A role for neutral sphingomyelinase activation in the inhibition of LPS action by phospholipid oxidation products

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Abstract **Previous studies from our laboratory and others presented evidence that oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (OxPAPC) and oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylethanolamine can inhibit lipopolysaccharide (LPS)-mediated induction of interleukin-8 (IL-8) in endothelial cells. Using synthetic derivatives of phosphatidylethanolamine, we now demonstrate that phospholipid oxidation products containing α,β-unsaturated carboxylic acids are the most active inhibitors we examined. 5-Keto-6-octendioic acid ester of 2-phosphatidylcholine (KOdiA-PC) was 500-fold more inhibitory than OxPAPC, being active in the nanomolar range. Our studies in human aortic endothelial cells identify one important mechanism of the inhibitory response as involving the activation of neutral sphingomyelinase. There is evidence that Toll-like receptor-4 and other members of the LPS receptor complex must be colocalized to the caveolar/lipid raft region of the cell, where sphingomyelin is enriched, for effective LPS signaling. Previous work from our laboratory suggested that OxPAPC could disrupt this caveolar fraction. These studies present evidence that OxPAPC activates sphingomyelinase, increasing the levels of 16:0, 22:0, and 24:0 ceramide and that the neutral sphingomyelinase inhibitor GW4869 reduces the inhibitory effect of OxPAPC and KOdiA-PC. We also show that cell-permeant C6 ceramide, like OxPAPC, causes the inhibition of LPS-induced IL-8 synthesis and alters caveolin distribution similar to OxPAPC. Together, these data identify a new pathway by which oxidized phospholipids inhibit LPS action involving the activation of neutral sphingomyelinase, resulting in a change in caveolin distribution. Furthermore, we identify specific oxidized phospholipids responsible for this inhibition.**—Walton, K. A., B. G. Gugiu, M. Thomas, R. J. Basseri, D. R. Eliav, R. G. Salomon, and J. A. Berliner. **A role for neutral sphingomyelinase activation in the inhibition of LPS action by phospholipid oxidation products.** ***J. Lipid Res.*** **2006.** 47: **1967–1974.**

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Both proinflammatory and anti-inflammatory effects of phospholipid oxidation products on endothelial cells have been demonstrated (1, 2). The proinflammatory effects lead to chronic monocyte-specific endothelial interactions. Several laboratories have demonstrated that treatment of endothelial cells and macrophages with oxidized LDL and minimally modified low density lipoprotein (MM-LDL) inhibits the acute inflammatory response mediated by lipopolysaccharide (LPS) action (2–4). Oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (OxPAPC), the major active component of MM-LDL, was previously found to inhibit the LPS-mediated induction of E-selectin (3) and interleukin-8 (IL-8) synthesis (4) in human aortic endothelial cells (HAECs) and in human umbilical vein endothelial cells (5). The effects of OxPAPC were mimicked by oxidized palmitoyl-2-arachidonoyl-*sn*-glycero-3 phosphatidylethanolamine (OxPAPE), indicating the importance of the oxidation at the *sn*-2 position of the phospholipid and the similarity in effects of choline- and ethanolamine-containing phospholipid (6). For the current studies, we used a more systematic approach to examine the effects of different OxPAPE lipids on LPS action to expand and compare these effects with the more limited studies we had done with OxPAPC. An important goal of the these studies was to identify the most active oxidized phospholipids in inhibiting LPS induction of IL-8.

Abbreviations: A-PE, 1-palmitoyl-2-azelayl-*sn*-glycero-3-phosphatidylethanolamine; HAEC, human aortic endothelial cell; HDdiA-PE, 9-hydroxy-10-dodecendioic acid ester of lysophosphatidylethanolamine; IL-8, interleukin-8; KDdiA-PE, 9-keto-10-dodecendioic acid ester of lysophosphatidylethanolamine; KOdiA-PC, 5-keto-6-octendioic acid ester of 2-phosphatidylcholine; KOdiA-PE, 5-keto-6-octendioic acid ester of 2-lysophosphatidylethanolamine; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MM-LDL, minimally modified low density lipoprotein; ON-PE, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphatidylethanolamine; OxPAPC, oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine; OxPAPE, oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylethanolamine; POVPC, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphatidylcholine; sCD14, soluble CD14; TNF-α, tumor necrosis factor-α.

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A second goal of the current studies was to examine the mechanism by which OxPAPC inhibits LPS action in HAECs. Several mechanisms have been suggested by which OxPAPC or MM-LDL inhibits the LPS-induced transcription of downstream targets. At the extracellular level, OxPAPC has been shown to block the interaction of LPS with lipopolysaccharide binding protein (LBP) and CD14 when these proteins are bound to dishes (5). This competition for binding to LBP or CD14 presented as a soluble molecule, as they would be in vivo, is a possible inhibitory mechanism that would act predominantly at the extracellular level. A goal of the current studies was to test the importance of these competitive effects in the OxPAPC inhibition of LPS-induced transcription of IL-8.

Studies from our laboratory have demonstrated that oxidized phospholipids also have effects on LPS action at the cellular level, because OxPAPC inhibition of LPS action was observed in cells in which the OxPAPC was removed before LPS addition (4). These studies suggested that inhibition of the assembly of the LPS receptor complex in a lipid raft/caveolar fraction might be involved in the cellular inhibitory action of OxPAPC. It has been demonstrated previously that activation by LPS requires the recruitment of Toll-like receptor 4 and MD-2 to lipid rafts/caveolar areas of the cell enriched in sphingomyelin (4, 7–10). Oxidized LDL, MM-LDL, and an OxPAPC component, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphatidylcholine (POVPC), were shown to trigger an early activation of the sphingomyelin-ceramide pathway, as shown by both sphingomyelin hydrolysis and ceramide formation (11–13). The current studies tested the hypothesis that an increase in sphingomyelinase activity plays a role in the ability of phospholipid oxidation products to inhibit the LPS induction of IL-8 and alter the caveolar/lipid raft fraction.

EXPERIMENTAL PROCEDURES

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. (Alabaster, AL), or Sigma and oxidized as described previously (14). Oxidized ethanolamine phospholipids were synthesized as described previously (15). Mass analysis, liquid chromatography-mass spectrometry, tandem mass spectrometric analysis, and quantitation of oxidized phospholipids were performed as described previously (16). LPS from *Escherichia coli* O111:B4 (a natural, smooth strain) was obtained from List Biological Laboratories, Inc. Tumor necrosis factor-α (TNF-α) was obtained from R&D Systems. Soluble CD14 (sCD14) was obtained from Dr. Moshe Arditi (Cedar-Sinai, Los Angeles, CA). Recombinant human LBP was obtained from XOMA. C6 ceramide, C6 dihydroceramide, GW4869, and desipramine were obtained from Sigma.

Cell culture

HAECs (17) were isolated and cultured as described previously.

Cell fractionation

Cells were fractionated on sucrose gradients and fractions run using Western blotting as described previously (18). Characterization of these fractions is shown in our previous publication (19). Briefly, 5–100 mm dishes of HAECs were scraped in ice-cold PBS, pelleted, resuspended in 0.5 ml of 0.5 M Na_2CO_3, and passed through a 21 gauge needle 12 times. Cells were then sonicated on ice three times for 20 s each and then mixed with 0.5 ml of 90% (w/v) sucrose in 25 mM MES, pH 6.5, and 150 mM NaCl. Equal amounts of protein from this lysate-sucrose mixture were transferred to the bottom of a 5 ml thin-walled centrifuge tube (Beckman; No. 344057) and layered sequentially with 1 ml of 35% sucrose-0.25 M Na_2CO_3, 1 ml of 25% sucrose-0.25 M Na_2CO_3, and 1 ml of 5% sucrose-0.25 M Na_2CO_3. Samples were centrifuged for 16 h at 48,000 rpm in an SW-55 rotor at 4°C. Fractions were collected by manually pipetting 12–400 μl fractions from the top of the centrifuge tube and stored at −20°C or assayed immediately. Fractions were run for Western blotting, and the amount of caveolin 1 in fractions 1–3 (which had the highest amount of caveolin 1) was quantitated by densitometry. As an additional check on equal loading, total caveolin levels were also determined in the whole cell lysate.

IL-8 assays

After treatment, supernatants were collected and assayed for IL-8 or monocyte chemoattractant protein-1/JE using Quantikine ELISA kits (R&D Systems).

Sphingomyelinase assays

The Amplex Red® Sphingomyelinase Assay Kit (Molecular Probes) was used to monitor sphingomyelinase activity. Acid sphingomyelinase activity was measured at acid pH and neutral sphingomyelinase at neutral pH during the sphingomyelin hydrolysis step. Samples were read in a fluorescence microplate reader with absorption and fluorescence emission set at 560 and 590 nm, respectively.

Ceramide measurement by electrospray ionization tandem mass spectrometry

Cells from three 100 mm dishes of HAECs either untreated or treated with OxPAPC were pooled and briefly sonicated. Cells (2.5 mg of protein) and 300 pmol of C8 ceramide were added as a standard for loss during extraction. Lipids were then extracted by a modified Bligh and Dyer method and dried under a stream of argon gas. Using triplicate injections, electrospray ionization tandem mass spectrometry was used to measure different ceramide species from this crude cellular extract as described previously (20). A Perkin-Elmer Sciex API III triple quadrupole mass spectrometer fitted with an Ion SprayTM source was used in the positive ion mode for analysis. Spectra were collected by scanning with 0.3 Da step size and a scan speed of ~6 s at an orifice voltage of 65 V. Tandem mass spectrometry spectra were recorded in the fragment ion mode with argon as the collision gas (CGT = 100) at an orifice voltage of 85 V. The amount of ceramide was calculated as the ratio of the area of the tandem mass spectrometry peak to the area of the internal standard. This experiment was performed on three different cell isolates. For verification of the tandem mass spectrometry identification of particular ceramides, authentic standards were used.

Statistics

The data are presented as means ± SD.

RESULTS

Identification of major inhibitory oxidized phospholipids

To identify the most potent phospholipid oxidation products responsible for inhibiting the LPS induction of IL-8, we examined a series of synthetic phosphatidyletha-

nolamines with palmitic acid residue at the *sn*-1 position and oxidation epitopes at the *sn*-2 position (**Fig. 1A**). 1-Palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphatidylethanolamine (ON-PE), 9-hydroxy-10-dodecendioic acid ester of lysophosphatidylethanolamine (HDdiA-PE), 1-palmitoyl-2-azelayl-*sn*-glycero-3-phosphatidylethanolamine (A-PE), and 9-keto-10-dodecendioic acid ester of lysophosphatidylethanolamine (KDdiA-PE) were derived from 1-palmitoyl-2-linoleyl-*sn*-glycero-3 phosphatidylethanolamine, whereas the other synthetic lipids were derived from 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylethanolamine. All were tested at a concentration of 4 μg/ml. For comparison, 50 μg/ml OxPAPC and OxPAPE were also tested. Cells were treated with 4 ng/ml LPS in the presence or absence of oxidized phospholipids. After 4 h, IL-8 levels in the medium were determined. 4-Keto-5-heptendioic acid ester of lysophosphatidylethanolamine, 5-keto-6-octendioic acid ester of 2-lysophosphatidylethanolamine (KOdiA-PE), KDdiA-PE, and HDdiA-PE, which contain an α,β-unsaturated carboxylic acid at the *sn*-2 position, were the most active inhibitors tested, reducing LPS-induced IL-8 synthesis by 80–90% at 4 μg/ml (Fig. 1B). The aldehyde-containing phospholipids 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphatidylethanolamine and ON-PE inhibited LPS action by 50–60%, whereas the carboxylic acid-containing derivatives lacking the α,β-unsaturation, A-PE and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidyl ethanolamine, only inhibited LPS action by ~40%. Because we obtained a high level of inhibition with KOdiA-PE and similar results were seen with phosphatidylcholine and phosphatidylethanolamine derivatives, a dose-response determination was made comparing OxPAPC with KOdiA-PC (Fig. 1C). Although OxPAPC showed significant inhibition at >25 μg/ml, KOdiA-PC showed significant inhibition at 50 ng/ml and thus was 500-fold more active than OxPAPC.

The role of CD14 and LPB in the inhibitory action of OxPAPC

To determine the role of CD14 and LBP in the inhibition of LPS induction of IL-8 by OxPAPC, we co-treated HAECs with OxPAPC and LPS in the presence and absence of sCD14. Although sCD14 strongly stimulated LPS action, it did not reverse the inhibitory effect of OxPAPC (**Fig. 2A**). Concentrations as high as 200 μg/ml sCD14 were unable to prevent the inhibitory effect of 100 ng/ml KOdiA-PE (LPS + sCD14 = 63 ± 4; LPS + sCD14 + KOdiA-PE = 8 ± 0.6 IL-8 pg/ml). Exposure of cells to soluble LBP also significantly increased LPS action but did not inhibit the effect of OxPAPC on LPS action (Fig. 2B).

OxPAPC-induced sphingomyelinase activation regulates OxPAPC inhibition of LPS induction of IL-8

We first determined the ability of OxPAPC to activate sphingomyelinase using an Amplex Red® sphingomyelinase assay; both neutral and acid sphingomyelinase activities were determined. The assays were performed on lysates from HAECs treated for 4 h with control medium, medium containing 50 μg/ml OxPAPC, or medium containing 20 ng/ml TNF-α as a positive control. As shown in **Fig. 3A**, OxPAPC treatment, as well as TNF-α, significantly increased both neutral and acidic sphingomyelinase activity. We then directly measured levels of ceramide formed in response to OxPAPC. We quantitated the ceramide species produced by OxPAPC treatment using electrospray ionization tandem mass spectrometry. OxPAPC treatment (50 μg/ml for 4 h) significantly increased C16:0, C22:0, and C24:0 (Fig. 3B), whereas C24:1 was unchanged (data not shown). To determine whether these changes in enzymatic activity play a role in the OxPAPC inhibition of LPS action, we used specific inhibitors of neutral sphingomyelinase (GW4869; 15 μM) and acid sphingomyelinase (desipramine; 10 μM). Assays were performed to verify that the concentrations of inhibitor used were sufficient to inhibit the induction of sphingomyelinase activity by OxPAPC. For these experiments, cells were pretreated for 1 h with GW4869, desipramine, or vehicle control before treatment with control medium or medium containing 50 μg/ml OxPAPC for 4 h. The OxPAPC-induced neutral sphingomyelinase activity was reduced by >90% with GW4869 but not with desipramine, whereas the acid sphingomyelinase activity was reduced by 90% with desipramine but not with GW4869 (data not shown). We next tested the effect of these inhibitors on the ability of OxPAPC to inhibit LPS induction of IL-8. Cells were pretreated for 1 h with vehicle control, GW4869, or desipramine. Then, control medium, OxPAPC, OxPAPC + LPS, KOdiA-PC, or KOdiA-PC + LPS was added to the wells. After 4 h, medium was collected and IL-8 levels were determined (**Fig. 4A**). GW4869 significantly inhibited the ability of OxPAPC and KOdiA-PC to decrease the LPS induction of IL-8, but desipramine was ineffective (Fig. 4B).

We hypothesized that because of its known ability to alter cell membrane composition, ceramide might mimic the effect of OxPAPC. Cell-permeant C6 ceramide (10 and 20 μM) was able to reduce LPS-induced IL-8 synthesis to control levels, whereas the less permeant C6 dihydroceramide had only a modest effect (**Fig. 5**). Ceramide alone minimally affected levels of IL-8 synthesis in untreated cells and did not increase lactate dehydrogenase release over the 4 h treatment time used for these studies (data not shown). We had previously demonstrated that OxPAPC disrupted caveolae, decreasing the levels of caveolin 1 in the buoyant fraction (19). We now demonstrate that C6 ceramide had a similar effect to OxPAPC on caveolin 1 distributions: for caveolin in fractions 1, 2, and 3, control = 71 ± 6%, OxPAPC = 44 ± 4%, and ceramide = 42 ± 4%.

DISCUSSION

The potent activity of oxidized LDL and its component lipids to inhibit LPS action has been well documented in vivo and in vitro (4, 5, 21). Several groups have previously demonstrated that oxidized phospholipids have both proinflammatory and anti-inflammatory effects on endothelial cells. The proinflammatory effects, mediated by 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidylcholine, POVPC, and 1-palmitoyl 2-glutaroyl-sn

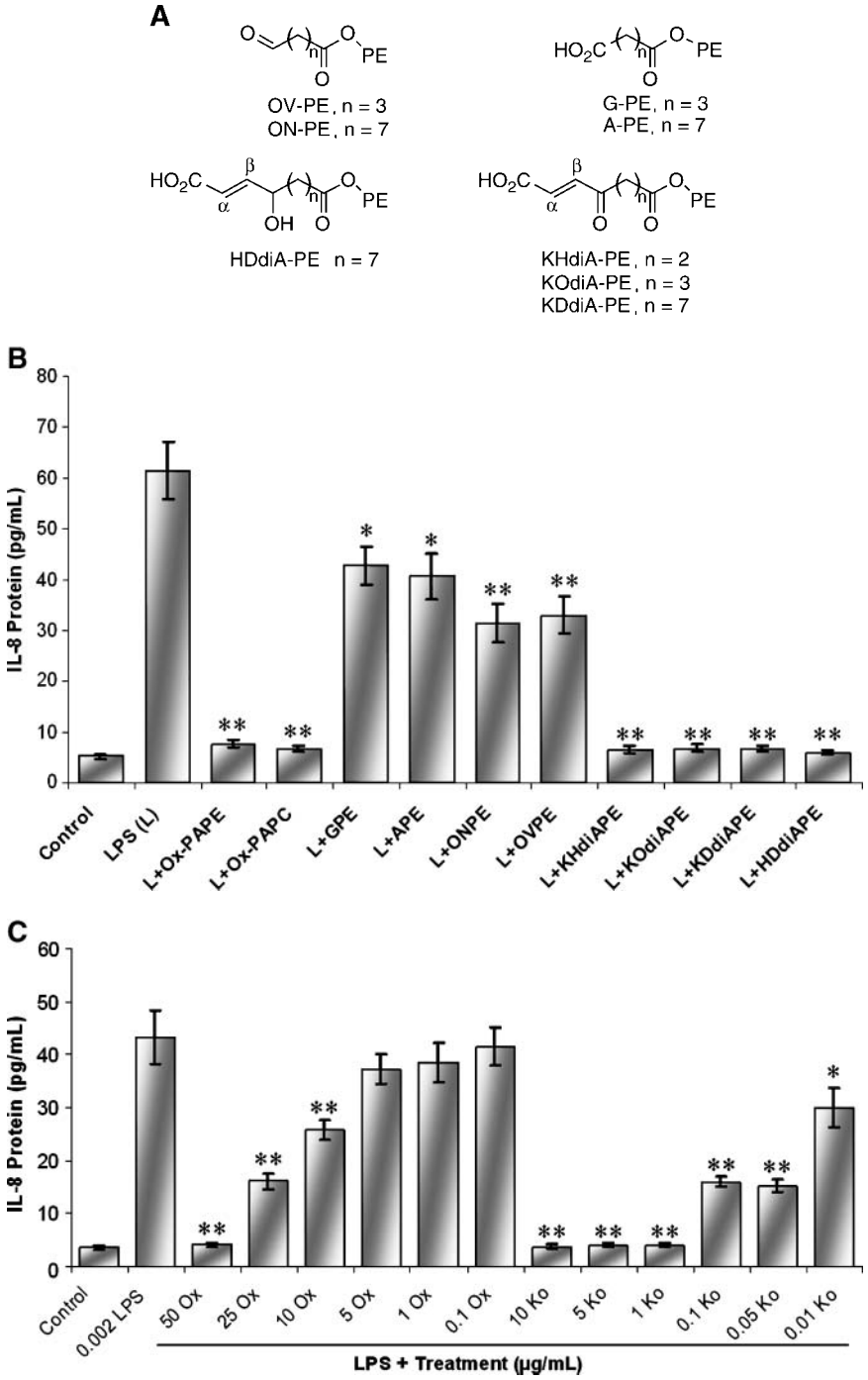

Fig. 1. Comparison of the inhibition of lipopolysaccharide (LPS) action by individual oxidized phosphatidylethanolamine species. A: Structures of synthetic oxidized ethanolamine phospholipids used in inhibition studies. PE represents a lysophosphatidylethanolmine (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphatidylethanolmine) residue. B: Inhibition of LPS-induced interleukin-8 (IL-8) synthesis by oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (OxPAPC), oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylethanolamine (OxPAPE), and seven synthetic oxidized phospholipids shown in A. Human aortic endothelial cells (HAECs) were treated with medium alone (control), 4 ng/ml LPS (L), LPS + 50 μg/ml OxPAPC or OxPAPE, or LPS + 4 μg/ml 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidylethanolamine (G-PE), 1-palmitoyl-2-azelayl-*sn*-glycero-3-phosphatidylethanolamine (A-PE), 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphatidylethanolamine (ON-PE), 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphatidylethanolamine (OV-PE), 4-keto-5-heptendioic acid ester of lysophosphatidylethanolamine (KHdiA-PE), 5-keto-6-octendioic acid ester of 2-lysophosphatidylethanolamine (KOdiA-PE), 9-keto-10-dodecendioic acid ester of lysophosphatidylethanolamine (KDdiA-PE), or 9-hydroxy-10-dodecendioic acid ester of lysophosphatidylethanolamine (HDdiA-PE) for 4 h, and IL-8 levels in the medium were determined. All phospholipids at 4 μg/ml significantly inhibited the ability of LPS to stimulate the synthesis of IL-8 ($P < 0.01$) compared with cells treated with LPS alone. C: Dose-response curve for the inhibitory effect of OxPAPC and KOdiA-PC on LPS-induced IL-8 synthesis. Cells were treated with control medium or 2 ng/ml LPS with or without the addition of OxPAPC (Ox) or KOdiA-PC (Ko) at the indicated concentrations for 4 h. IL-8 levels in the medium were then determined. Data are given as means ± SD. Each data point was determined in quadruplicate. ** $P < 0.01$, * $P < 0.05$ compared with LPS alone. The results shown represent one of three experiments that gave similar results.

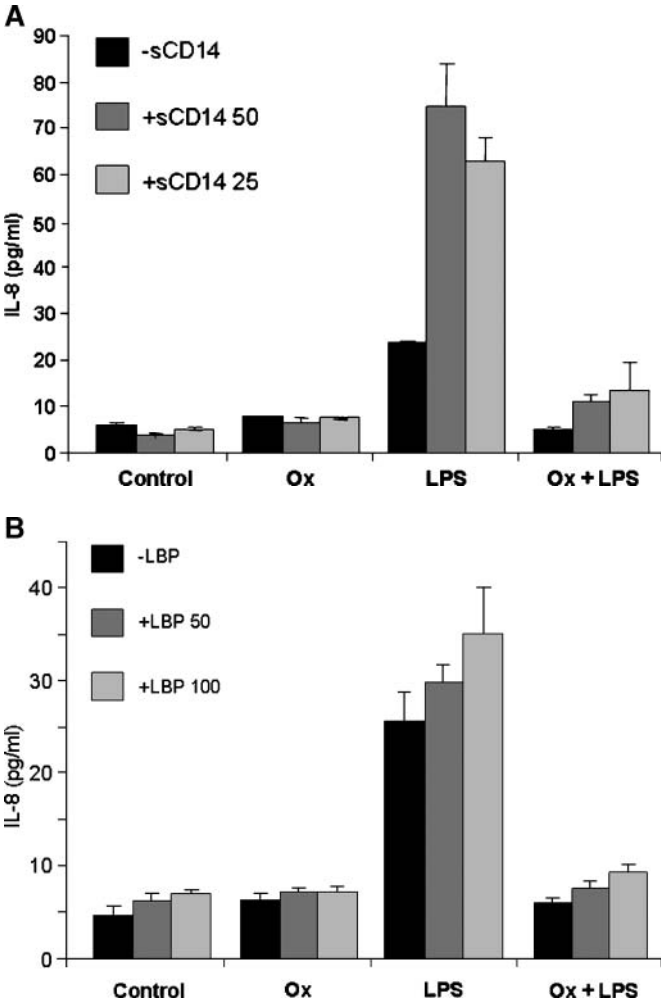

Fig. 2. Effect of soluble CD14 (sCD14; A) and lipopolysaccharide binding protein (LBP; B) on the inhibition of LPS-induced IL-8 synthesis by OxPAPC. HAECs were treated with medium alone (control), 15 μg/ml OxPAPC (Ox), 4 ng/ml LPS, or 15 μg/ml OxPAPC plus 4 ng/ml LPS (Ox + LPS) in the presence of 0, 25, or 50 ng/ml sCD14 (A) or 0, 50, or 100 ng/ml LBP (B) for 4 h. Medium was then used for the IL-8 assay. There was no significant effect of LBP or sCD14 on OxPAPC inhibition. Values shown are means ± SD of quadruplicate determinations. These results are representative of three experiments that gave similar results.

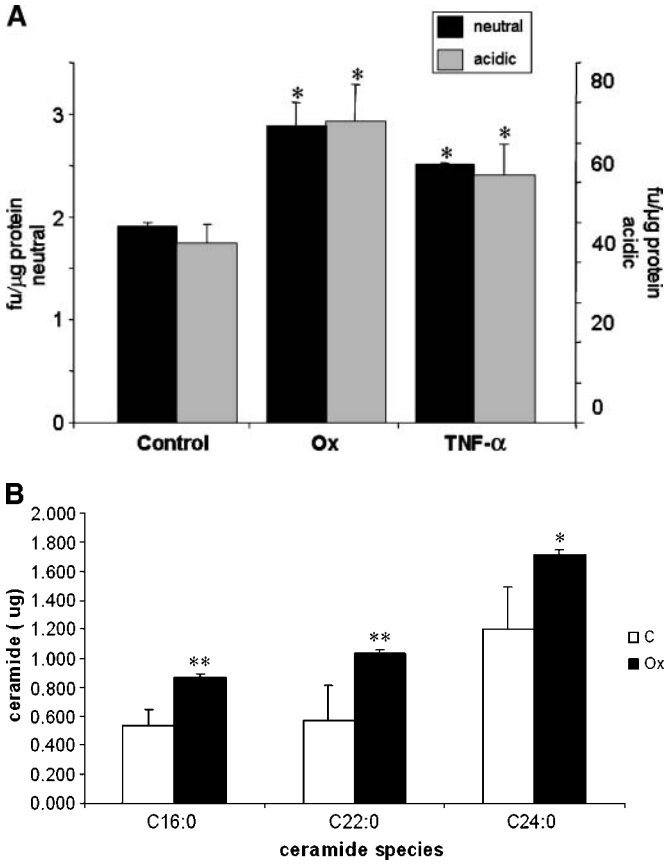

Fig. 3. Effect of OxPAPC on neutral and acidic sphingomyelinase activity (A) and ceramide production (B). A: Cell lysates from HAECs treated with medium alone (control), 50 μg/ml OxPAPC (Ox), or 20 ng/ml tumor necrosis factor-α (TNF-α) for 4 h were used in the Amplex Red® sphingomyelinase assay for acid or neutral sphingomyelinase. Data are given as fluorescence units (fu)/μg protein. B: Cell lysates from HAECs treated with medium alone [control (C)] or 50 μg/ml OxPAPC (Ox) for 4 h were assayed for ceramide production using electrospray ionization tandem mass spectrometry. For both A and B, values are means ± SD of quadruplicate experiments. ** $P < 0.01$, * $P < 0.05$ for OxPAPC-treated cells compared with controls. For A, the data are representative of three experiments; for B, the data represent four separate cell isolates examined with tandem mass spectrometry and values pooled.

(italisized)-glycero-3-phosphatidyl choline (PGPC), activate monocyte endothelial interactions (1, 22). To better characterize the oxidized phospholipids that inhibit LPS action, we examined a series of candidate synthetic lipids derived from oxidized polyunsaturated fatty acyl-phosphatidylethanolamines. We demonstrate that phospholipid oxidation products containing α,β-unsaturated carboxylic acids (KHdiA, KOdiA, KDdiA, and HDdiA) are much more potent inhibitors of the LPS induction of IL-8 than are phospholipids containing short-chain aldehydes or acids, such as oxovaleryl (OV) and glutaryl (G) (Fig. 1B). 1-Palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidylcholine, the most active proinflammatory phospholipid, did not inhibit the ability of LPS to induce IL-8 (data not shown), and OVPE, another active inducer, was minimally effective at inhibiting LPS action. Thus, different oxidation products are most potent in regulating the proinflammatory and anti-inflammatory effects of oxidized phospholipids.

Several mechanisms have been implicated in this inhibitory response to LPS in different cell types and for different LPS target genes. The current studies focused on the mechanism by which oxidized phospholipids inhibit LPS induction of IL-8 synthesis in HAECs. We focused on IL-8 because of its importance in the inflammatory response to LPS. One mechanism by which OxPAPC could inhibit LPS action was identified by Bochkov and coworkers (5), who reported that OxPAPC inhibited the binding of LPS to immobilized LBP and CD14. On the basis of these data, they suggested that OxPAPC might sequester the components of the LPS receptor complex extracellularly, thus inhibiting LPS induction of E-selectin.

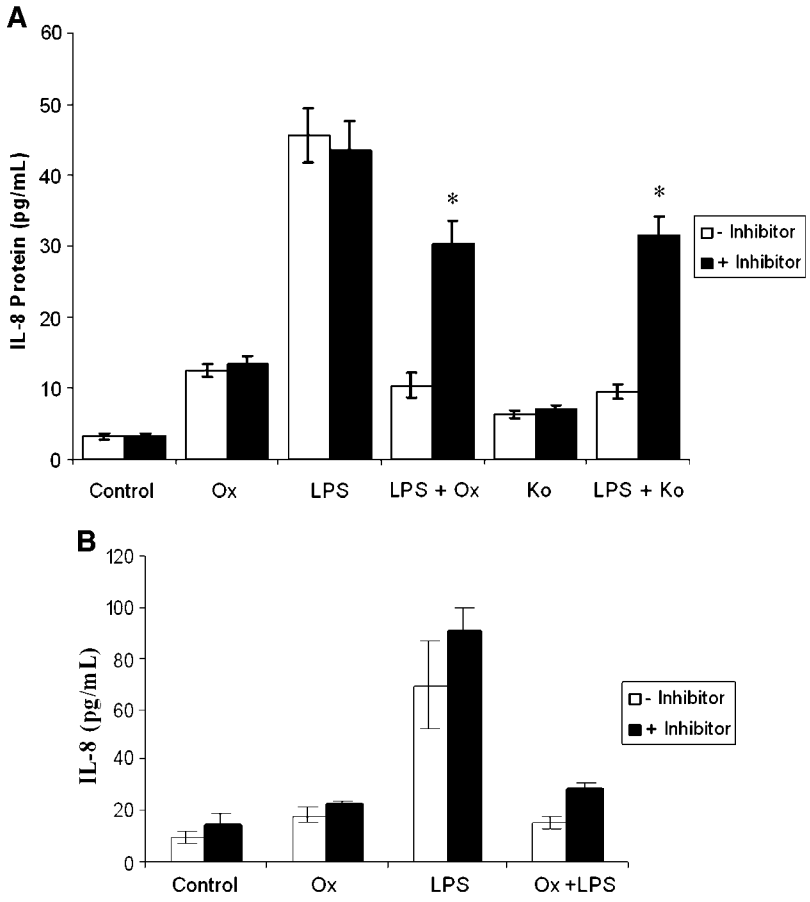

Fig. 4. Effect of sphingomyelinase inhibitors on the ability of OxPAPC (Ox) or KOdiA-PC (Ko) to inhibit LPS induction of IL-8 synthesis. A: The effect of GW4869, a neutral sphinomyelinase inhibitor, was examined. B: The effect of desipramine, an acid sphingomyelinase inhibitor, was examined. HAECs were pretreated with vehicle control, GW4869 (15 μM), or desipramine (10 μM) for 1 h followed by treatment with medium alone (control), 50 μg/ml OxPAPC, or 100 ng/ml KOdiA-PC with or without 4 μg/ml LPS for 4 h. IL-8 levels in the medium were determined. Values are given as means ± SD of triplicate determinations. * $P < 0.01$ compared with treatment without inhibitor. These results are representative of three experiments that gave similar results.

However, although addition of sCD14 and to a lesser extent LBP significantly increased LPS action (probably because the amount of CD14 and LBP in the medium or cells is rate-limiting), our studies demonstrate that addition of sCD14 and LBP did not significantly reduce OxPAPC's inhibition of LPS-induced IL-8 synthesis (Fig. 2A, B).

The current studies present evidence that activation of neutral sphingomyelinase plays an important role in the ability of OxPAPC to inhibit LPS action. Previous studies have demonstrated that MM-LDL and POVPC activate neutral sphingomyelinase in smooth muscle cells (13). We provide evidence that, in HAECs, OxPAPC treatment also activates neutral and acidic sphingomyelinases (Fig. 3A) and concurrently increases ceramide levels (Fig. 3B). Importantly, we show that a neutral sphingomyelinase inhibitor, GW4869, although decreasing OxPAPC-mediated neutral sphingomyelinase activity, reduces the inhibitory activity of OxPAPC and KOdiA-PC on LPS induction of IL-8 (Fig. 4A). In contrast, an acidic sphingomyelinase inhibitor, desipramine, although decreasing OxPAPC-mediated acidic sphingomyelinase activity, did not significantly reduce OxPAPC's inhibitory effect on LPS action (Fig. 4B). These results indicate that neutral sphingomyelinase is necessary for the inhibitory effect of oxidized phospholipids, whereas acidic sphingomyelinase plays no significant role. Others have demonstrated significant differences in the roles of these two sphingomyelinases (23).

Although we are the second group to report the activation of neutral sphingomyelinase by OxPAPC, the mechanism of activation is not yet known. One mechanism for the activation may relate to arachidonate release, which has been shown previously to activate neutral sphingomyelinase (23). We have previously demonstrated the release of arachidonate in response to OxPAPC (24). A second mechanism may involve increased oxidative stress that occurs in response to OxPAPC and results in hemoxygenase induction (25). It has been demonstrated by several laboratories that blocking the generation of reactive oxygen species inhibits the induction of neutral sphinomyelinase activity by several signal transduction pathways (26).

There are a number of products of neutral sphingomyelinase that might be responsible for the OxPAPC effect on LPS action. We tested the role of one of these products, ceramide. We present evidence that cell-permeant C6 ceramide, at nontoxic concentrations, inhibits LPS-induced IL-8 synthesis (Fig. 5A). The role of ceramide in LPS action has been examined previously by several different groups (27–31). LPS itself does not appear to increase ceramide levels; however, downstream effectors of LPS, such as TNF and IL-1, clearly increase ceramide (27). The effects of ceramide on LPS inflammatory action differ with the cell type, time of treatment, and target molecule examined. In some studies, ceramide has been shown to inhibit the effects of LPS on nitric oxide synthesis and cyclooxygenase 2

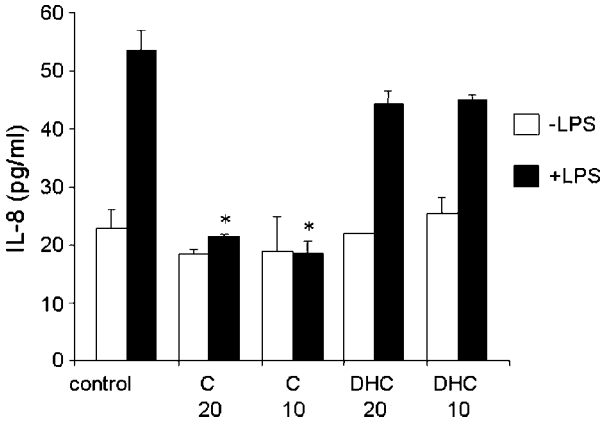

Fig. 5. Effect of ceramide on LPS-induced IL-8 synthesis and on HAECs were treated with medium alone (control), C6 ceramide (C) at 10 or 20 μM, or C6 dihydroceramide (DHC) at 10 or 20 μM with or without 4 ng/ml LPS for 4 h. Values shown are means ± SD of triplicate determinations. * $P < 0.01$ compared with cells treated with LPS alone. These results are representative of four experiments that gave similar results.

expression (28, 29), whereas in other studies, it was shown to potentiate the expression of these molecules (30, 31). Based on our own experience with the use of ceramide and sphingomyelinase inhibitors in endothelial cells, the effect of this pathway can vary with the levels of the agents used. For the studies reported here, we used concentrations of ceramide and sphingomyelinase inhibitor that did not alter the ability of LPS to induce IL-8 in HAECs after 4 h of treatment. At higher concentrations, we found potentiating effects on LPS action. Our group previously demonstrated that treatment of endothelial cells with OxPAPC decreased the number of caveolae and the amount of caveolin-1 in the buoyant fractions. This also appeared to alter the mobilization of Toll-like receptor 4 to the buoyant fraction by LPS. We now demonstrate that C6 ceramide also results in caveolin-1 redistribution. Degradation of sphingomyelin to ceramide has been demonstrated to cause alterations to rafts and caveolae and to alter downstream signaling (32, 33). Furthermore, ceramide and LPS assemble different proteins into raft fractions (34). It has been demonstrated directly that lipid raft integrity is essential for LPS cellular activation, because raft-disrupting drugs, such as nystatin, inhibit LPS-induced TNF-α secretion (8). We hypothesize that the major effect of ceramide in HAECs, under the conditions used for these studies, is to disrupt caveolae, resulting in deficient assembly of LPS receptor complex.

Among the phospholipids tested, phospholipids containing α,β-unsaturated fatty acids have previously been demonstrated to bind most avidly to CD36 (35). Furthermore, both CD36 and scavenger receptor class B type I have been demonstrated to play a role in the action of bacterial products (36, 37). Others have demonstrated low levels of CD36 in large vessel endothelial cells, and we have shown previously that CD36 is not involved in the OxPAPC inhibition of LPS action (4). CD36 is a scavenger receptor shown to bind oxidized LDL and certain oxidized phospholipids. However, oxidized LDL has been shown to bind to multiple scavenger receptors, some of which have been shown to have cell-signaling properties. It is thus possible that the binding of oxidized phospholipids to another scavenger receptor may be responsible for the inhibition of LPS action by the bioactive phospholipids identified in this study. Oxidized phospholipids may compete for binding of LPS to this scavenger receptor, and/or oxidized phospholipids may activate sphingomyelinase by binding to this receptor.

In summary, the current studies demonstrate that the inhibitory effect of OxPAPC on LPS induction of IL-8 is mediated, at least in part, by the activation of neutral sphingomyelinase. Our studies further suggest that the sphingomyelinase product ceramide may mediate at least some of the inhibitory effect of OxPAPC by altering the caveolar lipid raft fraction, which mediates the assembly of the LPS receptor complex. Our data do not exclude the possibility that other products of sphingomyelinase may also be involved in the inhibitory effect of OxPAPC. These studies identify specific phospholipid oxidation products containing α,β-unsaturated fatty acids as potent inhibitors of LPS action. Thus, the formation and destruction of these lipids may play an important role in the response to bacterial infection.

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