tag (**Fig. 6**). This would render them separable using avidin-biotin affinity chromatography. Because the avidin affinity enrichment is denaturing (washes with detergents and extraction from beads by boiling in reducing Laemli sample buffer), we hypothesized that probably only covalently bound biotinylated lipids would be visible on the streptavidin-HRP Western blot. We identified using standard proteomic techniques the main component of the specific band on the streptavidin-HRP Western blot as CKAP4, a type II transmembrane protein located mainly in the endoplasmic reticulum/Golgi intermediate compartment but also found in significant amounts on the surface of vascular smooth muscle cells, where it regulates tissue plasminogen activator. In our fluorescence microscopy studies, we found that Ox-PAPE-*N*-biotin labeling has a perinuclear distribution, consistent with the location of CKAP4 (Fig. 5). The nonspecific band was identified as an intrinsic biotin binding protein, propionyl-CoA carboxylase, a mitochondrial protein.

The proteins identified from the avidin affinity-purified proteins obtained from HAECs treated with Ox-PAPE-*N*-biotin (see supplementary Table I) were not verified to be biotinylated by reverse immunoprecipitation, with the exception of CKAP4. Immunoprecipitation of CKAP4 from HAECs treated with Ox-PAPE-*N*-biotin was determined to be biotinylated when analyzed by Western analysis with streptavidin-HRP (data not shown). We suspect that the peptides identified by mass spectrometry were from parts of the protein that were not modified by the biotinylated oxidized phospholipids, because the presence of the lipid would probably make the peptide unassignable. Alternatively, the biotinylated oxidized phospholipids may have dissociated from the proteins during the processing for mass spectrometric analysis. Work is in progress, and will be reported elsewhere, to fully identify all of the proteins that appear to specifically associate with Ox-PAPE-*N*-biotin. Once the proteins with which oxidized phospholipids interact are identified, standard molecular biology techniques will be used to elucidate the pathways through which they regulate inflammatory processes in HAECs in response to oxidized phospholipids. Previously, it was reported that structurally similar oxidized phospholipids differentially regulate the endothelial binding of monocytes and neutrophils (47). The work presented here suggests that the different biological responses to individual oxidized phospholipids may be attributable to specific lipid-protein interactions.

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