Oxidized phospholipids mediate occludin expression and phosphorylation in vascular endothelial cells

Lucas DeMaio,1 Mahsa Rouhanizadeh,1 Srinivasa Reddy,2 Alex Sevanian,2‡ Juliana Hwang,2 and Tzung K. Hsiai1,2

1Department of Biomedical Engineering and Division of Cardiovascular Medicine, 2Division of Nephrology, Department of Molecular Pharmacology and Toxicology, University of Southern California; and 3Departments of Medicine and Medical Molecular Pharmacology, School of Medicine, University of California, Los Angeles, California

Submitted 26 May 2005; accepted in final form 31 August 2005


First published September 19, 2005; doi:10.1152/ajpheart.00554.2005—Oxidized 1,2-α-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC), a component of minimally modified LDL, induces production of proinflammatory cytokines and development of atherosclerotic lesions. We tested the hypothesis that OxPAPC alters expression, phosphorylation, and localization of tight junction (TJ) proteins, particularly occludin, a transmembrane TJ protein. OxPAPC reduced total occludin protein and increased occludin phosphorylation dose dependently (10–50 μg/ml) and time dependently in bovine aortic endothelial cells. OxPAPC decreased occludin mRNA and reduced the immunoreactivity of zonula occludens-1 at the cell-cell contacts. Furthermore, OxPAPC increased the diffusive flux of 10-kDa dextran in a dose-dependent manner. O2⁻ production by bovine aortic endothelial cells increased nearly twofold after exposure to OxPAPC. Also, enzymatic generation of O2⁻ by xanthine oxidase-lumazine and H₂O₂ by glucose oxidase-glucose increased occludin phosphorylation, implicating reactive oxygen species as modulators of the OxPAPC effects on occludin phosphorylation. Superoxide dismutase and/or catalase blocked the effects of OxPAPC on occludin protein content and phosphorylation, occludin mRNA, zonula occludens-1 immunoreactivity, and diffusive flux of 10-kDa dextran. These findings suggest that changes in TJ proteins are potential mechanisms by which OxPAPC compromises the barrier properties of the vascular endothelium. OxPAPC-induced disruption of TJs, which likely facilitates transmigration of LDL and inflammatory cells into the subendothelial layers, may be mediated by reactive oxygen species.

oxidized 1,2-α-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; reactive oxygen species

Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC), a biologically active component of minimally modified LDL in atherosclerotic lesions (60), was used as a surrogate to OxLDL in the present study. OxPAPC also induces monocyte binding to endothelial cells (7, 19, 35) and, on the basis of its pattern of gene induction, likely plays important roles in angiogenesis, atherosclerosis, and inflammation (19, 43).

Atherosclerotic lesions predominate within arterial bifurcations and areas of curvature, suggesting that local variations in hemodynamic forces (i.e., low fluid wall shear stress and flow reversal) underlie the pathogenesis of atherosclerosis (12, 15, 30). The origin of oxidized lipids remains unclear, but there is evidence of their existence in the circulation, leading to accelerated LDL oxidation (46, 62) and, presumably, the formation of biologically active oxidized species such as OxPAPC. Furthermore, oscillatory flow, which occurs at arterial bifurcations, induces the production of superoxide anion (O₂⁻) by the vascular endothelium and the oxidative modification of native LDL (25, 37). Therefore, the endothelium may be more susceptible to reactive oxygen species (ROS) and oxidized lipids in the atherosclerosis-prone regions associated with disturbed flow. In the present study, we propose that oxidized lipids such as OxPAPC may compromise the integrity of the blood-tissue barrier by altering the organization of the endothelial tight junction (TJ).

The TJ, the most apical intercellular junction, forms the rate-limiting barrier to water and solute flux through the intercellular cleft. Altered expression and organization of TJ proteins may account for the accumulation of lipid in the arterial intima. TJs consist of an assembly of peripheral membrane-associated and transmembrane proteins. Zonula occludens-1 (ZO-1), a peripheral membrane-associated protein, is a member of the membrane-associated guanylate kinase family (40). Membrane-associated guanylate kinase proteins have PDZ (PSD-95/Dlg/ZO-1) and guanylate kinase-like domains, both of which are protein-protein-interacting domains; therefore, ZO-1 is believed to play a central role in the organization and assembly of transmembrane proteins (17). Occludin, the first transmembrane TJ protein identified (20), forms a rate-limiting transport structure within the intercellular cleft (24, 28, 38, 61). Occludin contains two extracellular loops that are believed to form a junctional seal (1). Occludin confers adhesiveness when expressed in fibroblasts (53), and microinjection of occludin

† Deceased 17 February 2005.

Address for reprint requests and other correspondence: L. DeMaio, Dept. of Biomedical Engineering and Division of Cardiovascular Medicine, DRB 398, USC, Los Angeles, CA 90089 (e-mail: ldemaio@usc.edu).
decreases paracellular transport in Xenopus oocytes (10). Furthermore, antisense oligonucleotides to occludin in human arterial endothelial cells increase solute flux (28), and loop-binding peptides decrease transendothelial electrical resistance and occludin content in Xenopus epithelial cells (61).

In the present study, we examined the effects of OxPAPC on the TJ proteins occludin and ZO-1. We demonstrate that treatment of bovine aortic endothelial cell (BAEC) monolayers with OxPAPC reduced expression of occludin mRNA and protein, increased occludin phosphorylation, and increased diffusive flux [i.e., diffusive permeability coefficient (Pd)] of 10-kDa dextran. Total ZO-1 protein was unaffected by OxPAPC; however, the immunoreactivity of ZO-1 at the cell-cell contacts appeared significantly more disorganized after 4 h of exposure to OxPAPC. Superoxide dismutase (SOD) and/or catalase attenuated the effects of OxPAPC on ZO-1 localization, occludin protein and phosphorylation state, occludin gene, and Pd of 10-kDa dextran, implicating an important role for ROS in the regulation of TJ protein expression, phosphorylation, and endothelial permeability.

METHODS

Cell culture. Primary BAECs (Cell Applications, San Diego, CA) between passages 5 and 7 were seeded at a density of 2.5 × 10^5 cells/cm^2 onto 35-mm tissue culture dishes. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (DMEM-10% FCS) and incubated at 37°C with 5% CO_2. On the day of the experiment, DMEM-10% FCS was replaced with DMEM without FCS. Occludin protein content as detected by Western blot was not observable until 3 days after plating of BAECs, and monolayers were confluent at ±1 day after plating.

Preparation of OxPAPC. PAPC (Sigma-Aldrich, St. Louis, MO) was oxidized by transfer of 1 mg in 100 μl of chloroform to a clean 16 × 25 mm^2 glass test tube and evaporation of the solvent under a stream of nitrogen. The lipid residue was allowed to autoxidize during exposure to air for 24–48 h. The extent of oxidation was monitored by positive electrospray ionization-mass spectrometry in the positive mode (60).

Immunoblot for ZO-1 protein, occludin protein, and occludin phosphorylation. After OxPAPC treatment, monolayers were washed with ice-cold PBS and lysed in an SDS-based extraction buffer composed of 0.2% SDS, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 2 mM EDTA, 10 mM HEPES, 10 mM NaF, 1 mM NaVO_4, 1 mM benzamidine, and 0.2 mM PMSF. Insoluble material was separated from the lysate by centrifugation in a microfuge at 10,000 g for 10 min. Equal protein was loaded onto 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked with 5% milk in Tris-buffered saline + Tween 20, and immunoblotted with rabbit antioccludin polyclonal antibody (1:1,000 dilution; Zymed Laboratories, San Francisco, CA) or rat anti-ZO-1 monoclonal antibody (clone R40-76, provided by Dr. Bruce Stevenson, Department of Cell Biology and Anatomy, University of Alberta, Edmonton, AB, Canada) at a dilution of 1:2. The blot was then incubated with anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (1:4,000 dilution) or anti-rat HRP-conjugated secondary antibody (1:4,000 dilution; Amershams, Piscataway, NJ). Cross-reactivity of all antibodies with BAECs has been established. Occludin and ZO-1 protein content was detected by chemiluminescent detection (Pierce, Rockford, IL). Occludin, which is phosphorylated on serine and threonine residues, appears as doublet bands in BAECs, with the top band in the higher phosphorylation state (3, 13).

Cytotoxicity/viability assay. BAECs were cultured on 22-mm^2 sterile glass coverslips inside 35-mm petri dishes containing 2 ml of DMEM-10% FCS. At 3 days after the cells were plated, the medium was replaced with DMEM without serum and treated with 50 μg/ml OxPAPC for 4 h. Then BAECs were washed with Dulbecco’s PBS, and 150 μl of the combined LIVE/DEAD assay reagents (Molecular Probes, Eugene, OR) were added to the surface of the coverslip. Our working solution of assay reagents contained 1 μM ethidium homodimer-1 and 0.8 μM calcein-AM in Dulbecco’s PBS. These two probes measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. Monolayers were incubated for 45 min at room temperature and mounted onto microscope slides. Slides were viewed with an Olympus BX60 microscope equipped with epifluorescence optics.

Vascular endothelial O_2^- generation in response to OxPAPC. Extracellular O_2^- production by BAECs in response to OxPAPC treatment was determined spectrophotometrically by measurement of the SOD-inhibitable reduction of cytochrome c at 550 nm (model DU 640, Beckmann) as described previously (26). Briefly, BAECs cultured on sterile glass coverslips were exposed to 50 μg/ml OxPAPC in DMEM containing 100 μM acetylated ferricytochrome c. Control samples were maintained in DMEM containing 100 μM cytochrome c. At 1-h intervals for up to 4 h, aliquots of supernatant were taken for absorbance measurements at 550 nm. The specificity of cytochrome c reduction by O_2^- was established by comparing reduction rates in the presence and absence of 60 μg/ml SOD. The rates for SOD-inhibited cytochrome c reduction were corrected for O_2^- formation using the extinction coefficient for cytochrome c (ε_{550} = 2.1 × 10^4 M^-1 cm^-1).

Effect of O_2^- and H_2O_2 generators on occludin content and phosphorylation. O_2^- or H_2O_2 was generated by enzymatic reactions to mimic the rate of ROS produced by BAECs during exposure to OxPAPC. To determine the direct effect of O_2^- generation on occludin protein and phosphorylation state, BAEC monolayers in DMEM containing 500 μM lumazine were exposed to 0, 10, and 20 mU/ml xanthine oxidase at 37°C. After 4 h, BAEC monolayers were lysed and prepared for immunoblot of occludin protein and phosphorylation. Rates of O_2^- generation were also determined spectrophotometrically by measurement of the SOD-inhibitable reduction of cytochrome c at 550 nm. Briefly, samples were maintained in a 96-well plate with DMEM containing 100 μM cytochrome c alone and with 60 μg/ml SOD, and absorbance was measured every 2 min for up to 1 h. The rates for SOD-inhibited cytochrome c reduction were corrected for O_2^- formation using the extinction coefficient for cytochrome c (see above).

To determine the direct effect of H_2O_2 generation on occludin protein and phosphorylation, BAEC monolayers in DMEM containing 4.5 g/l glucose were exposed to 0, 10, and 20 mU/ml glucose oxidase. Rates of H_2O_2 production in DMEM without serum were determined spectrophotometrically with Amplex red (Molecular Probes), a fluorogenic substrate with very low background fluorescence, which reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin (41). To generate a standard curve, 10 μM Amplex red reagent and 1 U/ml HRP were added to DMEM containing known amounts of H_2O_2 or glucose oxidase at various concentrations. Fluorescence measurements (excitation wavelength = 530 nm, emission wavelength = 590 nm) were performed with a spectrophotofluorometer (model LS-5, PerkinElmer Life Sciences, Boston, MA) equipped with a thermal-controlled and magnetic stirring sample compartment. After subtraction of background fluorescence, H_2O_2 rates of production (μM/min) were calculated from the standard curve.

Quantitative real-time RT-PCR. After BAECs were exposed to OxPAPC and/or catalase + SOD, total RNA was isolated using an RNaseasy kit (Qiagen). Primers used for real-time RT-PCR were taken from rat occludin cDNA: GGTGCGAGTCCTGGCG (5’1, 1,784 nt) and CTGGTGATCTGAGATGGTAGGTA (3’, 1,831 nt) (5). Real-time RT-PCR was performed at 50°C for 2 min and 95°C for 10 min, and then run for 40 cycles between 95°C for 15 s and 60°C for 1 min on the real-time RT-PCR Engine (MJ Research Opticon). Measurements were normalized with GAPDH by ΔΔC_{t} method,
where \( C_t \) is the threshold cycle number at which the fluorescence has increased significantly over the background (25).

**Immunochemistry.** BAECs, grown on Transwell filters, were fixed with 1% paraformaldehyde for 10 min, permeabilized with PBS containing 0.2% Triton X-100 for 10 min, and blocked with PBS containing 10% BSA and 0.1% Triton X-100 for 1 h. BAECs were then incubated with anti-ZO-1 primary antibody (1:1 dilution) for 1 h, washed five times with PBS containing 0.1% Triton X-100, and then incubated with donkey anti-rat Cy3 antibody (1:200 dilution; Jackson Immunoresearch Laboratories, West Grove, PA). Filters were carefully removed from Transwell casing and mounted onto a glass slide and coverslip using Aqua Poly Mount (Polysciences, Warrington, PA). A Leica TCS SP MP inverted confocal microscope connected via fiber optic to a Spectra-Physics Integrated Two-Photon Laser System at the UCLA Brain Research Institute was used to capture digital images. All images within an experiment were captured in an identical fashion.

**Measurement of diffusive dextran flux.** For permeability measurements, BAEC monolayers were grown on 0.4-μm-pore, 24.5-mm-diameter Transwell polycarbonate filters (Costar, Cambridge, MA) for 7 days. Filters were incubated with 1 ml of 15-mm diameter Transwell polycarbonate filters (Costar, Cambridge, MA) for 3 days. On the day of the experiment, DMEM-10% FCS was replaced with phenol red-free DMEM without FCS. OxPAPC and/or SOD + catalase treatment were introduced on the apical side of the membrane. Dextran flux was measured by application of 7.5 μM 10-kDa Oregon green-dextran (Molecular Probes) to the apical chamber of inserts with a confluent BAEC monolayer. A trace concentration (≈2 nM) of Oregon green-dextran was added to the basolateral compartment so that the linear relation between dextran concentration and fluorescent intensity was maintained at low concentrations. At the start and each hour during the 4-h experiment, 75-μl samples were taken from the basolateral compartment. A sample was taken from the apical compartment at the last time point; the amount of fluorescence did not change over the 4-h time course on the apical side. Fluorescence of aliquots was quantified using a Tecan GENios Pro fluorescence plate reader. 

**Statistical analysis.** Data were analyzed by analysis of variance (ANOVA) or Student-Newman-Keuls multiple comparisons test to examine differences among groups. The minimum level of statistical significance was taken as \( P < 0.05 \).

**RESULTS**

**Effect of OxPAPC on occludin protein and phosphorylation.** Altered expression of TJ proteins within arterial bifurcations or curvatures, where disturbed flow develops, may promote the trapping of oxidized lipid into the subendothelial layers. To determine whether oxidized lipids affect TJ protein content, BAECs were lysed after 4 h of exposure to 50 μg/ml OxPAPC, subjected to SDS-PAGE, and immunoblotted for ZO-1 (Fig. 1A) and occludin (Fig. 1B). ZO-1 protein content was not altered by OxPAPC at 4 h. In endothelial cells, occludin migrates as two major bands, ≈60 and 62 kDa, which have been termed occludin-α and occludin-β, respectively. Total occludin protein (α + β) was reduced after 4 h of exposure to OxPAPC; the blot in Fig. 1B was reprobed for GAPDH reactivity, which was not altered by OxPAPC. These experiments were performed twice, and the results were identical. Multiple experiments examining the dose response and time course of occludin protein changes after OxPAPC exposure are shown in Figs. 2, 3, and 6.

The dose response of occludin protein downregulation and occludin phosphorylation upregulation by OxPAPC was also examined by Western blot (Fig. 2A). Total occludin protein was reduced to 52 ± 11%, 34 ± 19%, and 31 ± 11% of control after 4-h exposures to OxPAPC at 10, 25, and 50 μg/ml (Fig. 2B). The difference in protein levels between control and OxPAPC-treated BAECs was significant (\( P < 0.01 \), \( n = 3 \)); there was no significant difference among the different concentrations of OxPAPC, suggesting a saturation of its biological effect at the physiologically representative concentration as measured in minimally modified LDL (60). The effect of OxPAPC on occludin phosphorylation was also examined by Western blot. It was previously shown that alkaline phosphatase treatment collapses the slower-migrating occludin band(s) to a single band in BAEC monolayers, bovine retinal endothelial cell monolayers, and retinal capillaries (3, 13); therefore, the slower-migrating 62-kDa band (occludin-β) in BAECs exists at a higher phosphorylation state than the 60-kDa band (occludin-α). Occludin phosphorylation in BAECs exposed to the various concentrations of OxPAPC was quantified as the ratio of occludin-β to occludin-α content (Fig. 2C). Occludin phosphorylation increased in BAECs treated with 25 μg/ml OxPAPC compared with BAECs treated with 10 μg/ml OxPAPC (\( P < 0.05 \)). Exposure to 50 μg/ml OxPAPC significantly increased the ratio of occludin-β to occludin-α compared with the three other treatment conditions (i.e., control and 10 and 25 μg/ml OxPAPC, \( P < 0.001 \), \( n = 3 \)).

![Fig. 1. Oxidized L-α-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) decreased occludin protein but not zonula occludens-1 (ZO-1) protein. A: bovine aortic endothelial cell (BAEC) lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted for ZO-1 (rat monoclonal antibody, clone R40-76) after 4 h of exposure to 50 μg/ml OxPAPC. OxPAPC did not alter total ZO-1 content in BAEC monolayers. B: immunoblot showing occludin reactivity from BAECs exposed to 50 μg/ml OxPAPC for 4 h. In endothelial cells, occludin migrates as 2 major bands, at 60 and 62 kDa, which have been termed occludin-α and occludin-β, respectively. Total occludin protein (α + β) was reduced after 4 h of exposure to OxPAPC. Blot was reprobed for GAPDH reactivity, which was not altered by OxPAPC. C, control.](https://ajpheart.physiology.org/content/290/2/H676/F1)
Oxidized lipids alter tight junctions

The time course of occludin protein changes in response to OxPAPC was investigated in BAEC monolayers by Western blot (Fig. 3A). Occludin protein was significantly reduced to 42 ± 6% and 18 ± 3% after 1 and 4 h, respectively (P < 0.01, n = 3; Fig. 3B). The ratio of occludin-β to occludin-α remained relatively constant in monolayers exposed to OxPAPC for shorter durations (Fig. 3C). After 4-h exposures, however, BAECs exhibited a higher expression of occludin-β than occludin-α, indicating an increase in occludin phosphorylation. Specifically, the ratio of occludin-β to occludin-α after a 4-h exposure increased 5.8 ± 1.4-fold relative to control (P < 0.01, n = 3).

To eliminate the possibility that cell death was causing alterations in occludin protein, the effect of a 4-h exposure to OxPAPC on the viability and cytotoxicity of BAEC monolayers was examined using the LIVE/DEAD two-color fluorescence assay. Our maximum concentration of OxPAPC, 50 μg/ml, produced a uniform green fluorescence inside the cells that was comparable to untreated live cells (n = 3; data not shown). OxPAPC did not produce the distribution of red fluorescence that was observed in monolayers treated with 70% methanol for 30 min.

Generation of ROS in response to OxPAPC. Hwang et al. (25) reported that oxidation of LDL is in part due to O$_2^-$ formation via membrane-bound NADPH oxidase. In the present study, the rates of O$_2^-$ production also increased in response to 50 μg/ml OxPAPC (Fig. 4A). More specifically, OxPAPC increased O$_2^-$ production by 1.8 ± 0.1-, 1.7 ± 0.2-, 1.8 ± 0.2-, and 2.0 ± 0.3-fold after 1, 2, 3, and 4 h, respectively: 0.25 ± 0.02, 0.30 ± 0.02, 0.34 ± 0.03, and 0.37 ± 0.05 μM·min$^{-1}$·10$^6$ cells$^{-1}$ at 1, 2, 3, and 4 h, respectively (P < 0.05, n = 5). The addition of SOD stopped O$_2^-$ production over time, despite the presence of OxPAPC: 0.02 ± 0.02, 0.05 ± 0.01, 0.07 ± 0.02, and 0.06 ± 0.02 μM·min$^{-1}$·10$^6$ cells$^{-1}$ at 1, 2, 3, and 4 h, respectively. On the basis of these measurements, it is likely that OxPAPC also increases H$_2$O$_2$ formation at a rate near 1 μM/min in a 35-mm plate (growth area = 10 cm$^2$, cell density = 2.5 × 10$^5$ cells/cm$^2$), with the assumption of 1:1 stoichiometry between O$_2^-$ dismutation and H$_2$O$_2$ formation.

To directly investigate the contribution of ROS to changes in occludin phosphorylation and/or occludin content in response to OxPAPC treatment, specific O$_2^-$ (lumazine-xanthine oxidase)- and H$_2$O$_2$ (glucose-glucose oxidase)-generating systems were added to BAEC monolayers (see METHODS). We observed a concentration-dependent generation of O$_2^-$ and H$_2$O$_2$ on coincubation with xanthine oxidase (plus 500 μM lumazine) and glucose oxidase, respectively, at increasing concentrations (Fig. 4B). Higher concentrations of xanthine oxidase than glucose oxidase are required to produce ROS at a rate similar to OxPAPC (i.e., 1 μM/min). The effect of O$_2^-$ and H$_2$O$_2$ generators on occludin protein and phosphorylation state was also examined by Western blot (Fig. 4C); BAEC monolayers were exposed to xanthine oxidase (10 and 20 mU/ml)-lumazine and glucose oxidase (10 and 20 mU/ml)-glucose at 37°C. After 4 h, BAEC monolayers were lysed and prepared for immuno-
ROS may modulate the effects of OxPAPC on occludin phosphorylation.

Effect of OxPAPC on occludin gene expression. OxPAPC may influence the expression of important gene families that play a general role in inflammation, atherosclerosis, and wound healing (36, 43). To determine the effect of 50 μg/ml OxPAPC on occludin gene expression, quantitative real-time RT-PCR was performed and normalized by GAPDH (Fig. 5A). The influence of SOD (292 U/ml) and/or catalase (500 U/ml) on OxPAPC-mediated gene expression of occludin was also investigated to further implicate a role for ROS and their scavengers in the regulation of the TJ. Occludin mRNA was significantly reduced to 38 ± 6% of control after 4 h of exposure to OxPAPC (P < 0.05, n = 4). In monolayers treated with OxPAPC and SOD or catalase, no significant attenuation of the OxPAPC-induced reduction in the occludin gene was observed. However, in monolayers treated with OxPAPC and SOD + catalase, the OxPAPC response was significantly blocked (P < 0.05), indicating that SOD and catalase were required to attenuate the OxPAPC-induced reduction in the occludin gene. Figure 5B shows the effect of antioxidant enzymes on occludin mRNA in the absence of OxPAPC. Occludin mRNA was dramatically increased in monolayers exposed to SOD (15 ± 3-fold relative to control), catalase (18 ± 6-fold relative to control), and SOD + catalase (26 ±
1-fold relative to control). These results indicate that the depletion of ROS increases expression of the occludin gene and protein (Fig. 6).

Effect of catalase and SOD on OxPAPC-induced changes in occludin content and phosphorylation. We investigated the influence of SOD and catalase to implicate a role for scavengers of ROS in the regulation of occludin in the presence and absence of 50 μg/ml OxPAPC. The effect of catalase (500 U/ml) on OxPAPC-mediated occludin expression was examined by immunoblot (Fig. 6A). Four hours of exposure to OxPAPC significantly reduced occludin content to 73 ± 7% of control (P < 0.05, n = 3), whereas in monolayers treated with catalase and OxPAPC for 4 h, occludin content was restored to the level of the control sample and was significantly greater than in OxPAPC-treated monolayers (P < 0.01, n = 3; Fig. 6B). The protective effect of catalase on occludin content was statistically significant (P < 0.01). Catalase significantly increased occludin content in monolayers not exposed to OxPAPC (P < 0.001). These results suggest that occludin levels are influenced by the presence of H2O2 mediated by vascular endothelium.

The effect of catalase (500 U/ml) on occludin phosphorylation in the presence or absence of 50 μg/ml OxPAPC was also examined in the same Western blots (Fig. 6C). OxPAPC increased the ratio of occludin-β to occludin-α by 2.5 ± 0.6-fold relative to control. The increase in occludin phosphorylation induced by OxPAPC was significantly higher than that induced by all other treatments (P < 0.05, n = 3). The ratio of occludin-β to occludin-α in monolayers treated with OxPAPC and catalase was nearly identical to that in control monolayers.

In parallel, the effect of SOD (292 U/ml) on BAEC occludin content and phosphorylation in the presence or absence of 50 μg/ml OxPAPC was also examined by immunoblot (Fig. 6D). OxPAPC significantly reduced occludin content to 30 ± 5% of control (Fig. 6E; P < 0.01, n = 3). Total occludin protein was significantly elevated when monolayers unexposed to OxPAPC were treated with SOD (P < 0.05, n = 3). The addition of SOD + catalase to OxPAPC-treated monolayers also blocked the OxPAPC-induced reduction in occludin protein (data not shown). The effect of SOD on occludin phosphorylation in the presence or absence of OxPAPC (Fig. 6F) was very similar to the effect of catalase on occludin phosphorylation (Fig. 6C); OxPAPC increased the ratio of occludin-β to occludin-α by 2.9 ± 1.0-fold relative to control, and SOD tended to attenuate the OxPAPC-induced increase in occludin phosphorylation. These results indicate that ROS (O2· and H2O2) were implicated in the ratio of occludin-β to occludin-α.

Effect of OxPAPC on immunoreactivity of ZO-1. The effect of OxPAPC on the cellular localization of ZO-1 was investigated by immunofluorescence confocal microscopy. The reactivity of ZO-1 in monolayers exposed to OxPAPC for 4 h (Fig. 7B) was less intense and localized at the cell–cell contacts in a more discontinuous fashion than in untreated monolayers (Fig. 7A). The addition of SOD (Fig. 7C), catalase (Fig. 7D), and SOD + catalase (Fig. 7E) to monolayers exposed to OxPAPC attenuated the OxPAPC-induced disorganization of ZO-1 reactivity at the cell–cell contacts. The addition of SOD + catalase to the apical chamber did not alter ZO-1 reactivity in monolayers unexposed to OxPAPC compared with control monolayers (data not shown). In general, occludin immunoreactivity along the BAEC borders was markedly more discontinuous than the observed ZO-1 immunoreactivity (data not shown).

Effect of OxPAPC on Pd. The cleft between adjacent endothelial cells is the primary pathway for the transport of water and hydrophilic solutes into the underlying tissue (39). Discontinuities in the TJ strand allow for paracellular transport of solutes, including 10-kDa dextran. Pd of 10-kDa dextran across BAEC monolayers under various treatment conditions is shown in Fig. 8. The average Pd of control monolayers was 7.5 ± 0.4 × 10−6 cm/s over the 4-h time course. OxPAPC increased Pd in a dose-dependent fashion. At 50 μg/ml, Pd increased 1.7 ± 0.2-fold compared with control. The increased flux of dextran through OxPAPC-treated monolayers was significant at 50 μg/ml OxPAPC (P < 0.05, n = 4). The addition of SOD to monolayers exposed to 50 μg/ml OxPAPC did not significantly reduce Pd. However, the addition of catalase and catalase + SOD significantly attenuated the OxPAPC-induced increase in Pd (P < 0.05, n = 4). Despite the increased expression of occludin stimulated by SOD and catalase in the absence of OxPAPC, neither of these enzymes significantly altered Pd compared with control monolayers. It is possible that SOD + catalase increases occludin protein in the cyto-
plasm but does not directly affect TJ function or the trafficking and assembly of TJ proteins at the cell-cell contacts after 4 h.

**DISCUSSION**

Information regarding the effects of oxidative stress on the organization of vascular TJ proteins is very limited. Here, we report that OxPAPC, an oxidized phospholipid, alters TJ organization in BAECs. After 4 h of exposure to OxPAPC, ZO-1 immunoreactivity at the cell-cell contacts appeared more disorganized and less intense, occludin protein content was reduced, occludin phosphorylation was increased, and the flux of 10-kDa dextran ($P_d$) was increased. OxPAPC also induced a similar reduction in occludin mRNA over the identical 4-h time course. Besides reducing occludin synthesis, it is also possible that OxPAPC increases its degradation. Occludin is a functional target of the E3 ubiquitin-protein ligase Itch (52), a member of the HECT domain-containing ubiquitin-protein ligases, which may be activated in the presence of OxPAPC to increase occludin internalization and degradation. The data presented here indicate that oxidized phospholipids induce TJ disassembly in aortic endothelial cells and, thus, may provide a basis for the accumulation of lipid in the subendothelial layers.

The altered expression and organization of TJ proteins and the subsequent increase in permeability in response to OxPAPC are similar to that caused by vascular endothelial growth factor (VEGF) and shear stress. In brain microvessel endothelial cells, Wang et al. (58) showed that VEGF disrupted occludin and ZO-1 organization and reduced occludin protein content but did not affect ZO-1 content. Antonetti et al. (3, 4)

---

**Fig. 7.** Effect of OxPAPC and/or antioxidant enzymes on ZO-1 immunoreactivity. OxPAPC (50 μg/ml) reduced ZO-1 reactivity at cell-cell contacts. Addition of SOD (292 U/ml), catalase (500 U/ml), and SOD + catalase blocked OxPAPC-induced reduction in ZO-1 reactivity at cell border. A: ZO-1 reactivity in control monolayers. B: ZO-1 reactivity in monolayers exposed to OxPAPC for 4 h. C–E: ZO-1 reactivity in monolayers exposed to OxPAPC and SOD, OxPAPC and catalase, and OxPAPC and catalase + SOD, respectively. Scale bar, 20 μm.

**Fig. 8.** Effect of OxPAPC and/or antioxidant enzymes on diffusional permeability ($P_d$) of BAEC monolayers to 10-kDa dextran. OxPAPC increased $P_d$ in a dose-dependent manner (10–50 μg/ml). OxPAPC was used at 50 μg/ml when coincubated with SOD (292 U/ml) and/or catalase (500 U/ml). Addition of catalase or catalase + SOD blocked the increase in $P_d$ stimulated by 50 μg/ml OxPAPC. *$P < 0.05$. 

---

AJP-Heart Circ Physiol • VOL 290 • FEBRUARY 2006 • www.ajpheart.org
reported that VEGF increased the serine/threonine phosphorylation of occludin within 15 min and reduced occludin protein in bovine retinal endothelial cells after 6 h. In BAECs, shear stress was also shown to increase occludin phosphorylation within 5 min and reduce occludin content over longer time periods; ZO-1 protein, on the other hand, was unaffected by shear (13). In these studies, increases in endothelial permeability and hydraulic conductivity (water flux) occur over a time course similar to the observed increase in occludin phosphorylation in response to VEGF and shear stress. These responses precede reductions in occludin protein. Several reports have demonstrated that the distribution and function of occludin may be controlled by its phosphorylation, suggesting the levels of phosphooccludin may be a key determinant of the barrier properties of the TJ complex (for review see Ref. 18). The similar alterations to the TJ complex in response to VEGF and shear stress may be expected, because VEGF and shear stress stimulate the Flk-1/Cbl/Akt signaling pathway (59). In the present study, OxPAPC also reduced total occludin protein but not total ZO-1 protein. Therefore, ZO-1 protein levels, in contrast to occludin levels, are most likely not regulated by oxidized lipids, VEGF, or shear stress. The distribution of ZO-1 and its association with the TJ, however, may be affected by oxidants. The selective reduction in occludin protein is also supported by the finding that tyrosine phosphatase inhibition induces occludin proteolysis but does not alter the expression of ZO-1, cadherin, and β-catenin in human umbilical endothelial cells (55).

Despite evidence that inflammatory mediators such as VEGF, shear stress, and OxPAPC regulate the levels of occludin and ZO-1 protein in a similar fashion, the specific mechanism by which OxPAPC mediates TJ disorganization may be distinct from VEGF and shear stress. A significantly longer time course (i.e., hours) is required for OxPAPC than for VEGF or shear stress (i.e., minutes) to increase occludin phosphorylation. Furthermore, it has been shown that OxLDL inhibits VEGF-induced endothelial cell migration, although an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway (9) and VEGF-induced phosphorylation of occludin was prevented by expression of a dominant-negative protein for Akt in BAECs (42). Also, Kuzuya and colleagues (32) showed that VEGF prevents OxLDL-induced endothelial cell damage via an intracellular glutathione-dependent mechanism that occurs through the KDR/Flk-1 receptor.

OxLDL has been shown to induce O$_2^-$ formation and activate NAD(P)H oxidase in the endothelium (45, 49). Similarly, OxPAPC increased expression of the NADPH oxidase subunit Nox4 and increased O$_2^-$ formation (44). Therefore, ROS may mediate some of the effects of OxPAPC on the TJ. Our findings indicate that the generation of ROS after OxPAPC addition is a cumulative process that could alter the activity or expression of kinases or phosphatases that regulate occludin’s phosphorylation state. Attenuation of the OxPAPC-induced increase in occludin phosphorylation and $P_0$ of 10-kDa dextran was more effectively produced by catalase than SOD, suggesting that H$_2$O$_2$ formation may mediate the effect of OxPAPC on occludin phosphorylation by virtue of its capacity to act as an oxidant. Also, occludin phosphorylation appeared to be more sensitive to the direct formation of H$_2$O$_2$ by glucose oxidase than to the formation of O$_2^-$ by xanthine oxidase-lumazine. H$_2$O$_2$-mediated changes in occludin phosphorylation state could arise through O$_2^-$ dismutation by activation of specific kinases such as PKC, which has been shown to directly phosphorylate Ser$^{338}$ of occludin (2); furthermore, H$_2$O$_2$ has been shown to stimulate the tyrosine phosphorylation of PKC isoforms through activation of Src family kinases (57). Previous studies also showed that H$_2$O$_2$ inhibits protein tyrosine phosphatases by inducing reversible oxidation of the catalytic cysteine residues (14, 29); thus it is possible that ROS-induced phosphatase inhibition may also stimulate the phosphorylation of occludin. O$_2^-$ may undergo other reactions, such as reductive interaction with proteins and metals or reaction with nitric oxide (11), that diminish its dismutation to H$_2$O$_2$. Also the generation of ROS by glucose oxidase or xanthine oxidase did not appear to downregulate occludin protein as effectively as OxPAPC. OxPAPC activates a number of signal transduction pathways (6) (e.g., PKC, PKA, Raf/MEK1,2/Erk-1,2 MAP kinase cascade, JNK MAP kinase, and transient protein tyrosine phosphorylation) that may increase occludin degradation (52) to a greater extent than the ROS generators.

Although the signaling pathway(s) utilized by OxPAPC is not well understood, it is likely that OxPAPC alters occludin expression and phosphorylation and that this response, at least in part, involves the increased production of ROS. Disruption of endothelial TJs by oxidized lipids likely provides the basis for the increased transmigration of plasma proteins, such as LDL, into the subendothelial layers. Future investigation into the roles of antioxidant enzymes in TJ biology may facilitate the development of therapeutic strategies for vascular oxidative stress and inflammatory responses.

GRANTS

This study was supported by an American Heart Association Postdoctoral Fellowship (L. DeMaio), American Heart Association Grant BGLA 0265166U (T. K. Hsiai), National Institutes of Health Grants KO8 HL-08689-01A1 (T. K. Hsiai), HL-50350 (T. K. Hsiai and A. Sevanian), and RO1 AG-19789 (A. Sevanian), and National Heart Foundation/American Heart Assistance Foundation Grant H2003-028 (T. K. Hsiai).

REFERENCES


37. Steinberg D and Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? Circulation 105: 2107–2111, 2002.


