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Supplementary Material

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Oxidized Phospholipids Are More Potent Antagonists of Lipopolysaccharide than Inducers of Inflammation

Olga V. Oskolkova,* Taras Afonyushkin,* Beatrix Preinerstorfer,† Wolfgang Bicker,‡ Elena von Schlieffen,* Eva Hainzl,* Svitlana Demyanets,§ Gernot Schabacker,* Wolfgang Lindner,† Alexandros D. Tselepis,¶ Johann Wojta,§ Bernd R. Binder,* and Valery N. Bochkov*

Polyunsaturated fatty acids are precursors of multiple pro- and anti-inflammatory molecules generated by enzymatic stereospecific and positionally specific insertion of oxygen, which is a prerequisite for recognition of these mediators by cellular receptors. However, nonenzymatically oxidized free and esterified polyunsaturated fatty acids also demonstrate activities relevant to inflammation. In particular, phospholipids containing oxidized fatty acid residues (oxidized phospholipids; OxPLs) were shown to induce proinflammatory changes in endothelial cells but paradoxically also to inhibit inflammation induced via TLR4. In this study, we show that half-maximal inhibition of LPS-induced elevation of E-selectin mRNA in endothelial cells developed at concentrations of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) 10-fold lower than those required to induce proinflammatory response. Similar concentration difference was observed for other classes and molecular species of OxPLs. Upon injection into mice, OxPAPC did not elevate plasma levels of IL-6 and keratinocyte chemoattractant but strongly inhibited LPS-induced upregulation of these inflammatory cytokines. Thus, both in vitro and in vivo, anti-LPS effects of OxPLs are observed at lower concentrations than those required for their proinflammatory action. Quantification of the most abundant oxidized phosphatidylcholines by HPLC/tandem mass spectrometry showed that circulating concentrations of total oxidized phosphatidylcholine species are close to the range where they demonstrate anti-LPS activity but significantly lower than that required for induction of inflammation. We hypothesize that low levels of OxPLs in circulation serve mostly anti-LPS function and protect from excessive systemic response to TLR4 ligands, whereas proinflammatory effects of OxPLs are more likely to develop locally at sites of tissue deposition of OxPLs (e.g., in atherosclerotic vessels). The Journal of Immunology, 2010, 185: 7706–7712.

Inflammation is orchestrated by multiple positive and negative feedbacks mediated by extracellular messengers. An important mechanism producing inflammatory mediators is enzymatic oxidation of polyunsaturated fatty acids (PUFAs) leading to formation of PGs, thromboxanes, leukotrienes, and resolution-inducing compounds, such as lipoxins, protectins, and resolvins. However, accumulating data show that nonenzymatically oxidized lipids also demonstrate multiple activities relevant for inflammation. Several families of nonenzymatic PUFAs oxidation products were shown to stimulate or inhibit inflammatory reactions. These include PUFAs fragmentation species [e.g., hydroxynonenal (1, 2)], hydroxides, and hydroperoxides of PUFAs (3–5), and isoprostanes (6–9). In addition to free oxidized PUFAs, esterified oxidized residues also demonstrate pro- and anti-inflammatory activities (10–12). In this work, we analyzed modulation of LPS-induced inflammatory reactions by nonenzymatically oxidized phospholipids (OxPLs). The majority of cell-associated PUFAs are esterified in phospholipids (PLs). Nonenzymatic peroxidation generates PLs containing a mixture of oxidized residues (12). OxPLs were characterized as the major component of minimally oxidized low-density lipoprotein responsible for its ability to induce synthesis of cytokines and chemokines (IL-6, IL-8, MCP-1, etc.) and stimulate adhesion of monocytes to endothelial cells (ECs) (12, 13). Further studies demonstrated that OxPLs induce an inflammatory response mediated by adhesion of monocytes to ECs expressing P-selectin and CS-1–fibronectin (11, 14). It was hypothesized that OxPLs are initiators of chronic monocyte inflammation characteristic of atherosclerosis (15). However, under different biological conditions,
OxPLs can also induce anti-inflammatory effects. In particular, various classes and molecular species of OxPLs were shown to inhibit action of LPS and TLR2 ligands in vitro and in vivo (11, 16–21). Several cell types were shown to be targets of the anti-LPS action of OxPLs, including human ECs (11, 16), blood monocytes (21), monocyte-derived macrophages (22), human and mouse macrophage cells lines (17), dendritic cells (23), skin fibroblasts (21), and epithelial cells transfected with TLR4 and TLR2 together with coreceptors (17, 21). The mechanisms of the anti-LPS effect of OxPLs seem to be complex. Available data suggest that OxPLs inhibit both recognition of LPS by LPS-binding protein, CD14, and MD-2 (17, 21), as well as cell-associated events such as coupling of TLR4 to intracellular signaling adapters (22, 24).

Induction of pro- and anti-inflammatory (anti-LPS) effects by the same OxPLs raises a question about the integration of opposite activities during inflammation induced by TLR4 ligands. In this work, we studied relative impact of each of these activities at different concentrations of OxPLs. To this end, we compared concentration dependencies of proinflammatory effects of OxPLs on ECs with their ability to suppress inflammatory reactions to LPS. The results show that induction of TLR4 develops at significantly lower concentrations of OxPLs than those required for their proinflammatory action. Injection of OxPLs into mice did not change plasma levels of inflammatory cytokines but significantly inhibited their induction by LPS. Thus, OxPLs antagonize activation of TLR4 at concentrations lower than those required for induction of inflammatory reactions in ECs in vitro and systemic inflammation in vivo. In other words, OxPLs are more potent as anti-LPS agents compared with their intrinsic proinflammatory activity.

Materials and Methods

Materials

Synthetic 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoglycerol (PAPG), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (phosphatidic acid; PAPA), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine (PAPS), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC, 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-O-alkylideneoctanoyl-sn-glycero-3-phosphocholine (DTPC), and 1,2-dinonanoyl-sn-glycero-3-phosphocholine (DNPC) were obtained from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) and 1-palmitoyl-2-(9-oxo)nonanoyl-sn-glycero-3-phosphocholine (PONPC) were from Cayman Chemicals (Ann Arbor, MI). Dry lipids were oxidized by exposure to air until ∼20% of the lipid remained intact and the rest was oxidized. Oxidized lipids were dissolved in chloroform, purged with argon, and stored at −70°C. Oxidation was monitored by TLC and electrospray ionization-mass spectrometry (12). Mass-spectra of OxPLs have been published previously (21). 1-Palmitoyl-2-(5-oxovaleryl)sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PPOPC), a mixture of 1-palmitoyl-2-(12-hydroperoxylecysa-5,8,10,14-tetraenoyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-(15-hydroperoxycosa-5,8,11,13-tetraenoyl)-sn-glycero-3-phosphocholine (PAPC-OOH), and 1-palmitoyl-2-(5,6-epoxy isoprostane E2)-sn-glycero-3-phosphocholine (PEIPC)-enriched fraction were prepared as described previously (25). Noncleavable analogue of PAPC was synthesized as described previously (21). Concentration of PLs was determined by phosphorus assay (26). Organic solvents were of analytical grade. Diethylether, triaminediethylether, and 1 N sulfuric acid. Absorbance was measured at 450 nm. Quantification was performed using standard solutions of mouse IL-6 and KC (R&D Systems) that were analyzed in parallel to the plasma samples.

Isolation of mouse aortas and blood collection from Apco−/− mice

Apco−/− male mice (18 mo old) were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood was collected retro-orbitally using EDTA as anticoagulant. BHT (0.01% w/v final concentration) was added to blood prior to plasma separation. Isolation of aortic arch including a part of thoracic aorta was performed after heart perfusion through the left ventricle with PBS supplemented with DTPA (2 mM) and BHT (0.01%, w/v). Tissue pieces of 5.4–9.0 mg wet weight were washed with PBS/DTPA/BHT, placed into cryotubes, overlaid with argon, and stored at −70°C.

Extraction of total lipids from biological samples

Human plasma samples were obtained by centrifugation of EDTA-anticoagulated blood and stored at −70°C. Before lipid extraction, 100 μl human plasma was diluted with 400 μl water containing 2 mM DTPA and 0.01% BHT. Atherosclerotic plaques were obtained from symptomatic patients undergoing carotid endarterectomy. The samples were snap-frozen in liquid nitrogen in the surgery room and stored at −70°C until lipid extraction. Human material was obtained and processed according to the recommendations of the Ethical Commission of the Medical University of Vienna, which included obtaining informed consent in accordance with the Declaration of Helsinki. Pieces of atherosclerotic vessels (20 to 200 μg wet weight) were transferred from storage vessels into a mortar placed within a glove-box filled with argon, poured with liquid nitrogen, and powdered by pestle.

Extraction of total lipids from plasma, homogenized atheroma, or aorta samples was performed by a modified Folch extraction procedure using a 20-fold excess of organic solvents (27). All solutions were supplemented with 0.01% BHT. Water solutions additionally contained 2 mM DTPA. Extraction was performed in tubes purged with argon and tightly closed. Phase separation cartridges (PTS, 6 ml; Macherey-Nagel, Germany) were prewashed with 2 mM DTPA (pH 7) were used for separation of organic and aqueous phases. Internal standard DTPC (40 ng per sample) or DNPC (10 ng per sample) was added to each sample at the beginning of the procedure. The lipid extracts were evaporated under a stream of argon at 30°C and stored at −70°C until analysis.

Solid-phase extraction of PLs from total lipid extracts

To prevent artificial oxidation, all solutions were supplemented with antioxidant (BHT, 0.01%). Solid-phase extraction (SPE) cartridges and column samples were placed in a glass chamber filled with argon; positive pressure of argon was used for elution from cartridges instead of vacuum. For purification of human samples, Isolute NH2-SPE cartridges (100 mg; Separtis, Grenzach-Wyhlen, Germany) were prewashed with 3 ml methanol and 3 ml hexane. Lipid extracts from 100 μl human plasma were applied in 0.5 ml chloroform and, cartridges were washed with 3 ml acetic acid (5%, v/v) in diethyl ether/BHT (0.01%, w/v) to remove neutral lipids and acylglycerols. PLs were eluted with 3 ml methanol/2 ml water/1 ml BHT. The solvent was evaporated under a stream of argon at 30°C, and the samples were stored at −70°C prior to analysis by HPLC-tandem mass spectrometry (HPLC-MS/MS). For mouse samples, silica cartridges (DSC-Si, 50 mg; Sigma-Aldrich) were used to isolate PLs from total lipid extracts. Cartridges were preconditioned with 2 ml chloroform/BHT (0.01%, w/v). The same solvent was used for application of samples (2 ml) and elution of neutral lipids (2 × 1 ml). PLs were eluted with 1 ml methanol/5% acetic acid (v/v)/BHT (0.01%, w/v). Upon addition of 2 ml chloroform/BHT and 1 ml 0.7 M formic acid and brief vortexing, two phases were formed. The lower phase was collected and dried under argon.

Quantification of IL-6 and keratinocyte chemoattractant cytokines in murine plasma

Concentrations of IL-6 and keratinocyte chemoattractant (KC) were determined in murine plasma diluted 1:100 with 1% BSA/PBS using ELISA in 96-well plates (Nunc-ImmunoModule F8 Maxisorp; Thermofisher Scientific, Roskilde, Denmark) precoated with IL-6 or KC capture Abs (R&D Systems), streptavidin-linked HRP (dilution 1:200; R&D Systems), and 3,3′,5,5′-tetramethylbenzidine as the peroxidase substrate (KPL, Gaithersburg, MD), the reaction was stopped by addition of 35 μl 1 N sulfuric acid. Absorbance was measured at 450 nm. Quantification was performed using standard solutions of mouse IL-6 and KC (R&D Systems) that were analyzed in parallel to the plasma samples.
flow. Analysis of eluates by mass spectrometry demonstrated that the yield of POVPC, PGPC, PONPC, and PAzPC was >80% in PL fractions obtained either on NH₄ or silica cartridges.

**Quantitative determination of OxPLs in biological samples by HPLC-MS/MS**

Fragmented phosphatidylycerolines (PCs) were separated by HPLC using a 1200 series liquid chromatography system from Agilent (Waldbronn, Germany). PL fractions from human samples (from 100 μl plasma or 10 mg tissue) were reconstituted in 100 μl of a solution of 10 mM ammonium acetate in methanol/water (80:20, v/v) and 40 μl thereof was injected onto a Luna C18(2) HPLC column (3 μm, 150 × 2.0 mm; Phenomenex, Torrance, CA), which was kept at 25°C. Chromatographic separation was carried out using solutions A (10 mM ammonium acetate in methanol/water, 80:20, v/v) and B (10 mM ammonium acetate in methanol) with a linear gradient starting from 25% B in A to 100% B over 20 min followed by isotropic elution with 100% B for the next 15 min. Mobile phase was supplied at a flow rate of 0.2 ml/min. The HPLC column was coupled to a 4000 QTrap triple quadrupole linear ion trap hybrid mass spectrometer system equipped with a Turbo V electrospray ion source (Applied Biosystems, Foster City, CA). The following general settings were used: electrospray ionization voltage was 4300 V, temperature of the ion source was 500°C, and entrance potential was 10 V. Nitrogen was used as nebulizer, heater, and curtain gas with the pressure set at 60, 50, and 10 psi, respectively. Compound-specific MS/MS parameters (i.e., declustering potential, collision energy, and cell exit potential) were optimized for every analyte individually using the instrument’s automated fragmentation optimization tool (Supplemental Table I).

PL fractions from mouse samples (from 35 to 51 μl mouse plasma or 5.4 to 11.4 mg tissue) were reconstituted in 100 μl 85% aqueous methanol containing 5 mM ammonium formate and 0.1% formic acid, and 10 μl thereof was injected onto a Kinetex C18 column (50 × 3.0 mm, 2.6 μm; Phenomenex), which was kept at 20°C. Chromatographic separation was carried out using solutions C (5 mM ammonium formate and 0.1% v/v formic acid in water) and D (5 mM ammonium formate and 0.1% v/v formic acid in methanol) with a gradient: 0 min, 85% D; 2 min, 85% D; 3 min, 95% D; 11 min, 100% D; 16 min, 100% D; 16.1 min, 85% D; 20 min, 85% D. Mobile phase was supplied at a flow rate of 0.4 ml/min from 0 to 3 min; it was then linearly increased to 0.6 ml/min until 11 min run time, kept constant at 0.6 ml/min until 19 min run time, and it was reduced linearly to 0.4 ml/min until 20 min run time. Detection was carried out using the mass spectrometer as above in positive ion mode by selected reaction monitoring using a specific PC product ion (m/z 184, which corresponds with the cleaved phosphocholine residue) using nitrogen as collision gas. The general settings were as follows: electrospray ionization voltage was set to 4500 V and the temperature of the ion source was 550°C; nitrogen was used as nebulizer, heater, and curtain gas with the pressure set at 50, 30, and 20 psi, respectively. Selected reaction monitoring settings were as follows: declustering potential = 120 V, entrance potential = 10 V,
collision energy = 53 V, cell exit potential = 14 V. Data acquisition was performed with the Analyst software, version 1.5 (Applied Biosystems). Concentrations of analytes in biological samples were determined from calibration curves plotted as ratio of analyte peak area to DTPC (human samples) or peak area to DNPC (mouse samples) versus analyte amount using standard solutions of POVPC, PGPC, PAzPC, and PONPC.

**HUVECs treatment with LPS and OxPLs, RNA isolation, and gene expression analysis**

HUVECs were obtained and cultured as described (28). After an overnight incubation in 7% FCS, the cells were pretreated with indicated concentrations of OxPLs for 20 min in medium 199 containing 2% FCS and 20 mM HEPES followed by incubation with or without LPS (final concentration 50 ng/ml). Before adding to cells, aliquots of OxPLs in chloroform were dried under a stream of argon with simultaneous vortexing to remove the solvent. OxPLs were further resuspended by vigorous vortexing in medium 199 supplemented with 2% FCS and 20 mM HEPES. Thus, no organic solvent was added to cells. Stimulation was performed in quadruplicate. After incubation at 37˚C for 4 h, the medium was aspirated and TRIzol reagent (Invitrogen, Carlsbad, CA) was added. Equal volumes from identically treated quadruplicate samples were combined to compensate for sample-to-sample variation. Nine hundred nanograms of total RNA were reverse-transcribed using murine leukemia virus reverse transcriptase (MuLV; Applied Biosystems) and oligo(dT)16 primers. RT-qPCR was performed using the Light Cycler instrument and the Fast Start SYBR Green I kit (Roche, Basel, Switzerland). In all assays, cDNA was amplified using a standard program (10-min denaturing step; 55 cycles of 5 s at 95˚C, 5 s at 65˚C, and 15 s at 72˚C; melting point analysis in 0.1˚C steps). Target gene expression was quantified as described (29). The expression of mRNA of target genes was normalized to the expression of β2-microglobulin mRNA. In all experiments, β2-microglobulin–normalized level of expression in control (mock-treated) cells was taken as 1. Sequences of primers are given in the Supplemental Table II.

**Statistical analysis**

The results are representative of two to four independent experiments giving similar results. Statistical analysis was performed using two-tailed Student t test.

**Results**

To compare concentration dependencies of proinflammatory and anti-LPS activities of OxPLs, upregulation of E-selectin in ECs was chosen as a classical inflammatory reaction induced by LPS that allows for sensitive monitoring of TLR4 activation. Previously, we showed that OxPLs inhibited LPS-induced accumulation of both E-selectin mRNA and protein in HUVECs (21). Therefore, only expression of E-selectin mRNA was measured in this work. In parallel, we quantified mRNA of IL-8, which is an important inflammatory cytokine upregulated by OxPLs in ECs both at mRNA and protein levels (30, 31).

Different classes of OxPLs obtained by oxidation of precursors having the same sn-1 (palmitoyl) and sn-2 (arachidonoyl) residues but different polar head groups demonstrated both anti-LPS and proinflammatory activities (Fig. 1). We found that half-maximal inhibition of LPS-induced elevation of E-selectin mRNA was observed at concentrations ~10-fold lower than those required to induce IL-8 mRNA under identical experimental conditions. Both

![FIGURE 3. Proinflammatory and anti-LPS effects of OxPLs containing different PUFAs. Quadruplicate samples of HUVECs were treated with OxPLs obtained by oxidation of precursor PCs containing one palmitoyl residue and one of linoleoyl (18:2), arachidonoyl (20:4), or docosahexaenoyl (22:6) residues. Stimulation of cells and quantification of gene expression were performed as described in Fig. 1. In addition to the experiments presented, similar results were obtained in three (OxPAPC) independent experiments. Structures of the precursor PLs are shown in insets.](http://www.jimmunol.org/)

![FIGURE 4. Proinflammatory and anti-LPS effects of individual OxPCs. Quadruplicate samples of HUVECs were treated with OxPCs containing either nonfragmented oxidized sn-2 residue (hydroperoxide [PAPC-OOH], epoxyisoprostane [PEIPC]) or oxidatively fragmented sn-2 residue (oxovaleryl [POVPC], glutaroyl [PGPC]). Stimulation of cells and analysis of E-selectin and IL-8 expression were performed as described in Fig. 1. In addition to the experiments presented, similar results were obtained in one (PGPC) independent experiment. Structures of PLs are shown in insets.](http://www.jimmunol.org/)
activities were essentially independent of the type of polar head group, suggesting that oxidation of major PL classes produces species with reproducible combination of proinflammatory and anti-LPS activities.

In experiments presented in Fig. 1, either induction of IL-8 by OxPLs, or—in separate cell culture dishes—inhibition by OxPLs of LPS-induced E-selectin expression was studied. Because IL-8 is induced both by LPS and OxPLs, we analyzed changes in IL-8 mRNA levels in cells treated with a combination of a fixed concentration of LPS and increasing concentrations of OxPLs. Nonmonotonic U-shaped concentration dependence of IL-8 induction shows that low concentrations of OxPLs inhibited elevation of IL-8 induced by LPS, whereas higher concentrations of OxPLs upregulated this cytokine (Fig. 2). Similarly, low concentrations of OxPAPC inhibited LPS-induced upregulation of CXCL1 and CXCL3, whereas at higher concentrations OxPAPC by itself was capable of inducing CXCL1 and CXCL3 (Supplemental Fig. 1). These data illustrate dual-phase activity of OxPLs, namely their anti-LPS effects at lower concentrations and proinflammatory action at higher concentrations with respect to genes induced by both OxPLs and LPS (e.g., IL-8, CXCL1, and CXCL3).

The ratios between concentrations inducing anti-LPS and proinflammatory effects were similar for oxidized phosphatidylcholines (OxPCs) containing different oxidized PUFA’s; that is, linoleic (ω-6, 18:2), arachidonic (ω-6, 20:4), and docosahexaenoic (ω-3, 22:6) acid (Fig. 3). The data suggest that the number of double bonds does not significantly influence the balance between pro- and anti-inflammatory species generated by peroxidation. Furthermore, the results show that neither anti-LPS nor proinflammatory activity critically depend on formation of isoprostanes because oxidation of PL-esterified linoleic acid, which cannot generate cyclic endoperoxide and prostanooids, nevertheless produced OxPLs with prominent anti-LPS and proinflammatory activities.

Because peroxidation of PUFA-PL generates a mixture of oxidized products, each of which may have different combination of pro- and anti-inflammatory properties, we compared anti-LPS and proinflammatory activities of representative molecular species of OxPC. Full-length species such as hydroperoxide-PC and isoprostane-PC (PAPC-OOH, PEIPC), as well as OxPCs with fragmented residues (PGPC, POVPC) demonstrated both proinflammatory and anti-LPS activities (Fig. 4). The data show that at least for tested molecular species, the anti-LPS and proinflammatory activities are intrinsic properties of the same molecule.

**FIGURE 5.** Comparison of concentrations of OxPLs inducing half-maximal inhibition of LPS or induction of IL-8. Half-maximal effective concentrations of OxPLs were determined graphically from the concentration dependence curves presented in Figs. 1, 3, and 4. If saturation of effect was not achieved at maximal concentrations used in a particular experiment, maximal observed effect was taken as 100% for determination of EC50. Mean values ± SD are shown.

**FIGURE 6.** Effects of OxPAPC on LPS-induced elevation of inflammatory cytokines in mouse plasma. C57BL/6 mice were injected i.p. with 0.35 ml sterile saline (n = 9), OxPAPC (200 μg per animal, n = 9), LPS (1 mg/kg animal weight, n = 8), or a combination of LPS and OxPAPC (n = 8). Blood samples were collected after 2 and 4 h, and plasma levels of KC (A) and IL-6 (B) were determined by ELISA. The results are representative of three independent experiments.

**FIGURE 7.** Concentrations of fragmented OxPCs in human (A) and mouse (B) plasma and atherosclerotic vessels. Total lipids were extracted from human plasma (n = 10), human carotid endarterectomy samples (n = 4), ApoE−/− mouse plasma (n = 6), or ApoE−/− mouse aorta samples (n = 6) using a modified Folch procedure. PLs were isolated and quantified by HPLC-MS/MS as described in Materials and Methods.
A summary on the concentration dependencies of anti-LPS versus proinflammatory activities of different classes and species of OxPLs is presented on Fig. 5. The data consistently show 10-fold difference between the two activities thus supporting our conclusion about higher potency of OxPLs as LPS antagonists compared with their proinflammatory effects.

We further analyzed the balance of anti-LPS and proinflammatory activities of OxPLs in vivo. To this end, systemic levels of mouse analogue of human IL-8, KC, were quantified after i.p. injection of OxPAPC and LPS into mice. OxPAPC did not elevate plasma levels of KC/IL-8, whereas it strongly inhibited LPS-induced elevation of this cytokine (Fig. 6A). Similar regulation was observed for another sensitive marker of systemic inflammation, IL-6 (Fig. 6B). The data suggest that effects of LPS in vivo can be inhibited by concentrations of OxPLs that do not induce systemic inflammation. Thus, similar to our in vitro findings, anti-LPS effects of OxPLs at systemic level are more potent than their proinflammatory action.

The data described above allow hypothesizing that the net effect of OxPLs in LPS-induced inflammation depends on their concentration. Quantification of the most abundant oxidatively fragmented PCs in human and mouse plasma demonstrated that individual species were present at concentrations in the range 0.1–1 μM (Fig. 7). These data are in good agreement with previous estimates of OxPC levels in human and mouse plasma (32, 33). Thus, total concentrations of fragmented OxPCs together with nonfragmented OxPCs and OxPLs with other head groups are likely to reach low micromolar levels that are close to TLR4-inhibiting concentrations. Compared with plasma, in human atherosclerotic plaques or aortas of aged Apoe knockout mice the levels of OxPCs (normalized to tissue wet weight) were several-fold higher (Fig. 7). The elevation was paralleled by higher degree of oxidation of precursor PLs in atherosclerotic vessels compared with that in plasma both in human and murine samples (data not shown). Taking into account that arterial fragments contained atheroma-free areas, local concentrations of OxPLs in the lesion may be significantly higher. Indeed, previous studies demonstrated accumulation of OxPCs in rabbit atherosclerotic vessels at concentrations of the order 10 to 100 μM/kg wet weight (12, 19). Our data (Figs. 1, 3, 4) and previous publications consistently show that such concentrations of OxPCs and other classes of OxPLs induce prominent proinflammatory effects (19).

Discussion

In this work, a contradiction concerning opposite pro- and anti-inflammatory activities of OxPLs in LPS-induced inflammation was analyzed. Previously published experiments characterizing anti-LPS and proinflammatory activities of OxPLs were performed under variable experimental conditions and therefore they did not consistently show whether OxPLs exert these effects at the same or different concentrations. This study was specifically designed to compare concentration dependencies of the two effects. An important finding presented in this work is that potencies of the anti-LPS and proinflammatory effects of OxPLs on ECs significantly differ. We show that major classes and molecular species of OxPLs inhibit action of LPS at concentrations that are 10-fold lower than those inducing proinflammatory effects such as elevation of IL-8 in ECs.

The study was performed on ECs that were chosen as a cell type intimately involved in inflammation and convenient for analysis of TLR4 activation. Available data show that OxPLs induce similar anti-LPS effects in other cell types, as illustrated by the inhibition of LPS effects on isolated monocytes or human blood ex vivo (21). Our in vivo data suggest that OxPLs inhibit activation of various cell types mediating systemic response to LPS. In support of this notion, we show in this study that OxPAPC strongly inhibited LPS-induced elevation of inflammatory cytokines in murine plasma. Because OxPAPC alone did not elevate plasma levels of IL-6 and KC, the data additionally point to higher potency of OxPLs as LPS antagonists compared with their proinflammatory action. Furthermore, these results show that OxPLs can inhibit effects of LPS not only in cell culture medium in vitro but also in the presence of physiological levels of lipids and proteins circulating in blood plasma.

Simultaneous analysis of proinflammatory and anti-LPS effects performed in this work confirmed that several individual molecular species of OxPCs demonstrated both anti-LPS and proinflammatory activities, although at different concentrations. Therefore, anti-LPS and proinflammatory activities of OxPAPC, OxPAPS, and other natural mixtures of OxPLs generated by peroxidation of a single precursor cannot be explained solely by the action of molecular species demonstrating selective pro- or anti-inflammatory activity. At least for those individual molecular species tested in this work, both activities are intrinsic properties of the same molecule. It is likely that similar structural determinants of OxPLs (e.g., oxidized sn-2 residues) are necessary for inhibition of TLR4 pathway by binding to CD14 and MD-2 (17, 21) and alternately for inducing inflammatory effects via independent mechanisms that have to be characterized.

Quantification of OxPLs showed that human and mouse plasma contains submicromolar to low micromolar levels of total oxidatively fragmented PCs. Thus, plasma levels of OxPLs are insufficient to induce proinflammatory effects but close to the threshold concentrations above which they demonstrate anti-LPS activity. Therefore, accumulation of OxPLs due to oxidation of lipoproteins during acute inflammation (34) may elevate anti-LPS activity in plasma and thus counteract inflammation at the systemic level. Alternately, significantly higher levels of OxPLs were detected locally at sites of chronic inflammation, such as atherosclerosis (Refs. 12, 19, and this work). Concentration-dependent transition from anti-LPS to proinflammatory activity of OxPLs described in this work allows hypothesizing that relatively low levels of OxPLs present in the circulation serve an anti-LPS function and may protect from excessive systemic response to TLR4 ligands. In contrast, proinflammatory effects of OxPLs are more likely to develop locally at sites of lipid oxidation and accumulation (e.g., in atherosclerotic vessels, inflamed lung tissue, or leprosy lesions, all characterized by tissue deposition of OxPLs) (12, 19, 35, 36). According to this hypothesis, OxPLs paradoxically might be capable of simultaneously inducing pro- and anti-inflammatory effects within different compartments of the same organism.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure S1. Low concentrations of OxPAPC inhibit LPS-induced CXCL1 and CXCL3 mRNA expression, whereas high concentrations of OxPAPC upregulate these genes. HUVECs were preincubated in medium 199 containing 2% FCS with 10, 30 and 100 µM OxPAPC for 20 minutes, followed by addition of medium with or without LPS (final concentration 50 ng/ml). Incubation was stopped by medium aspiration and addition of the Trizol reagent. RNA isolation and analysis of expression of CXCL1 (A) and CXCL3 mRNA (B) was performed. All expression levels were normalized to β2-microglobulin. Normalized expression of CXCL1 and CXCL3 in mock-treated cells (medium alone) was taken as 1. mRNA expression in the cells treated with LPS alone is expressed as 100%. Statistical analysis was performed using two-tailed, paired t-test.
### Supplementary Table 1. HPLC-MS/MS parameters of the analytes.

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<tr>
<td>PAzPC</td>
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<td>7.6</td>
<td>81</td>
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<td>PLPC</td>
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<td>184.1</td>
<td>25.5</td>
<td>56</td>
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<td>184.1</td>
<td>25.2</td>
<td>101</td>
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</tr>
</tbody>
</table>

Abbreviations: DP, declustering potential; CE, collision energy; CXP, cell exit potential.
**Supplementary Table 2.** Primer sequences for qRT-PCR.

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>E-Selectin</td>
<td>5'-GGT TTG GTG AGG TGT GCT C-3'</td>
<td>5'-TGA TCT TTC CCG GAA CTG C-3'</td>
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<tr>
<td>IL-8</td>
<td>5'-CTC TTG GCA GCC TTC CTG ATT-3'</td>
<td>5'-TAT GCA CTG ACA TCT AAG TTC TTT AGC A-3'</td>
</tr>
<tr>
<td>β₂-Microglobulin</td>
<td>5'-GAT GAG TAT GCC TGC CGT GTG-3'</td>
<td>5'-CAA TCC AAA TGC GGC ATC T-3'</td>
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<tr>
<td>TLR4</td>
<td>5'-AAT CCC CTG AGG CAT TTA GG-3'</td>
<td>5'-CAC AGC CAC CAG CTT CTG TA-3'</td>
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<td>CXCL1</td>
<td>5'-AGG GAA TTC ACC CCA AGA AC-3'</td>
<td>5'-CAC CAG TGA GCT TCC TCC TC-3'</td>
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<tr>
<td>CXCL3</td>
<td>5'-GCA GGG AAT TCA CCT CAA GA-3'</td>
<td>5'-GGT GCT CCC CTT GTT CAG TA-3'</td>
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