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Oxidized Phospholipid Inhibition of Toll-like Receptor (TLR) Signaling Is Restricted to TLR2 and TLR4

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The generation of reactive oxygen species is a central feature of inflammation that results in the oxidation of host phospholipids. Oxidized phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC), have been shown to inhibit signaling induced by bacterial lipopeptide or lipopolysaccharide (LPS), yet the mechanisms responsible for the inhibition of Toll-like receptor (TLR) signaling by OxPAPC remain incompletely understood. Here, we examined the mechanisms by which OxPAPC inhibits TLR signaling induced by diverse ligands in macrophages, smooth muscle cells, and epithelial cells. OxPAPC inhibited tumor necrosis factor-α production, iNOS degradation, p38 MAP kinase phosphorylation, and NF-κB-dependent reporter activation induced by stimulants of TLR2 and TLR4 (Pam3CSK4 and LPS) but not by stimuli of other TLRs (poly(I-C), flagellin, loxoribine, single-stranded RNA, or CpG DNA) in macrophages and HEK-293 cells transfected with respective TLRs and significantly reduced inflammatory responses in mice injected subcutaneously or intraperitoneally with Pam3CSK4. Serum proteins, including CD14 and LPS-binding protein, were identified as key targets for the specificity of TLR inhibition as supplementation with excess serum or recombinant CD14 or LBP reversed TLR2 inhibition by OxPAPC, whereas serum accessory proteins or expression of membrane CD14 potentiated signaling via TLR2 and TLR4 but not other TLRs. Binding experiments and functional assays identified MD2 as a novel additional target of OxPAPC inhibition of LPS signaling. Synthetic phospholipid oxidation products 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine inhibited TLR2 signaling from ~30 μM. Taken together, these results suggest that oxidized phospholipid-mediated inhibition of TLR signaling occurs mainly by competitive interaction with accessory proteins that interact directly with bacterial lipids to promote signaling via TLR2 or TLR4.

The generation of reactive oxygen species by phagocytes is central to the antimicrobial effectiveness of the inflammatory response. Activated polymorphonuclear cells in particular express enzymes such as NADPH oxidase and myeloperoxidase, which together generate a range of oxidants, including superoxide, hydrogen peroxide, and hypochlorous acid, each of which may be released from these cells to cause oxidative damage not only to invading microorganisms but also to host molecules in surrounding tissues (1). Phospholipids containing polyunsaturated fatty acid chains, such as the abundant phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), are particularly susceptible to oxidation by such mediators (2, 3), and products of PAPC oxidation have been shown to accumulate at sites of inflammation (3–6) and in cells treated with stimuli such as IL-1β, TNF-α, or long wave ultraviolet radiation (7–9). Oxidation of PAPC leads to the formation of a mixture of products, ranging from epoxyisoprostanes to truncated chain derivatives that are collectively termed OxPAPC, which is a widely used model for the investigation of oxidized phospholipid (OxPL) function (10–12).

To date, much research has focused on the potential of OxPLs to augment inflammatory events by mechanisms such as the up-regulation of IL-8 or promotion of monocyte binding to endothelial cells, particularly in the context of the chronic inflammatory processes of atherosclerosis (6, 13). However, OxPLs have also been shown to possess diverse anti-inflammatory properties. For example, OxPAPC was shown to induce peroxisome proliferator-activated receptor-α-dependent signaling, expression of heme oxygenase-1 and MAP kinase phosphatase-1, each of which has established antiinflammatory properties (14–16). More directly, it was shown that OxPAPC potently inhibits inflammation-related tissue damage and death caused by endotoxemia in mice (17). As a result, it has been proposed that host recognition of OxPLs may represent an endogenous feedback mechanism that serves to limit the

The abbreviations used are: PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; OxPAPC, oxidized PAPC; OxPL, oxidized phospholipid; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; LBP, LPS-binding protein; MBCD, methyl-β-cyclodextrin; HASMC, human arterial smooth muscle cell; ssRNA, single-stranded RNA; POYPC, 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine and PGPC, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine; LPS, lipopolysaccharide; TNF, tumor necrosis factor; MAPK, mitogen-activated protein (MAP) kinase; IL, interleukin; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; ANOVA, analysis of variance; Pam3CSK4, tripalmitoyl cysteineyl lipopeptide; MITT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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potential damage caused by inflammation-induced oxidants (17).

Such feedback mechanisms are now understood to play key roles in the resolution of inflammation, a process that is increasingly viewed as active and well orchestrated. Examination of resolving exudates has identified a range of lipid-based mediators of resolution, such as the lipoxins and resolvins, which each promote resolution (18, 19). However, despite recent progress in understanding the roles for enzymatically oxidized lipids in resolution, the potential role of oxidized lipids created by nonenzymatic routes, such as by reactive oxygen species-mediated peroxidation, has received comparatively little attention.

Because stimulation of Toll-like receptor (TLR) signaling has been shown to play a central role in the induction of inflammation, oxidant release, and tissue damage in many different disease models (21–23), it is notable that OxPAPC potently inhibits its ability of LPS to stimulate TLR4-dependent signaling in macrophages, dendritic cells, and endothelial cells (3, 10, 11, 17, 24, 25). The mechanisms by which OxPAPC inhibits LPS signaling are currently under debate, and numerous potential models have been put forward. For example, it has been suggested that the generation of cAMP in response to products of phospholipid oxidation could inhibit NF-κB-induced gene transcription stimulated by LPS in endothelial cells (10). More recently, it was shown that OxPLs can bind to CD14 and LPS-binding protein (LBP), both of which serve to enhance cellular sensitivity to LPS (17). Alternatively, it has been suggested that the capacity of OxPLs to deplete cellular cholesterol and thereby disrupt lipid-rafts or caveolae could also contribute to TLR inhibition, on the assumption that recruitment of TLRs to rafts may be a requirement for effective TLR-signaling (11, 26). Finally, using synthetic derivatives of OxPLs, it was shown that the production of ceramide via neutral sphingomyelinase activation may also contribute to inhibition of LPS signaling and that phospholipid oxidation products containing α,β-unsaturated carboxylic acids are among the most efficient inhibitors of LPS signaling derived from PAPC oxidation (27).

However, despite this progress, very little has been learned about the mechanisms by which OxPLs may inhibit signaling induced by other TLRs. Although it was shown that treatment of macrophages with OxPAPC reduces the secretion of IL-8 in response to the TLR2 ligand Mycobacterium tuberculosis lipoprotein (11) and that high concentrations of OxPAPC may reduce the amount of TNF-α secreted in response to the TLR9 agonist CpG DNA (24), no studies have yet addressed the mechanisms by which these or other TLRs beyond TLR4 are inhibited by OxPLs. We, therefore, aimed to establish the potential of OxPLs to inhibit signaling induced by other TLRs and to investigate the mechanisms responsible for these effects.

**EXPERIMENTAL PROCEDURES**

**Reagents**—PAPC, methyl-β-cyclodextrin (MBCD), nystatin, MTT, DMEM, RPMI, and fetal calf serum were purchased from Sigma. Escherichia coli R1 LPS was a kind gift of Professor Ian Poxtton (University of Edinburgh). TLR ligands PamC5K0, poly(I:C), Bacillus subtilis flagellin,loxoribine, ssRNA, and endotoxin-free CpG DNA were from Invivogen. Recombinant soluble CD14 was from R&D Systems. LBP purified from human plasma was obtained from Hycult Biotechnology. Synthetic 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) were obtained from Avanti Polar Lipids. Endotoxin contamination of reagents was ruled out by the use of limulus amebocyte lysate assays (Quadratech) or challenge of TLR4-transfected HEK-293 cells. OxPAPC was prepared by air oxidation of native PAPC as described previously (4). Briefly, 1 mg of PAPC in chloroform was evaporated to dryness under nitrogen in a glass tube. The lipid film was then exposed to air in darkness for 72 h before resuspension in 2 mg/ml in chloroform and stored under nitrogen at −80 °C. The extent of oxidation of PAPC was monitored by electrospray mass spectrometry and showed a similar profile to that described previously (4, 28). Aliquots of stock OxPAPC were dried under nitrogen in Eppendorf tubes immediately before use and vortexed into prewarmed medium (37 °C) for 30 s before the addition to cells.

**Cells and Media**—The human monocytic THP-1 cell-line (ECACC 88081201) was cultured in RPMI, 10% FCS. Before challenge in all experiments, THP-1 cells were treated with 100 nM phorbol 12-myristate 13-acetate for 72 h to allow differentiation to adherent macrophages. Murine HEK-293 cells (ECACC 85120602) and the transformed human arterial smooth muscle cell line HEK-293 cells (ECACC 85120602) and the transformed human arterial smooth muscle cell line HEK-293 cells (ECACC 85120602) and the transformed human arterial smooth muscle cell line HEK-293 cells (ACBRI #443, Applied Cell Biology Research Institute, Kirkland, WA) were maintained in DMEM, 10% FCS.

**Challenge of Cells with Pathogen-associated Molecular Patterns (PAMPs)**—THP-1 macrophages were challenged with medium alone (DMEM, 1% FCS) or 1–100 ng/ml PamC5K0 or 0.1–10 ng/ml LPS in the presence or absence of 30 μg/ml OxPAPC. J774A.1 macrophages were challenged with medium alone or 250–1000 ng/ml B. subtilis flagellin with or without 30 μg/ml OxPAPC. HASMC were challenged with medium alone (DMEM, 1% FCS) or 10–1000 ng/ml poly(I:C). Alternatively, cells were challenged with 10 ng/ml PamC5K0, 10 ng/ml LPS, 500 ng/ml flagellin, or 1 μg/ml poly(I:C) with or without 30 μg/ml OxPAPC. In other experiments J774A.1 cells were challenged with 1 μM loxoribine, 2.5 μg/ml ssRNA, 10 μg/ml CpG DNA with or without 30 μg/ml OxPAPC. Supernatants from challenged cells were assessed for IL-8 content by enzyme-linked immunosorbent assay (R&D DuoSet) after 18 h or TNF-α content after 4 h. For serum dependence experiments, macrophages were washed three times in serum-free medium before challenge with PAMPs at the indicated concentrations in serum-free medium or serum supplemented with 1% FCS.

**Western Blotting**—To investigate the impact of OxPAPC on intracellular signaling pathways stimulated by TLRs, THP-1 macrophages were plated in 12-well plates (5 × 10^5 cells per well) and challenged with medium alone, 250 ng/ml TNF-α (Peprotech), or 10 ng/ml LPS with or without 50 μg/ml OxPAPC co-treatment for 20 min before lysis. Alternatively, J774A.1 cells were challenged with medium alone (DMEM, 1%
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**FIGURE 1. Effect of OxPAPC on bacterial lipopeptide-, LPS-, and flagellin-induced TNF-α production.** Human THP-1 macrophages (A, B, D, and E) or murine J774.1 macrophages (C and F) were challenged with PAMPs in the presence or absence of OxPAPC, and the TNF-α content of culture supernatants was measured 4 h after challenge. Cells were challenged with the indicated concentrations of the TLR2 agonist Pam3CSK4, the TLR4 agonist E. coli LPS, or the TLR5 agonist B. subtilis flagellin in the presence or absence of 30 μg/ml OxPAPC (A–C). Alternatively, cells were challenged with 100 ng/ml Pam3CSK4, 10 ng/ml LPS, or 1 μg/ml flagellin with the indicated concentrations of OxPAPC (D–F). Results shown are means of triplicate cultures ± S.D. and are representative of at least three similar experiments. **p < 0.01 versus without OxPAPC (ANOVA).

FCS), 10 ng/ml Pam3CSK4, 10 ng/ml LPS, 1 mM loxoribine, or 10 μg/ml CpG DNA with or without 50 μg/ml OxPAPC co-treatment for 20 min before lysis. Samples were boiled with 0.1 mM dithiothreitol and separated by reducing denaturing PAGE before blotting to nitrocellulose. Blots were then probed with antibodies to IκBα (Santa Cruz), phospho-p38 MAPK (Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz) and visualized with enhanced chemiluminescence kit (Pierce).

**Reporter Assays**—For transfection assays, HEK-293 cells were plated in 96-well plates at 2 × 10⁴ cells per well and transfected after 24 h using Genejuice (Novagen) according to the manufacturer’s instructions. Amounts of construct per well were 30 ng of human TLRs 2, 4 (co-expressing MD-2), or 5 (Invivogen), 30 ng of pCD14, 20 ng of transfection efficiency control renilla luciferase-reporter construct (pRL-TK), and 10 ng of firefly luciferase-reporter construct driven by NF-κB-dependent promoter (pELAM) cloned into pGL3 (Promega) as described previously (28), with the balance made up with empty pCDNA3. Cells were grown for 2–3 days before post-transfection at an 18 h challenge with PAMPs in the presence or absence of 10–30 μg/ml OxPAPC. Reporter levels were normalized to co-transfected renilla reporter expression. Promoter expression is represented as fold induction relative to cells cultured in medium alone ± S.D. To determine requirements of membrane CD14 expression, cells were transfected with TLRs ±30 ng per well of pCD14 or pCDNA3. All constructs used other than pRL-TK were of human origin.

**Serum Lipid Shuttle Protein Experiments**—HEK-293 cells transfected with TLR2 or TLR4-MD2 and NF-κB reporter were challenged with 100 ng/ml Pam3CSK4 or 1 ng/ml LPS with or without 30 μg/ml OxPAPC in DMEM supplemented with 1, 10, or 30% serum. Alternatively HEK-293-TLR2 cells were challenged with 100 ng/ml Pam3CSK4 in the presence or absence of MD2 protein was prepared by transfection of HEK-293 cells plated in 12-well plates (2 × 10⁴ cells per well) with 1 μg per well of a construct expressing FLAG- His-tagged MD2 (pEFBOS-MD2, a kind gift of Prof. K. Miyake). MD-2-containing supernatant was collected at 48 h. For MD2 immobilization, 100 μl per well of anti-FLAG monoclonal antibody (Sigma) was added to high binding immunoassay strips (Nunc Immunomodule) before washing 3 times in phosphate-buffered saline, 0.05% Tween and adding MD-2 supernatant for 2 h. Wells were then washed, and 500 ng/ml biotinylated LPS (Invivogen) was added in the presence of 0, 10, or 25 μg/ml native or oxidized PAPC for a further 2 h to allow binding to immobilized MD2. Wells were washed once again, and bound biotinylated LPS was measured by the addition of streptavidin-horseradish peroxidase (HRP) and colorimetric determination at 450 nm after the addition of HRP substrate 3,3′,5,5′-tetramethylbenzidine. For MD2 co-precipitation experiments, MD2 supernatant was incubated with 1 μg/ml biotinylated LPS with or without 15 min preincubation with 10 μg/ml unlabeled LPS or 50 μg/ml OxPAPC. Streptavidin-agarose beads (Sigma) were then added to mixtures and rotated overnight at 4 °C. Beads were pelleted by centrifugation (13,000 × g, 10 min), resuspended in SDS-PAGE loading buffer, boiled, and separated on 15% SDS-PAGE gels before transfer to nitrocellulose blots. Precipitated MD2 was visualized by probing blots with anti-FLAG antibody (Sigma). To assess functional effects of OxPAPC effects on MD2, MD2 supernatant was preincubated with 50 μg/ml OxPAPC for 30 min before the addition of 100 ng/ml LPS. Supernatants were then added to HEK-293 cells transfected with TLR4 and reporter constructs without MD2 cotransfection. Activation of NF-κB-dependent reporter was measured at 18 h as described above.

**Animal Experiments**—8-Week-old male BALB/c mice were injected subcutaneously with 50 μl of saline, 2.5 μg of 0.5 or 2.0 μg/ml recombinant sCD14 (R&D) or 0.3 or 1.0 μg/ml LBP purified from human plasma (Hycult biotechnology). After 18 h, expression of reporter was measured by luminometry as described above.

**Membrane Disruption and Cell Viability Assays**—Membrane cholesterol disrupting agents MBCD, nystatin, or OxPAPC were added to THP-1 macrophages at 0.032–10 mM, 3.2–1000 μM, or 10–300 μg/ml, respectively. 18 h later, cellular viability was monitored by MTT assay as described previously (30). Alternatively, THP-1 macrophages were challenged with 10 ng/ml Pam3CSK4 or LPS in the presence of MBCD or nystatin at 300–1200 or 1–5 μM, respectively. Supernatant TNF-α was measured at 4 h as described above.

**MD2 Binding Assays**—Soluble
Pam3CSK4 or 2.5 μg of Pam3CSK4 with 7.5 μg of OxPAPC (n = 3 per group). Skin around each injection site was removed from killed animals after 24 h, and extent of inflammatory infiltrate was assessed by hematoxylin and eosin staining. Polymorphonuclear granulocyte (PMN) infiltration into subcutaneous tissue was quantified by counting PMNs present in low power fields of Sudan black-stained sections. Alternatively, mice (n = 4 per group) were injected intraperitoneally with 200 μl of saline, 10 μg of Pam3CSK4, or 10 μg of Pam3CSK4 with 50 μg OxPAPC. Cells were counted in peritoneal lavage fluid (3 ml of phosphate-buffered saline) after 24 h using a hemocytometer. All experiments were carried out in accordance with the United Kingdom Home Office Guide on the Operation of Animals (Scientific Procedures) Act 1986.

RESULTS

OxPAPC Inhibits Signaling via TLR2 and TLR4 but Not Other TLRs—To establish whether the ability of OxPLs to inhibit TLR4 signaling extended to other TLRs, the capacity of OxPAPC to modulate cytokine release induced by defined PAMPs specific for other TLRs was assessed. As expected, OxPAPC inhibited the production of TNF-α by THP-1 macrophages in response to challenge with the TLR2 ligand Pam3CSK4 or the TLR4 ligand E. coli LPS in a dose-dependent manner, with maximal inhibition occurring at 30 μg/ml (Fig. 1).

However, TNF-α production in response to the TLR5 ligand flagellin was found to be unaltered by OxPAPC treatment (Fig. 1). Similar results were obtained with primary human monocyte-derived macrophages (not shown). TNF-α production by J774A.1 macrophages in response to the TLR7, TLR8, and TLR9 ligands loxoribine, ssRNA, and CpG DNA, respectively, was also unaltered by OxPAPC (Fig. 2). We found that human and murine macrophages produced very little or no TNF-α in response to the TLR3 ligand poly(I:C). However, poly(I:C) potently up-regulated the production of IL-8 by human aortic smooth muscle cells, and this response was not significantly altered by co-treatment with OxPAPC (Fig. 2). OxPAPC pre-treatment of cells for 1 h before challenge also had no effect on cytokine production in response to these PAMPs (data not shown).

To examine whether the specificity of OxPAPC inhibition of TLRs could be verified using alternative readouts of TLR-induced signaling, the activation of NF-κB dependent reporter transcription was examined in HEK-293 cells transfected with vector alone, TLR2, TLR5, or TLR4-MD2. These experiments were performed using a strain of HEK-293 that was found to express low levels of TLR3 and were, therefore, responsive to poly(I:C) but which did not express transcripts for TLRs 2, 4, or 5 and were entirely insensitive to LPS, bacterial lipopeptide or flagellin in the absence of TLR transfection (Ref. 28 and data not shown). As expected, OxPAPC dose-dependently inhibited TLR2- and TLR4-dependent induction of NF-κB dependent reporter activation in this system. However, TLR3- or TLR5-dependent induction of NF-κB was not altered by OxPAPC treatment (Fig. 3). In agreement with previous findings (11, 17), native (unoxidized) PAPC did not alter cytokine secretion or NF-κB reporter expression in response to PAMPs in these experiments (data not shown).

OxPAPC Inhibition of TLR2 and TLR4 Signaling Occurs Upstream of IkBα and p38 MAPK—To identify the point within the TLR-signaling cascades that may be targeted by OxPAPC for the specificity of TLR inhibition, PAMP-induced phosphorylation of p38 MAP kinase and degradation of IkBα was examined in THP-1 and J774A.1 macrophages. LPS-induced degradation of IkBα in these cells was blocked by OxPAPC treatment, whereas TNF-α dependent IkBα degradation was unaffected by co-culture with OxPAPC, indicating...
OxPAPC Inhibits TLR2 and TLR4 via CD14, LBP, and MD2

A

B

C

D

FIGURE 3. Effects of OxPAPC on TLR-dependent NF-κB reporter activation. Late passage HEK-293 cells, which expressed low levels of TLR3 transcripts but not mRNA for TLRs 2, 4, or 5 (data not shown), were transfected with CD14, NF-κB-sensitive reporter construct (pELAM), and transfection-efficiency control construct (pRL-TK) along with additional TLR2 (A), no TLRs (B), TLR4-MD2 (C), or TLR3 (D). After 48 h transfected cells were challenged with respective ligands (100 ng/ml Pam3CSK4, 10 ng/ml poly(I:C), 10 ng/ml LPS, or 1 μg/ml flagellin) and 0–30 μg/ml OxPAPC. NF-κB reporter activation was measured at 18 h and is presented as fold induction relative to control cultures challenged in medium alone. Means of triplicate cultures independently normalized for transfection efficiency are presented ± S.D. Results shown are representative of at least three similar experiments. **, p < 0.01 versus PAMP without OxPAPC (ANOVA).

that OxPAPC does not act as a nonspecific inhibitor of IκBα degradation (Fig. 4A). Stimulation of cells with the TLR2 ligand Pam3CSK4, like LPS, induced IκBα degradation and p38 MAPK phosphorylation, which were both inhibited by OxPAPC (Fig. 4B). However, IκBα degradation and p38 MAPK phosphorylation induced by ligands of TLRs 5, 7, or 9 (flagellin, loxoribine, and CpG DNA, respectively) was unaffected by OxPAPC (Fig. 4, B and C), confirming that OxPAPC does not act as a nonspecific inhibitor of IκBα degradation or p38 MAPK phosphorylation in macrophages. This further suggests that OxPAPC inhibition of TLR2- and TLR4-dependent signaling may occur upstream of these intracellular mediators. As before, native (unoxidized) PAPC had no effect on PAMP signaling (not shown).

Signaling via TLR2 and TLR4, but Not Other TLRs, Is Serum-dependent—Because TLRs 5, 7, 8, and 9 share intracellular signaling pathways that are thought to be very similar to those of TLR2 (31), we sought alternative mechanisms for the specificity of OxPAPC-mediated TLR inhibition. Because it has been shown that both LPS- and lipopeptide-induced cellular signaling is potentiated by serum-derived lipid-shuttle proteins (32), we sought to determine whether signaling via other TLRs may also require serum-derived accessory molecules. Although production of TNF-α by J774A.1 macrophages in response to LPS and Pam3CSK4 treatment was serum-dependent, TNF-α production in response to flagellin, loxoribine, ssRNA, and CpG DNA was found to be unaltered by the presence or absence of serum supplementation (Fig. 5 and data not shown). HASMC production of IL-8 in response to TLR3 stimulation with poly(I:C) was also unimpaired in the absence of serum (Fig. 5).

Signaling via TLR2 and TLR4, but Not TLR3 or TLR5, Is CD14-dependent—Signaling via TLR2 and TLR4 has been shown to be potentiated by the presence of CD14, which either in the soluble form (sCD14) or in a glycosylphosphatidylinositol-anchored membrane-bound form (mCD14), serves to catalyze the transfer of lipophilic PAMPs from bacterial membranes or LBP to the TLR2 or TLR4-MD2 receptor complexes (32, 33). To determine whether CD14 may also be required for signaling via other TLRs, stimulation of an NF-κB-dependent reporter was examined in the CD14-deficient HEK-293 cell line transfected with TLRs with or without a construct expressing membrane CD14. When challenged with PAMPs in the absence of serum (and, hence, any exogenous source of sCD14), co-expression of mCD14 markedly enhanced cellular sensitivity to LPS and Pam3CSK4, whereas it did not alter cellular sensitivity to the TLR3 and TLR5 ligands poly(I:C) or flagellin, respectively (Fig. 6).

OxPAPC Inhibition of TLR2 Signaling Is Dependent on Serum Components Including CD14 and LBP—In the presence of 1% serum, OxPAPC was found to be a potent inhibitor of TLR2- and TLR4-dependent signaling in transfected HEK-293 cells (Fig. 7). However, OxPAPC-mediated inhibition of TLR2 signaling was reversed completely by supplementation with 30% serum (Fig. 7A). Because OxPAPC has been shown to bind to CD14 (17) and sCD14 has been shown to play a role in lipopeptide signaling (32, 34, 35), we examined whether supplementation of the culture medium with recombinant sCD14 alone may also reverse OxPAPC-induced inhibition of TLR2 signaling. Consistent with a role for this protein, OxPAPC inhibition of Pam3CSK4 signaling was reversed by sCD14 supplementation in a dose-dependent fashion (Fig. 7C). Likewise, supplementation with exogenous human LBP also dose-dependently reversed OxPAPC inhibition of TLR2-dependent signaling (Fig. 7D), suggesting that both CD14 and LBP may be targets of OxPAPC inhibition of TLR2 signaling. Surprisingly, however, OxPAPC-mediated inhibition of LPS-induced signaling could not be reversed either by increased serum concentration or by supplementation with recombinant sCD14 or human LBP (Fig. 7B and data not shown).

Effects of Membrane-disrupting Agents on TLR Signaling—It has been well established that after binding of LPS to MD2, the stimulated TLR4-MD2 complex co-localizes to lipid-raft membrane fractions (26, 36). This observation has led to the suggestion that TLR localization to lipid-rafts or raft-like caveolae may be a requirement for effective LPS signaling and that
OxPAPC Inhibits TLR2 and TLR4 via CD14, LBP, and MD2

OxPAPC-mediated disruption of lipid-rafts may explain some of the ability of OxPAPC to inhibit TLR signaling (11, 27). We, therefore, aimed to examine further the potential of raft-disrupting agents to inhibit TLR-dependent signaling. MTT assays revealed that the two well established raft-disrupting agents, nystatin and MBCD, induced a dose-dependent reduction in cellular viability that was significant at concentrations higher than around 10 μM for nystatin or 1 mM for MBCD (Fig. 8A). OxPAPC had no effect on macrophage viability at concentrations up to 50 μg/ml, whereas 300 μg/ml OxPAPC induced a marked loss of cellular viability (Fig. 8B). When used at sublethal concentrations, neither nystatin nor MBCD inhibited TLR2- or TLR4-dependent induction of TNF-α production in THP-1 macrophages (Fig. 8, C and D). Inhibitors of established OxPAPC-induced signaling pathways that could mediate anti-inflammatory effects in cells, including RpCAMPs, H-89, MK886, and sodium orthovanadate, also failed to reverse OxPAPC-mediated inhibition of LPS signaling in macrophages (data not shown).

Effects of OxPAPC on LPS Binding to MD2—Because OxPAPC-mediated inhibition of LPS signaling could not be reversed by serum, CD14, or LBP and OxPAPC pretreated and washed cells have been shown to remain unresponsive to LPS (11), we next examined the possibility that a novel cellular target of OxPAPC may exist that is a component of the LPS-signaling cascade but not of the TLR2-signaling cascade. One such candidate is MD2, as the transfer of LPS monomers to MD2 is essential for the induction of cellular signaling by the TLR4-MD2 receptor complex, whereas it is not required for TLR2-dependent signaling (33). Furthermore, a hydrophobic cavity has recently been identified in a proposed structure of MD2 that is similar to that of CD14 and LBP (37), both of which have been shown to bind OxPAPC (17). For this reason we examined the potential of OxPAPC to competitively inhibit LPS binding to MD2. In co-precipitation assays of supernatants from HEK-293 cells expressing FLAG-tagged MD2 protein, biotinylated LPS efficiently recovered MD2 from the medium, whereas this was reduced by competitive inhibition with unlabeled LPS or 50 μg/ml OxPAPC (Fig. 9, A and B). Next, in immunoassays of mammalian cell-expressed recombinant MD2 bound to enzyme-linked immunosorbent assay plates, OxPAPC, but not native PAPC, was found to inhibit the binding of biotinylated LPS to MD2 in a dose-dependent fashion (Fig. 9C). Finally, it has been established that cells expressing TLR4 in the absence of MD2 are insensitive to LPS and that LPS sensitivity can be restored by supplementation with soluble MD2 before LPS challenge or with preformed LPS-MD2 complexes (38). As expected, HEK-293 cells expressing TLR4 in the absence of MD2

FIGURE 4. Effects of OxPAPC on intracellular signaling intermediates. A, THP-1 macrophages were challenged with medium alone (Ctrl), 250 ng/ml TNF-α, or 10 ng/ml LPS with or without 50 μg/ml OxPAPC co-treatment for 20 min before lysis. IκBα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content of lysates were measured by Western blot. B, alternatively, J774A.1 cells were challenged with medium alone (DMEM, 1% FCS), 10 ng/ml Pam3CSK4, 10 ng/ml LPS, or 1 μg/ml flagellin in the presence or absence of 50 μg/ml OxPAPC before measurement of IκBα degradation and p38 MAPK phosphorylation at 20 min. C, J774A.1 cells were challenged with 1 mM loxoribine (Lox) or 10 μg/ml CpG DNA with or without 50 μg/ml OxPAPC co-treatment for 20 min before lysis. Results shown are representative of at least three experiments.

FIGURE 5. Effect of serum on cytokine induction by ligands of TLRs 2, 3, 4, and 5. A and C, washed THP-1 macrophages were challenged with the indicated concentrations of Pam3CSK4 or LPS in the presence or absence of 1% serum. Supernatant TNF-α was measured at 4 h. B, HASMC production of IL-8 in response to poly(I: C) with or without serum measured at 18 h. D, J774A.1 macrophages were challenged with flagellin with or without serum, and TNF-α was measured at 4 h. Results shown are the means of triplicate cultures ± S.D. and are representative of at least three similar experiments. **, p < 0.01 versus serum (ANOVA).
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FIGURE 6. Effect of mCD14 expression on cellular sensitivity to ligands of TLR 2, 3, 4, and 5. HEK-293 cells were transfected with NF-κB-sensitive reporter construct (pELAM), transfection-efficiency control construct (pRL-TK), and TLR2 (A), no TLRs (B), TLR4-MD2 (C), or TLR5 (D) with or without co-transfection with membrane CD14. After 48 h transfected cells were challenged with indicated concentrations of Pam3CSK4, poly(I:C), LPS, or flagellin. NF-κB-dependent reporter activation was measured at 18 h and normalized to internal transfection efficiency control and is presented as -fold induction relative to control cultures challenged in medium alone. Means of triplicate cultures are presented ± S.D. and are representative of at least three similar experiments. **, p < 0.01 versus CD14 (ANOVA).

FIGURE 7. Effects of serum, LBP, and sCD14 on OxPAPC inhibition of TLR signaling. HEK-293 cells were transfected with NF-κB-sensitive reporter construct (pELAM), CD14, transfection-efficiency control construct (pRL-TK), and TLR2 (A, C, and D), or TLR4-MD2 (B), A and B, transfected cells were challenged with 100 ng/ml Pam3CSK4 or 1 ng/ml LPS with or without 30 μg/ml OxPAPC in DMEM supplemented with 1.10, or 30% serum. Alternatively, TLR2 transfectants were challenged with 100 ng/ml Pam3CSK4 and 30 μg/ml OxPAPC in 1% serum in the presence or absence of 0.5 or 2.0 μg/ml recombinant sCD14 (C) or 0.3 or 1.0 μg/ml LBP purified from human plasma (D). NF-κB-dependent reporter activation was measured at 18 h, normalized to internal transfection efficiency control, and is presented as -fold induction relative to control cultures challenged in medium alone. Means of triplicate cultures are presented ± S.D. and are representative of at least three similar experiments. **, p < 0.01 versus PAMP without OxPAPC (ANOVA).

co-transfection were insensitive to LPS but responded with increased NF-κB reporter activation in response to challenge with pre-assembled LPS-MD2 complexes. However, when complexes were prepared in the presence of both co-treatment (Fig 11D). In separate experiments the numbers of cells present in peritoneal lavage fluid from mice that were injected intraperitoneally with saline, Pam3CSK4, or Pam3CSK4 with OxPAPC were counted after 24 h. Pam3CSK4 treatment

OxPAPC and LPS, increased reporter activation was not observed (Fig. 9D).

Effects of Specific Phospholipid Oxidation Products on TLR2 Signaling—Because OxPAPC is a mixture of phospholipid oxidation products, we aimed to establish which products of PAPC oxidation may contribute to the inhibition of TLR2 signaling. Unoxidized PAPC, dipalmitoyl phosphatidylcholine, arachidonic acid, and lysophosphatidyl choline did not inhibit TLR2 signaling in transfected HEK-293 cells (Fig. 10A). Electrospray mass spectrometry of OxPAPC revealed a similar pattern of oxidation products to that described previously (4, 28), including products such as POVPc (m/z 594.4) and PGPC (m/z 610.4), which have been characterized previously (Fig. 10B) (4). POVPc and PGPC prepared by total organic synthesis significantly inhibited Pam3CSK4-induced TLR2 signaling, and LPS induced TLR4 signaling at concentrations ≥20 μg/ml (~30 μM, Fig. 10, C and D).

Effects of OxPAPC on TLR2-dependent Inflammation in Vivo—To confirm that the effects of OxPAPC on TLR2-dependent inflammation observed in vitro were relevant in vivo, two murine models of TLR2-dependent inflammation were examined. BALB/c mice were injected subcutaneously with 50 μl of saline, Pam3CSK4, or Pam3CSK4 with OxPAPC, and skin around each injection site was removed after 24 h for assessment of extent of inflammation. A dense inflammatory infiltrate was observed, particularly around the subcutaneous adipose tissue, in Pam3CSK4-injected animals that was reduced with OxPAPC co-treatment and absent in saline-injected skin samples (Fig 11, A–C). Quantification of polymorphonuclear granulocyte (PMN) influx into subcutaneous adipose tissues revealed a significant reduction in PMN influx with OxPAPC...
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Increased the number of cells present in peritoneal lavage fluid, and this increase was significantly reduced by OxPAPC co-treatment (Fig 11E).

DISCUSSION

The oxidation of host phospholipids by activated phagocytes is a common consequence of inflammatory events (3–6). Although many studies have focused on the potential of OxPLs generated by these and other processes to potentiate further pro-inflammatory mechanisms, such as the induction of IL-8 secretion or increased adherence of monocytes to endothelial cells (4, 8), more recent studies have identified numerous anti-inflammatory and protective pathways triggered by OxPLs (14–17). Together, these findings have led to the proposal that OxPLs may act as endogenously generated negative regulators of inflammatory signaling, particularly via their potent inhibition of LPS signaling (17). However, although the mechanisms by which OxPLs inhibit LPS-induced TLR4 signaling have been well studied, much less is known of the roles of OxPLs in the regulation of signaling initiated by other TLRs. In the absence of such studies and because most of the TLRs share very similar intracellular signaling pathways, it has been assumed by many researchers that OxPLs are likely to be inhibitors of all TLRs (24, 39).

Contrary to this expectation, however, we found that in a range of cell types and using a variety of markers of inflammatory signaling, OxPAPC inhibited only TLR2- and TLR4-MD2-dependent signaling but not signaling initiated by other TLRs. We note that it has been suggested previously that OxPAPC may additionally inhibit CpG-DNA-induced 1lxβ phosphorylation and TNF-α production from murine macrophages via TLR9 (24). However, we found that the high concentrations of OxPAPC used in this earlier study (100–300 µg/ml) caused a significant loss of viability in THP-1 macrophages (Fig 8), sug-

FIGURE 8. Effect of membrane cholesterol-disrupting agents on TLR signaling and cellular viability. THP-1 macrophages were exposed to indicated concentrations of methyl-β-cyclodextrin, nystatin, or OxPAPC for 18 h before assessment of cellular viability by MTT assay (A and B). Alternatively, THP-1 macrophages were challenged with medium, 10 ng/ml Pam3CSK4, or 10 ng/ml LPS (D) in the presence or absence of indicated concentrations of membrane cholesterol-disrupting agents methyl-β-cyclodextrin or nystatin. Supernatant TNF-α was measured at 4 h. Means of triplicate cultures are presented ± S.D. and are representative of at least three similar experiments. **, p < 0.01 versus untreated cells (ANOVA).

FIGURE 9. Effect of OxPAPC on binding of LPS to MD2. A, supernatant of cells transfected with MD2-FLAG was exposed to biotinylated LPS (B-LPS) with or without 15 min preincubation with unlabeled LPS or 50 µg/ml OxPAPC. LPS-MD2 complexes were precipitated with streptavidin-agarose beads, resuspended in SDS-PAGE loading buffer, and separated on 15% SDS-PAGE gels before transfer to nitrocellulose blots. Precipitated MD2 was visualized with anti-FLAG antibody. B, mean intensity of precipitated MD2 bands was determined by densitometry from three experiments ± S.D., p < 0.01 (**) and p < 0.001 (***)) versus control (Ctrl) (ANOVA). C, recombinant human MD2-FLAG was bound to immobilized anti-FLAG antibody, blocked with bovine serum albumin, and washed. Biotinylated LPS was then allowed to bind to bound MD2 in the presence or absence of the indicated concentrations of OxPAPC or native (unoxidized) PAPC. Relative capture of biotinylated LPS was quantified via binding of streptavidin-horseradish peroxidase binding to wells as measured by absorbance at 450 nm after the addition of 3,3’,5,5’-tetramethylbenzidine. **, p < 0.01 versus native PAPC (nPAPC)-treated (ANOVA). D, HEK-293 cells expressing NF-κB reporter and TLR4 without MD2 were exposed to medium (Ctrl), LPS, MD2 supernatant, MD2 supernatant exposed to 50 µg/ml OxPAPC for 30 min, or MD2 supernatant exposed to 50 µg/ml OxPAPC for 30 min before the addition of LPS for a further 30 min. Activation of reporter was measured at 18 h as described above and analyzed by ANOVA. Results shown are representative of at least three similar experiments.
gesting that the effects observed by this group may be related to nonspecific effects of OxPAPC toxicity at high concentrations rather than direct inhibition of TLR9 signaling.

The present study also confirms and extends the observations made by other groups that intracellular signaling intermediates downstream from TLR4 or receptors sharing similar signaling pathways are not obviously inhibited by OxPAPC (11, 17, 24). For example, the observation that p38 MAPK phosphorylation or IκBα degradation induced by ligands of TLRs 5, 7, 8, or 9 is unaffected by OxPAPC (Fig. 4) suggests that OxPAPC does not mediate anti-inflammatory effects via modulation of these mediators. Nevertheless, it should be noted that in certain cell types, such as dendritic cells, OxPAPC may exert immunosuppressive effects independently of its effects on direct inhibition of TLR-signaling pathways (25).

It has been suggested that the mechanism by which OxPAPC inhibits TLR signaling could involve the disruption of lipid rafts or raft-like caveolae in endothelial cells (11). This follows the well established observation that stimulation of TLR4-MD2 by LPS leads to the rapid recruitment of TLR4-MD2-LPS complexes to the lipid raft fraction of cell membranes (36, 40), and the resulting suggestion that recruitment to rafts may be a requirement for, rather than merely a consequence of ligand-induced TLR signaling (26, 36). A key contributor to the evidence supporting this hypothesis is the demonstration that raft-disrupting agents nystatin and MBCD-blunted TLR2- or TLR4-dependent signaling in macrophages and Chinese hamster ovary cells (26, 36). However, we found that the concentrations of raft-disrupting agents that were used in these studies (80 μM nystatin or 10 mM MBCD (26, 36)) induce a considerable degree of toxicity in THP-1 macrophages (Fig. 8). Using sublethal concentrations of these agents, we saw no evidence of inhibition of TLR2 or TLR4 signaling, whereas previous studies showed that the use of MBCD at similar concentrations (0.5–1.25 mM) led to efficient lipid raft disruption (41, 42). Thus, further studies will be required to clarify whether recruitment of TLRs to rafts is merely a consequence or a requirement of ligand-induced TLR-signaling. Likewise, further studies may be
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Effects of OxPAPC on TLR2-dependent inflammation in vivo. BALB/c mice were injected subcutaneously with 50 μl of saline (A), 2.5 μg of Pam3CSK4 (B), or 2.5 μg of Pam3CSK4 with 7.5 μg OxPAPC (C). The skin around each injection site was removed after 24 h, and extent of inflammatory infiltrate was assessed by H&E staining (n = 3 per group). The arrow indicates dense inflammatory infiltrate around subcutaneous adipose tissue in Pam3CSK4-treated animals. D, polymorphonuclear granulocyte influx into subcutaneous adipose tissue was counted in eight low power fields (mean ± S.D. shown). E, in separate experiments, BALB/c mice were injected intraperitoneally with 200 μl of saline, 10 μg of Pam3CSK4 (Pam3), or 10 μg of Pam3CSK4 with 50 μg of OxPAPC (n = 4 per group). Cells were counted in peritoneal lavage fluid after 24 h.

OxPAPC in our binding experiments (Fig. 9, A–C), this could reflect the nonphysiological origin and setting of the recombinant MD2 used in these experiments (i.e. in the absence of binding partner TLR4), as functional studies with MD2 in the presence of cell-expressed TLR4 showed a clearer inhibitory effect (Fig. 9D). Such a mechanism is not without precedent, as established inhibitors of LPS signaling, such as penta-acyl lipid A, Porphyromonas gingivalis LPS and the drug Eritoran have each been shown to mediate their inhibition of LPS signaling via competitive interaction with MD2 (37, 48–50). Thus, the model we propose is that the mechanisms by which OxPLs inhibit TLR signaling are similar to those of lipid A-related antagonists of TLR4, such that the main targets for competitive inhibition are CD14, LBP, and MD2. Because these molecules are not required for signaling by TLRs other than TLR2 and TLR4, this provides an explanation for the specificity of OxPL-mediated inhibition of TLRs.

Our efforts to identify specific phospholipid oxidation products present in OxPAPC that may contribute to TLR2 inhibition established that two prominent lipids present in OxPAPC, POVPC and PGPC, are each capable of inhibiting TLR2- and TLR4-dependent signaling at concentrations ≥20 μg/ml (~30 μM). Interestingly, other workers showed previously using electrospray ionization-mass spectrometry methods that POVPC and PGPC can reach concentrations of 40–60 μg/g of tissue during inflammation in rabbits (4). Thus, it is possible that the specific components of OxPAPC that we have identified to possess TLR inhibitory potential may reach concentrations sufficient to inhibit TLR2 and TLR4 function in vivo. Accordingly, we confirmed that OxPAPC inhibits TLR2-dependent inflammatory responses in vivo (Fig. 11) in a manner similar to that seen with LPS challenge of mice (17).

The relevance of the specificity of OxPAPC inhibition for only TLR2 and TLR4 remains to be established. Notably, ligands of TLR2 and TLR4 are normally encountered during the course of bacterial or fungal infections, whereas viral infections are typically thought to involve stimulation of other TLRs, such as TLRs 3, 7, or 8 (51). Thus, it is tempting to speculate that whereas OxPAPC may act as an efficient negative feedback regulator of inflammation during bacterial or fungal infections, this regulatory mechanism may not equally apply to viral infections. Accordingly, mice deficient in functional NADPH oxidase, a key contributor to reactive oxygen species formation by phagocytes and potentially therefore inflammation-induced OxPL formation, show exaggerated and temporally extended inflammatory responses to challenge with the TLR2 stimulant...
heat-killed Aspergillus fumigatus (52). Zymosan, also a TLR2 ligand, is widely used to promote the development of arthritis in mice, and it was shown that deficiency in NADPH oxidase also leads to excessive tissue damage in a murine model of zymosan-induced arthritis (53). Together, these findings suggest that phagocyte-derived oxidants may play a role in the negative feedback or resolution of inflammation that is distinct from their role in killing microbes. Indeed, several of the properties of OxPAPC, such as the nonphlogistic recruitment of monocytes, inhibition of TLR signaling, and the induction of heme oxygenase-1 expression are all consistent with a role in the promotion of resolution. The possibility that OxPLs may serve as mediators in this process is further supported by the recent identification of OxPLs as mediators of the anti-inflammatory properties of long wave ultraviolet radiation, which is used to treat inflammatory disorders of the skin (7).

In summary, the present studies establish that, contrary to expectation, OxPAPC is an inhibitor of only TLR2- and TLR4-dependent signaling, that this is mediated largely via interaction with accessory molecules including CD14, LBP, and MD2, and that this inhibition does not extend to other TLRs. Further examination of the anti-inflammatory properties and mechanisms of OxPLs could provide useful information for the development of novel therapeutic approaches to the treatment or prevention of TLR-mediated inflammatory diseases.

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