Specific Phospholipid Oxidation Products Inhibit Ligand Activation of Toll-Like Receptors 4 and 2


Objective—We have previously shown that phospholipid oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) inhibit lipopolysaccharide (LPS)-induced E-selectin expression and neutrophil binding in human aortic endothelial cells (HAECs). The current studies identify specific phospholipids that inhibit chemokine induction by Toll-like receptor-4 (TLR4) and -2 (TLR2) ligands in ECs and macrophages.

Methods and Results—Measurements of interleukin (IL)-8 and monocyte chemotactic protein-1 levels secreted from ox-PAPC– and LPS-cotreated ECs indicate that ox-PAPC inhibits activation of TLR4 by LPS. The effects of IL-1β and tumor necrosis factor-α, which utilize the same intracellular signaling molecules, were not inhibited. Cell fractionation and immunofluorescence analyses demonstrate that LPS induces membrane translocation of the LPS receptor complex to a lipid raft/caveolar fraction in ECs. Ox-PAPC inhibits this translocation and alters caveolin-1 distribution. Supporting an important role for caveolae in LPS action, overexpression of caveolin-1 enhanced LPS-induced IL-8 synthesis. Ox-PAPC also inhibits the effect of TLR2 and TLR4 ligands in human macrophages.

Conclusions—These studies report a novel mechanism that involves alterations to lipid raft/caveolar processing, by which specific phospholipid oxidation products inhibit activation by TLR4 and TLR2 ligands. These studies have broader implications for the role of ox-PAPC as a regulator of specific lipid raft/caveolar function. (Arterioscler Thromb Vasc Biol. 2003;23:1197-1203.)

Key Words: endothelial cells — oxidized phospholipids — lipopolysaccharide — Toll-like receptors — inflammation

Bacterial lipid–containing products that activate toll-like receptor (TLR) 4 and TLR2 are potent inducers of the acute inflammatory response and entry of neutrophils and monocytes into the vessel wall. Later on, neutrophil entry is inhibited while monocytes continue to enter the vessel wall until the infection is resolved. It has been shown that highly oxidized (ox-LDL) and minimally modified/oxidized LDL (MM-LDL) can inhibit the inflammatory effects of lipopolysaccharide (LPS).1,2 We and others have previously demonstrated that the major inhibitory lipids in MM-LDL are oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC).3 Ox-PAPC and 1 component phospholipid, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC), strongly inhibited LPS-mediated neutrophil binding and expression of E-selectin in endothelial cells (ECs), whereas native PAPC was ineffective.4 It was also shown that ox-PAPC components accumulate in apoptotic and necrotic cells5 and in interleukin (IL)-1β–treated cells.6 We further demonstrated that ox-PAPC components themselves induce a chronic inflammatory response by stimulating ECs to produce monocyte-binding molecules and chemotactic factors.7 These findings suggest that PAPC oxidation products, which accumulate in dying or cytokine-stimulated cells at sites of infection, might contribute to downregulation of the acute response to bacterial lipid–containing products and propagate more chronic inflammation at these sites. The current studies demonstrate that ox-PAPC inhibits the ability of TLR4 and TLR2 ligands to induce chemokine synthesis, identify the most potent inhibitory ox-PAPC components, and examine the mechanism of inhibition.

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Methods

Reagents

Tissue-culture media and reagents were obtained from Irvine Scientific, Inc. Fetal bovine serum was obtained from Hyclone. PAPC was obtained from Avanti Polar Lipids, Inc, and oxidized as described
POVPC was prepared as described previously. LPS from *Escherichia coli* O111:B4 was obtained from List Biological Laboratories, Inc. IL-1β and tumor necrosis factor (TNF)-α were obtained from R&D Systems. The TLR2 ligand, a 19-kDa *Mycobacterium tuberculosis* lipoprotein, was obtained from Dr Robert L. Modlin (UCLA). ELISA kits were purchased from R&D Systems (human IL-8 and monocyte chemotactic protein [MCP]-1) and Pharmingen (MCP-1/GE). Anti–caveolin-1 monoclonal antibodies were obtained from BD Transduction Laboratories. Anti-TLR4 monoclonal antibodies were obtained from Dr Suganya Viriyakosol (University of California at San Diego). Peroxidase-conjugated goat anti-mouse antibodies and fluorescein-conjugated donkey anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc.

**Cell Culture and Transfection**

Human (HAECs) and murine (MAECs) aortic endothelial cells and blood monocytes were isolated and cultured as previously described. Bovine aortic endothelial cells (BAECs) were obtained from VEC Technologies, Inc. For overexpression of caveolin-1, BAECs were transfected with 0.5 μg/well of the pcI-neo vector (Promega) containing the human caveolin-1 cDNA or vector alone with the use of Effectene (Qiagen). Immunofluorescence was performed to confirm the overexpression of caveolin-1.

**Biotinylated LPS Binding Assay**

LPS was biotinylated with the use of EZ-Link biotin-LC-hydrazide (Pierce). Binding reactions were performed on HAECs for 1 hour at room temperature in medium 199 containing 1% fetal bovine serum and HEPES. Bound biotin-LPS was detected with streptavidin peroxidase and o-phenylenediamine.

**Cell Fractionation and Determination of Caveolin-1, TLR4, and MD-2 in Membrane Fractions**

For analysis of caveolin-1 distribution, cells were lysed and cell membranes were fractionated with a sucrose gradient as described, except that the postnuclear supernatants were incubated in 1% Brij-58 for 1 hour at 4°C. For measurement of caveolar membrane TLR4 and MD-2, caveolar membranes were isolated as described previously. One microgram of each caveolar fraction was loaded onto nitrocellulose membranes by use of a slot blot apparatus. Bands were visualized with an enhanced chemiluminescence kit. Scans were quantified with a Molecular Dynamics densitometer to determine the relative band intensity in each fraction.

**Immunofluorescence**

BAECs grown on glass coverslips were treated with test additives and processed as described previously, except that the coverslips were incubated overnight at 4°C in primary antibody (4 μg/mL anti–caveolin-1 or 20 μg/mL anti-TLR4). Coverslips were examined with a Fluoview laser scanning confocal microscope (Olympus America). Images were generated and analyzed with the use of the Fluoview image analysis software (Olympus America).

**Statistics**

The data were analyzed with StatView software (Abacus Concepts) and are presented as mean±SD. The data are representative of at least 2 experiments that produced similar results.

**Results**

Ox-PAPC and Component Phospholipids Inhibit LPS-Induced Chemokine Synthesis

Previous studies documented the effect of ox-PAPC on E-selectin expression. To determine whether the inhibitory effect was more general, we examined the effect on chemokine synthesis. Coincubation of HAECs with LPS and ox-

**Figure 1.** Oxidized phospholipid inhibition of LPS-induced chemokine synthesis. A, HAECs were treated with medium (C), 30 μg/mL ox-PAPC (Ox), 4 ng/mL LPS (L), or 30 μg/mL ox-PAPC and 4 ng/mL LPS (Ox+L) for 4 hours. B and C, HAECs were treated with medium (C), 2 ng/mL LPS (L), or 2 ng/mL LPS and various concentrations of either POVPC or the m/z 664.4 component. *P<0.05 compared with LPS alone as calculated by 1-way ANOVA.

PAPC inhibited LPS induction of IL-8 and MCP-1 synthesis (Figure 1A); ox-PAPC treatment alone induced IL-8 and MCP-1 synthesis, as previously demonstrated. By fractionating ox-PAPC and testing purified lipids, we identified POVPC and the m/z 664.4 as the most potent inhibitory phospholipids in ox-PAPC, being active at concentrations in the nanomolar range (Figure 1B and 1C). We have previously shown that POVPC at 10 μg/mL caused a 40% increase in IL-8 and MCP-1. The m/z 664.4 component induced a similar increase (data not shown). Derivatization and tandem mass spectrometric analysis suggest that the m/z 664.4 component is a recently identified oxidative fragmentation product of PAPC, namely, 5-keto-6-octendioic acid ester of...
interferes with parts of the signaling pathway used by both TLR4 and IL-1R. We conclude that ox-PAPC is acting upstream of MyD88 and is interfering with the activation of the LPS receptor complex (consisting of CD14, TLR4, and MD-2) itself.

**Ox-PAPC Inhibits the Effect of TLR2 Ligands**

We also sought to determine whether ox-PAPC could inhibit the effect of Gram-positive bacterial products that act on TLR2. HAECs do not express detectable levels of TLR2 mRNA (data not shown). Therefore, monocyte-derived macrophages were used for these studies. Exposure to LPS or a 19-kDa *M tuberculosis* lipoprotein, a known TLR2 ligand, caused a 5- to 7-fold increase in IL-8 production (Figure 2C). As in HAECs, coinubcation of ox-PAPC and LPS resulted in a 75% decrease in IL-8 production compared with LPS alone. Coinubcation of ox-PAPC and the TLR2 ligand resulted in a 60% decrease in IL-8 production compared with lipoprotein alone, indicating that ox-PAPC can inhibit the effects of both TLR4 and TLR2 ligands. Similar to the effect in HAECs, ox-PAPC augmented IL-1β- and TNF-α-induced IL-8 responses in macrophages.

**Ox-PAPC Inhibits LPS Action at the Cellular Level**

We next addressed the question of whether ox-PAPC inhibition occurred cellulary or extracellularly. We determined that HAECs express CD14 (data not shown). Pretreatment of cells with antibody to CD14 inhibited LPS-induced IL-8 synthesis by 70%, whereas pretreatment of medium with antibody to CD14 was much less effective (data not shown). Thus, cellular CD14 accounts for the majority of LPS action in this cell type under the conditions used in our studies. Because lipoproteins have previously been shown to sequester LPS, thereby reducing their activity, we sought to determine whether ox-PAPC inhibits LPS action by sequestration of LPS itself or soluble components necessary for LPS action in this cell type. We determined that ox-PAPC does not block binding of biotinylated LPS to HAECs and actually stimulates binding (optical density at 450 nm: background (no ox-PAPC) 0.137; 0.005; LPS, 0.469; LPS plus ox-PAPC, 0.786; 0.005; representative of 7 experiments). To further address the possible extracellular effect of ox-PAPC, HAECs were pretreated with ox-PAPC for 30 minutes, rinsed, and then treated with LPS. In these experiments, pretreatment was as inhibitory as cotreatment (Figure 3A). The inhibition after pretreatment with ox-PAPC could not be explained by residual ox-PAPC present in the rinse medium, because the rinse medium from the ox-PAPC-pretreated cells did not inhibit LPS action. Pretreatment of macrophages with ox-PAPC was also inhibitory (Figure 3B). From these experiments, we conclude that in HAECs and macrophages, ox-PAPC is most likely inhibiting at the level of the membrane components of the LPS receptor complex.

**Ox-PAPC Alters Caveolar Processing and Inhibits LPS-Induced Redistribution of TLR4 and MD-2**

It has previously been demonstrated that LPS action in macrophages requires recruitment of TLR4 and MD-2 to lipid
rafts,21 where CD14 has been shown to reside.22 We hypothesized that in ECs, LPS signaling might require recruitment of TLR4 and MD-2 to caveolae, which have many similarities to lipid rafts. ECs in vivo display large numbers of caveolae, areas of the cell membrane enriched in cholesterol, sphingomyelin, and caveolin-1, although lower levels are seen in cell culture. Previous studies had demonstrated that ox-LDL disrupts caveolae, thereby displacing endothelial nitric oxide synthase.14 We hypothesized that ox-PAPC might also alter caveolae, thereby inhibiting LPS action. Western blotting of HAEC fractions demonstrated that treatment with LPS caused a shift in caveolin-1 distribution into fraction 1 and resulted in a slightly higher percentage of caveolin-1 in the most buoyant caveolar fractions (1 through 5) compared with control (Figure 4A). Ox-PAPC treatment caused a redistribution of caveolin-1 from the most buoyant caveolar fraction to internal membrane fractions, even in the presence of LPS. There was no change in total levels of caveolin-1. We also examined the distribution of TLR4 and MD-2 in HAECs by slot blot analysis of caveolar membrane fractions from a well-characterized, nondetergent method.14 In 3 separate experiments, we demonstrated that treatment of ECs with LPS also caused an increase in TLR4 and MD-2 in the caveolar membrane fraction (Figure 4B), which was blocked by ox-PAPC. There was no change in the total levels of TLR4 or MD-2 in cell lysates.

Western blotting of BAEC fractions also demonstrated that treatment with LPS caused a shift in caveolin-1 distribution into fraction 1 and resulted in a slightly higher percentage of caveolin-1 in the most buoyant caveolar fractions (1 through 5) compared with control (Figure 4A). Ox-PAPC treatment caused a redistribution of caveolin-1 from the most buoyant caveolar fraction to internal membrane fractions, even in the presence of LPS. PAPC did not cause this effect (data not shown).

Figure 3. Effect of ox-PAPC pretreatment on LPS- and lipoprotein-induced IL-8 synthesis. A, HAECs were treated with medium (C), 40 μg/mL ox-PAPC (Ox), 2 ng/mL LPS (L), or 40 μg/mL ox-PAPC and 2 ng/mL LPS (Ox+L) for 4 hours or pre-treated with medium or 40 μg/mL ox-PAPC for 30 minutes, rinsed with medium, and then incubated with LPS for 4 hours (c,r→L or Ox,r→L). The rinse medium was also used for a 30-minute pretreatment followed by incubation with LPS for 4 hours (c-rinse→L or Ox-rinse→L). *P<0.05 compared with LPS alone as calculated by 1-way ANOVA. B, Monocyte-derived macrophages were treated with medium (C), 40 μg/mL ox-PAPC (Ox), 5 μg/mL 19-kDa M. tuberculosis lipoprotein (T2), or 40 μg/mL ox-PAPC and 5 μg/mL 19-kDa M. tuberculosis lipoprotein (Ox+T2) for 4 hours or pre-treated with 40 μg/mL ox-PAPC for 30 minutes, rinsed with medium, and then incubated with 19-kDa M. tuberculosis lipoprotein for 4 hours (Ox,r→T2). *P<0.05 compared with 19-kDa M. tuberculosis lipoprotein alone as calculated by 1-way ANOVA.

Figure 4. Caveolin-1, TLR4, and MD-2 distribution in HAECs. A, Western blots from detergent sucrose gradient fractions isolated from HAECs treated with medium alone (C), 50 μg/mL ox-PAPC (Ox), 4 ng/mL LPS (L), or 50 μg/mL ox-PAPC and 4 ng/mL LPS (Ox+L) probed with anti–caveolin-1 antibodies. Densitometry values were plotted as percentage of the total density for each treatment group. B, Slot blots of 1 μg protein from the caveolar membrane fractions from HAECs treated with medium alone (C), 2 ng/mL LPS (L), or 50 μg/mL ox-PAPC and 2 ng/mL LPS (Ox+L) for 4 hours were probed with anti-TLR4 and anti–MD-2 antibodies. Slot blots shown are representative of 3 experiments.
Figure 5. Caveolin-1 and TLR4 distribution in BAECs and effect of caveolin-1 overexpression. A, Western blots from detergent sucrose gradient fractions isolated from BAECs treated with medium alone (C), 50 μg/mL ox-PAPC (Ox), or 50 μg/mL ox-PAPC and 4 ng/mL LPS (Ox+L) probed with anti-caveolin-1 antibodies. Densitometry values were plotted as percentage of the total density for each treatment group. Blots analyzed were within the linear range; blots shown were overexposed for increased contrast. B, 1 to 3, Representative immunofluorescence (objective lens, ×60) of BAECs treated with medium alone (C), 2 ng/mL LPS (L), or 50 μg/mL ox-PAPC and 2 ng/mL LPS (Ox+L) for 4 hours stained with anti-caveolin-1 antibody. B, 4 to 6, Representative immunofluorescence (objective lens, ×60) of BAECs treated as above but stained with anti-TLR4 antibody. No staining was observed with secondary antibody alone. C, BAECs were transfected with caveolin-1 cDNA or empty vector and treated with medium alone (control) or 1 ng/mL LPS for 18 hours. *P<0.05 compared with pcl-neo as calculated by 1-way ANOVA.

Discussion

The current studies demonstrate that in both ECs and macrophages, ox-PAPC inhibits the ability of bacterial lipids that target TLR4 and TLR2 to increase synthesis of chemokines. Furthermore, we identify KOdiA-PC and PPOPC as the major, specific phospholipids in ox-PAPC responsible for this inhibition.

Our studies addressed the mechanism of this inhibition and concluded that the inhibition of LPS action by ox-PAPC in these cell types was mediated by effects at the cellular level. This conclusion was based on the observation that pretreatment of cells with ox-PAPC followed by exposure to LPS was as effective as cotreatment to inhibit LPS action (Figure 3). To determine the cellular target of ox-PAPC inhibition, we first examined the effect of ox-PAPC on intracellular signaling from MyD88 to NF-κB activation. Our results indicate that ox-PAPC does not inhibit the pathway from MyD88 to NF-κB activation (Figures 2B and 2C), because no effect on IL-1β action, which shares this pathway, was observed. We also examined the effect of ox-PAPC on a 19-kD M tuberculosis lipoprotein, a known TLR2 ligand.9

Ox-PAPC inhibited the induction of IL-8 by the TLR2 ligand (Figure 2C), indicating that ox-PAPC is able to inhibit the action of other bacterial lipid–containing products. Recently, Lee and coworkers24 demonstrated that polyunsaturated fatty acids can inhibit TLR4 and TLR2 ligands. Their results, similar to ours, suggest that the molecular target for inhibition is TLR itself or its associated molecules, but not the components of the downstream pathways.

We provide evidence that ox-PAPC inhibits LPS action by preventing efficient recruitment of some of the components of the LPS receptor complex to the lipid raft/caveolae. We demonstrate that ox-PAPC causes a redistribution of caveolin-1 from the cell surface to internal membranes (Figures 4A and 5A). Importantly, we show that LPS stimulates redistribution of TLR4 and MD-2 from the more dense membrane fractions to the buoyant lipid raft/caveolar fraction (Figure 4B). Using immunofluorescence, we also demon-
strate that caveolin-1 and TLR4 distribution is altered by LPS treatment (Figure 5B). This change in localization, similar to the LPS-induced co-clustering of TLR4 with CD14 observed by Pfeiffer and coworkers,\textsuperscript{25} might be important for proper signaling induced by LPS. Supporting a role for lipid raft/caveolar localization in LPS action, we demonstrate that overexpression of caveolin-1, which has been shown to induce a 4-fold enrichment of cholesterol in caveolae,\textsuperscript{26} increased LPS-induced IL-8 synthesis (Figure 5C).

Triantafillou and coworkers\textsuperscript{21} previously demonstrated that TLR4 is present in macrophage lipid rafts on LPS stimulation and that raft-disrupting drugs such as nystatin can inhibit LPS action. Thus, our studies suggest that, like nystatin, ox-PAPC disrupts lipid raft/caveolar function, which leads to the inhibition of LPS action. We present evidence that the effect of ox-PAPC is not due to a detergent effect, because levels of individual phospholipids as low as 200 nmol/L were inhibitory, the inhibitory effects were rapidly reversible, PAPC at similar concentrations had no effect, and ox-PAPC did not inhibit the action of either IL-1\beta or TNF-\alpha. This lack of ox-PAPC toxicity was previously reported.\textsuperscript{4} Importantly, the TNF-\alpha receptor has been shown to localize to lipid rafts,\textsuperscript{27} indicating that ox-PAPC does not have a general inhibitory effect on agonists that signal through lipid raft/caveolar components. Because the inhibitory effects of ox-PAPC and component lipids were seen in both ECs and macrophages, our data point to lipid rather than caveolin protein effects as being most important in the inhibition of LPS action. Ox-PAPC lipid effects might involve an alteration in the level of sphingomyelin in caveolar membranes. Previous studies have shown that sphingomyelin, a major lipid raft/caveolar component, is hydrolyzed to ceramide in response to MM-LDL and ox-PAPC,\textsuperscript{28} ceramide has been demonstrated to inhibit LPS action.\textsuperscript{29} Other mechanisms could include changes in cholesterol content, as seen with ox-LDL.\textsuperscript{14}

Evidence for an extracellular effect of ox-PAPC in human umbilical vein ECs has recently been reported by Bochkov et al.\textsuperscript{1} They demonstrated that ox-PAPC inhibited LPS induction of E-selectin in human umbilical vein ECs and inhibited LPS-induced inflammation in vivo. In addition, they demonstrated that ox-PAPC inhibited the binding of LPS to immobilized LPS binding protein (LBP) and CD14. On the basis of these data, they suggested that ox-PAPC might extracellularly sequester these components of the LPS receptor complex, thus inhibiting its action. The disparity in their findings and those in the present studies might relate to a difference in the LPS preparations and the cell types used, because HAECS contain CD14 on the cell surface, whereas human umbilical vein ECs have been reported to lack this protein with increasing numbers of culture passages.\textsuperscript{30} Nevertheless, the Bochkov studies and the present studies suggest that ox-PAPC is an important inhibitor of LPS action and that several mechanisms might be responsible for this inhibition.

In summary, past studies on E-selectin\textsuperscript{4,31} and the current studies involving IL-8 and MCP-1 demonstrate that ox-PAPC is a strong inhibitor of LPS action. We identified POVP and a lipid of \textit{m/z} 664.4 (tentatively identified as KOdiA-PC) as the major inhibitory lipids in ox-PAPC. We also demonstrate that ox-PAPC is a strong inhibitor of TLR2 ligands. We describe a novel mechanism of ox-PAPC inhibition that involves changes in lipid rafts/caveolae. Ox-PAPC induces the synthesis of chemokines, as shown in Figure 1A, and stimulates monocyte but not neutrophil interactions with ECs\textsuperscript{4} while simultaneously inhibiting the acute inflammatory effects of LPS, as demonstrated in this study and others.\textsuperscript{4} Thus, the dual actions of this phospholipid enable it to contribute to a chronic inflammatory response. Overall, the present and past observations suggest that the inhibitory effects of ox-PAPC and its derivatives on activation of TLR4 and TLR2 might play a key role in altering the cascade of innate immunity to bacterial products.

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