

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart
Association®



Learn and Live SM

Multi-Hit Inhibition of Circulating and Cell-Associated Components of the Toll-Like Receptor 4 Pathway by Oxidized Phospholipids

Elena von Schlieffen, Olga V. Oskolkova, Gernot Schabbauer, Florian Gruber, Stephan Blüml, Melinda Genest, Alexandra Kadl, Claudia Marsik, Sylvia Knapp, Jesse Chow, Norbert Leitinger, Bernd R. Binder and Valery N. Bochkov
Arterioscler. Thromb. Vasc. Biol. 2009;29:356-362; originally published online Dec 26, 2008;

DOI: 10.1161/ATVBAHA.108.173799

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2009 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/29/3/356>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:

journalpermissions@lww.com

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

Multi-Hit Inhibition of Circulating and Cell-Associated Components of the Toll-Like Receptor 4 Pathway by Oxidized Phospholipids

Elena von Schlieffen, Olga V. Oskolkova, Gernot Schabbauer, Florian Gruber, Stephan Blüml, Melinda Genest, Alexandra Kadl, Claudia Marsik, Sylvia Knapp, Jesse Chow, Norbert Leitinger, Bernd R. Binder, Valery N. Bochkov

Objective—Oxidized phospholipids (OxPLs) that are abundant in atherosclerotic lesions are increasingly recognized as context-dependent lipid mediators demonstrating both pro- and antiinflammatory activities. Molecular mechanisms of their effects are largely unknown. Here we present novel information on the mechanisms whereby OxPLs modulate activation of TLR4 by lipopolysaccharide (LPS).

Methods and Results—We show, using several cell types and various inflammatory genes as readouts, that different classes and molecular species of OxPLs do not stimulate TLR4 but exert prominent inhibitory effects on LPS-induced reactions. Our data demonstrate that binding of OxPLs to the LPS-binding protein (LBP) and CD14 prevents recognition of LPS by these proteins, thus impairing activation of TLR4. In addition, OxPLs inhibited LBP- and CD14-independent activation of TLR4 by the synthetic TLR4 agonist E6020 indicating that in parallel with LBP and CD14, OxPLs target cell-associated steps in TLR4 cascade.

Conclusions—Our data suggest that OxPLs inhibit action of LPS via a multi-hit mechanism. These results support the notion that OxPLs are endogenous inhibitors of TLR4 produced in response to oxidative stress. (*Arterioscler Thromb Vasc Biol.* 2009;29:356-362.)

Key Words: oxidized phospholipids ■ lipopolysaccharide ■ TLR4 ■ LBP ■ CD14

Oxidized phospholipids (OxPLs) are generated as a result of oxidation of esterified polyunsaturated fatty acids (PUFAs). Oxidized phosphatidylcholines (OxPCs) were identified as proinflammatory components of LDL minimally modified by oxidation (mmLDL) and are increasingly recognized as lipid mediators with a broad spectrum of activities. Apart from OxPCs, oxidation of other classes of PUFA-PLs generates biologically active products that were detected in a number of pathologies including ischemia/reperfusion, radiation injury, cell aging, and apoptosis.¹⁻⁴ OxPCs are especially abundant in human and animal atherosclerotic lesions⁵⁻⁷ where they are thought to induce inflammatory

See accompanying article on page 337

reactions characteristic of atherosclerosis. In particular, OxPCs initiate mononuclear cell recruitment by inducing activation of adhesion molecules and production of MCP-1, IL-8, and other chemokines by endothelial cells (ECs).⁸ On the other hand, oxidized LDL and OxPLs demonstrate tissue-protective and antiinflammatory activities including upregulation of antioxidant enzymes or inhibition of inflammation

induced by bacterial lipopolysaccharide (LPS, endotoxin) via TLR4.^{9,10} The mechanisms of the antiendotoxin effects of OxPLs are only partially understood. In particular, it is not clear which chemical groups are important for the antiendotoxin activity of OxPLs and whether there are strict structural constraints for the activity. Furthermore, the data showing that certain effects of OxPLs are inhibited in TLR4 knockout animals^{11,12,13} suggest that OxPLs are not pure inhibitors/antagonists of TLR4 but may possess partial agonistic activity for TLR4. Last but not least, an important open question concerns the molecular targets of the antiendotoxin action of OxPLs. According to one hypothesis, OxPLs act at cell membrane level by preventing formation of TLR4 signaling complex in caveolae thus inhibiting downstream events. This inhibitory effect can result either from sequestration of membrane cholesterol by OxPLs, or from the activation of neutral sphingomyelinase leading to disruption of lipid rafts.^{13,14} In contrast, we postulated that OxPLs inhibit extracellular components of the TLR4 cascade, namely LPS-binding protein (LBP) and soluble CD14 (sCD14) that are present in plasma and extracellular fluid.⁹ This hypothesis is

Received July 11, 2008; revision accepted November 29, 2008.

From the Departments of Vascular Biology and Thrombosis Research (E.v.S., O.V.O., G.S., B.R.B., V.N.B.), Dermatology (F.G.), Internal Medicine III (S.B.), Medical and Chemical Laboratory Diagnostics (C.M.), and Internal Medicine I (S.K.), Medical University of Vienna, Austria; Eisai Research Institute (M.G., J.C.), Andover, Mass; and the Cardiovascular Research Center (A.K., N.L.), University of Virginia, Charlottesville.

Correspondence to Valery Bochkov, PhD, Center for Biomolecular Medicine and Pharmacology, Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Schwarzschanerstrasse 17, 1090 Vienna, Austria. E-mail valery.bochkov@meduniwien.ac.at

© 2009 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.108.173799

strongly supported by recent work.¹⁵ However, the mechanisms of inactivation of LBP and sCD14 by OxPLs until now were not studied.

In this work, we analyzed open questions related to the regulation of TLR4 by OxPLs. We show here that OxPLs having different polar head groups and oxidized *sn*-2 residues completely inhibit effects of LPS. We did not find evidence for TLR4 agonistic activity of OxPLs in several cell types that showed typical proinflammatory responses to LPS. Furthermore, we found that OxPLs inhibit both extracellular (LBP, sCD14) and cell-associated steps in recognition of LPS or activation of downstream signaling in ECs. In addition, the mechanisms of LBP and sCD14 inhibition were characterized. Our data support the notion that OxPLs bind to LBP and sCD14 thus preventing recognition of LPS and activation of TLR4.

Materials and Methods

Detailed description of materials and methods is presented in the supplemental materials (available online at <http://atvb.ahajournals.org>).

Lipids

Arachidonate-containing synthetic lipids were purchased from Avanti Polar Lipids. Dry lipids were oxidized by exposure to air and analyzed by mass spectrometry.⁷

HUVECs Treatment, RNA Isolation, and Gene Expression Analysis

HUVECs were preincubated in medium 199/7% fetal calf serum (FCS) overnight. Lipids were added in 2% FCS and 20 mmol/L Hepes in medium 199 twenty minutes before the addition of LPS to final concentration of 50 ng/mL. After 4 hours cells were harvested and RNA was isolated. Target gene expression was quantified by reverse transcription/quantitative real-time PCR and was normalized to β 2-microglobulin mRNA levels.

Nondenaturing Polyacrylamide Gel-Electrophoresis and Protein Staining

Phospholipids or ReLPS were resuspended in PBS containing EDTA and incubated either with recombinant sCD14 or plasma at 37°C with shaking. Equal volume of 2× sample buffer was added and samples were analyzed by nondenaturing gel-electrophoresis. Proteins were either stained with silver or transferred to PVDF membranes and blocked overnight in 5% dry milk/PBS, followed by incubation with anti-CD14 antibody and peroxidase-conjugated secondary antibody.

Results

Different Classes of OxPLs Inhibit Effects of LPS but Do Not Demonstrate Intrinsic Agonistic Activity for TLR4

We first tested whether the antiendotoxin activity of OxPLs depends on the type of phospholipid polar head group. Different classes of nonenzymatically oxidized palmitoyl-arachidonoyl-PLs (see mass-spectra in supplemental Figure I) inhibited LPS-induced elevation of E-selectin mRNA (Figure 1, A), as well as protein (supplemental Figure IIA). The inhibitory effects strongly depended on PL oxidation (supplemental Figure IIB), and were not attributable to lipid toxicity because the effects of TNF α or IL-1 β were not significantly inhibited by OxPLs (supplemental Figure IIC). Oxidation products of PLs containing PUFAs differing in the

number and location of double bonds also demonstrated inhibitory properties (supplemental Figure III). Furthermore, individual molecular species of oxidized phosphatidylcholine induced qualitatively similar inhibition of the effects of LPS (Figure 1B). Importantly, noncleavable analog of OxPAPC containing ether- and amide bonds that are resistant to phospholipases (see the structure and mass-spectrum in supplemental Figure IV) was comparable in potency with diacyl OxPAPC (Figure 1C). The latter data suggest that OxPLs inhibit effects of LPS acting as a whole PL molecule, and that cleavage of oxidized fatty acid residue is not necessary for the activity.

The data presented in Figure 1 show that in contrast to LPS, neither of tested OxPLs upregulated E-selectin in ECs. To check whether OxPLs might be agonistic for TLR4 in other cell types, or stimulate expression of other genes characteristic of acute inflammation, we used additional cell types and readout genes. OxPAPC suppressed LPS-induced activation of innate immune responses in blood cells as indicated by significant inhibition by OxPAPC of LPS-induced production of TNF α and IL-6 in human whole blood samples, whereas OxPAPC alone did not activate cytokine production (Figure 2A). Furthermore, treatment of LPS-stimulated human monocytes with increasing concentrations of OxPAPC reversed effects of LPS to basal level (Figure 2B). In addition, OxPAPC inhibited LPS-induced upregulation of inflammatory genes TNF α , IL-1 α , IL-1 β , and COX-2 in human dermal fibroblasts (Figure 2C). Again, OxPAPC alone did not stimulate expression of these genes. Finally, we tested the action of OxPAPC on HEK cells stably transfected with luciferase reporter driven by a fragment of E-selectin (ELAM) promoter. These cells responded to LPS only on cotransfection with TLR4 and MD-2 (Figure 2D). OxPAPC did not stimulate luciferase expression but strongly inhibited effect of LPS (Figure 2D). In summary, our data show that OxPAPC has no agonistic activity but consistently demonstrate antagonistic action on induction of TLR4 downstream genes in several cell types tested in our experiments.

OxPLs Inhibit Both Extracellular and Cell-Associated Events in TLR4 Activation

We next asked a question whether OxPLs inhibit extracellular components of TLR4 cascade (ie, LBP and sCD14), or cell-associated receptors/signaling mechanisms (membrane CD14, TLR4, MD-2, or downstream adaptors). To answer this question, we preincubated HUVECs with OxPAPC, washed them for up to 60 minutes with fresh medium 199/FCS (or 199/sCD14), and then stimulated with LPS in fresh medium 199/FCS (or 199/sCD14) but in the absence of OxPAPC. The removal of OxPAPC and addition of fresh medium 199/FCS (or 199/sCD14) lead to immediate reversal of the inhibition (Figure 3A), suggesting that soluble factors such as LBP or sCD14 are important targets of the antiendotoxin action of OxPAPC. This conclusion is additionally supported by recently published data showing that the addition of excess of sCD14 or LBP can partially overcome the antiendotoxin effect of OxPAPC.¹⁵

The reversal of the antiendotoxin effect on washing out OxPAPC was significant but incomplete (Figure 3A), raising

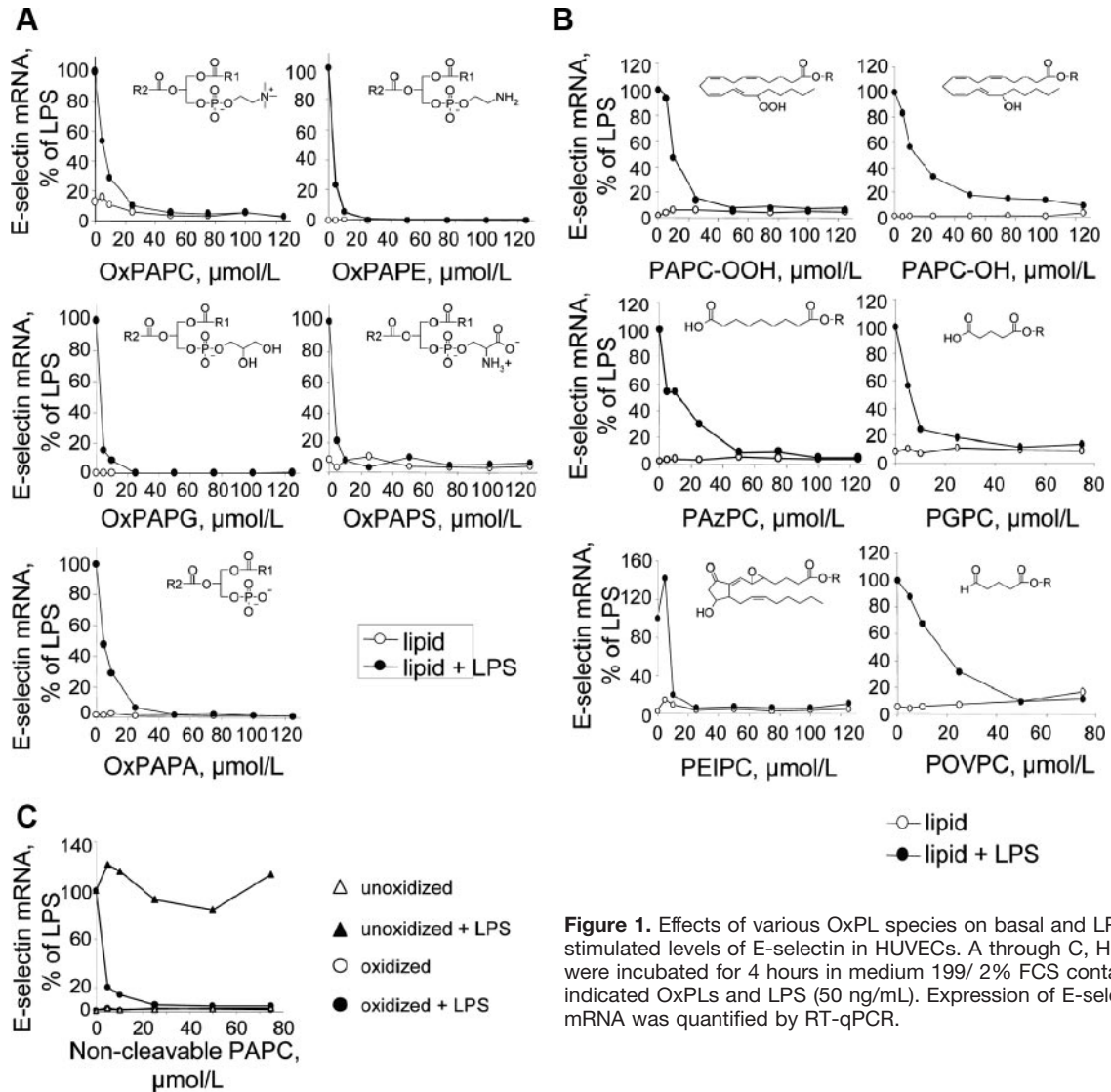


Figure 1. Effects of various OxPL species on basal and LPS-stimulated levels of E-selectin in HUVECs. A through C, HUVECs were incubated for 4 hours in medium 199/ 2% FCS containing indicated OxPLs and LPS (50 ng/mL). Expression of E-selectin mRNA was quantified by RT-qPCR.

a possibility that in addition to soluble factors, OxPAPC also inhibits cell-associated steps in LPS recognition or signaling. To test this hypothesis we applied compound E6020 at micromolar concentrations stimulating TLR4 independently of LBP and CD14.¹⁶ We found that E6020 stimulated luciferase reporter in serum-free medium (containing no LBP or sCD14); addition of recombinant sCD14 did not enhance the effect (Figure 3B, left panel). In contrast to E6020, the action of LPS was completely dependent on supplementation with sCD14 (Figure 3B, right panel). OxPAPC strongly inhibited activation of the reporter by E6020 (Figure 3B), suggesting that OxPAPC can inhibit cell-associated components of the LPS cascade. The identification of specific cellular targets mediating the antiendotoxin effects of OxPAPC (TLR4, MD-2, or downstream adaptors) was beyond the scope of this article because this question was addressed previously.^{13,14}

OxPLs Prevent Binding of LPS to LBP

Previously, we and others characterized LBP and CD14 as important targets of the antiendotoxin action of OxPLs.^{9,15}

However, the mechanisms of inactivation of LBP and CD14 were not studied. LPS is known to bind to lipoproteins^{17,18} and incorporate into phospholipid vesicles.¹⁹ Interaction with lipids masks LPS from recognition by cellular receptors and as a consequence prevents activation of TLR4 in vitro and in vivo.^{19,20} This mechanism of LPS inactivation can be referred to as LPS scavenging. On the other hand, certain forms of LPS and synthetic analogs of lipid A bind to CD14 but form inactive complexes with TLR4/MD-2 and do not stimulate signaling.²¹ Because these inactive LPS species compete with fully active LPS for binding to CD14 and TLR4/MD-2, this mechanism can be classified as receptor antagonism. To choose between the scavenging and antagonistic mechanisms of inhibition we applied previously described assay allowing to characterize LBP inhibitors.²² Preincubation of LBP immobilized in 96-well dishes with OxPAPC rapidly (within minutes) inhibited its ability to bind biotinylated LPS (bt-LPS, Figure 4A). We further pretreated immobilized LBP with OxPAPC, washed out OxPLs, and then added bt-LPS. In spite of the removal of the bulk of OxPLs by rinsing, OxPAPC-pretreated LBP was inactive and did not bind

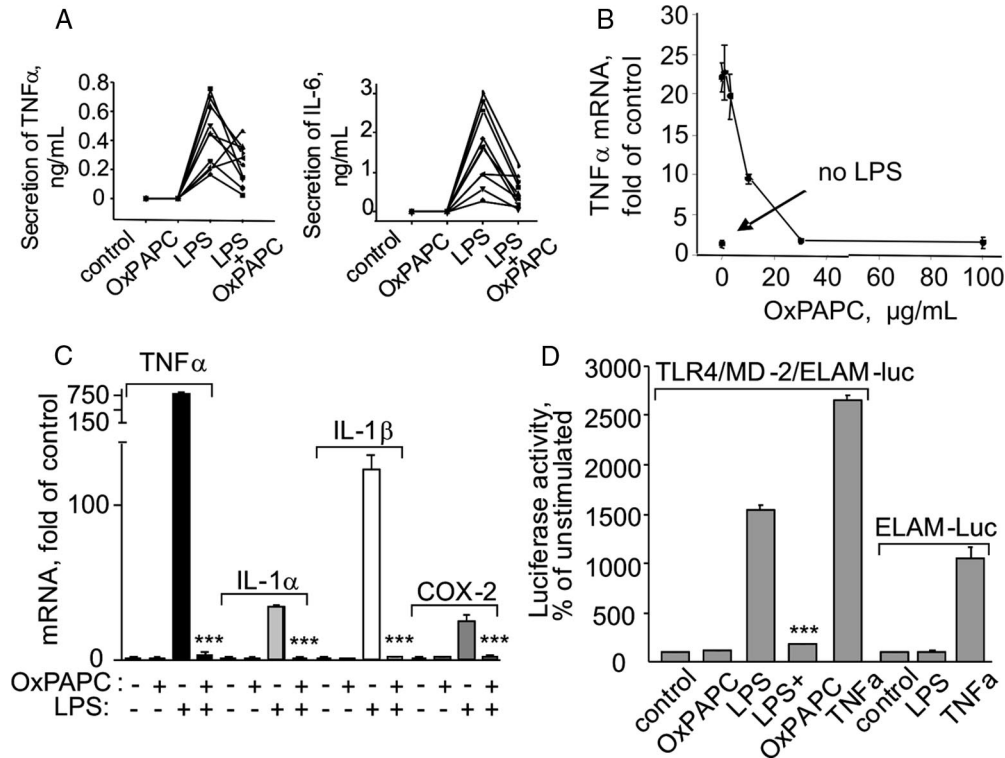


Figure 2. OxPLs demonstrate no TLR4 agonistic activity in various cell types. Effects of OxPAPC on basal and LPS-induced inflammatory activities were tested in human blood (A), blood mononuclear cells (B), human skin fibroblasts (C), and HEK293 cells stably transfected with TLR4, MD-2, and E-selectin promoter-luciferase reporter (D).

bt-LPS even after prolonged washing (Figure 4B). The effect was not attributable to the detachment of immobilized LBP as verified by unchanged binding of anti-LBP (Figure 4C). These results do not fit into the “scavenging” mechanism requiring that high concentrations of scavengers are present

in the medium, but rather support the model postulating that OxPLs form a complex with LBP and thus prevent binding of LPS, in analogy with the mechanism of ligand competition/receptor antagonism. In support of formation of a physical complex between OxPLs and LBP, we found that LBP bound to immobilized OxPAPC (Figure 4D). Furthermore, in a competitive assay the binding of LBP to immobilized LPS was prevented by different classes of OxPLs (Figure 4E); the efficiency of competition critically depended on lipid oxidation state (Figure 4F).

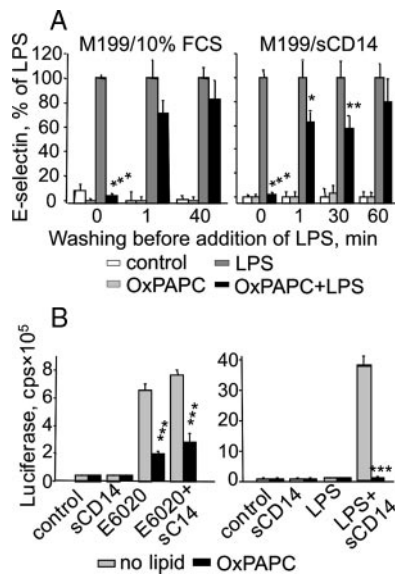


Figure 3. OxPLs inhibit both extracellular and cell-associated components of the TLR4 receptor cascade. A, Expression of E-selectin on HUVECs that were pretreated with OxPAPC, washed, and stimulated with LPS. B, Luciferase activity in HEK293 cells expressing MD-2, TLR4, and E-selectin promoter-luciferase reporter that were treated with OxPAPC, LPS, E6020, and sCD14.

OxPLs Form Physical Complexes With sCD14 and Prevent Interaction of LPS With sCD14

Because formation of physical complex between a ligand and receptor is a sine qua non of receptor antagonism, we further tested whether OxPLs in addition to LBP can also bind to sCD14. Preincubation of recombinant sCD14 with ReLPS resulted in faster anodic migration of ReLPS/sCD14 complex in nondenaturing gel as compared to sCD14 alone (Figure 5A), apparently because of acquisition of additional negative charge on phosphate groups of lipid A.²³ Similar enhancement of electrophoretic mobility was observed in the presence of oxidized anionic phospholipid-phosphatidylserine, but not neutrally charged phosphatidylcholine (Figure 5A). The effect was specific for sCD14 because OxPAPS did not influence mobilities of IgG or antithrombin III serving as control proteins (Figure 5B and supplemental Figure VA). The enhancement of electrophoretic mobility was characteristic of OxPAPS but not its unoxidized precursor PAPS, which induced only marginal changes in mobility of sCD14

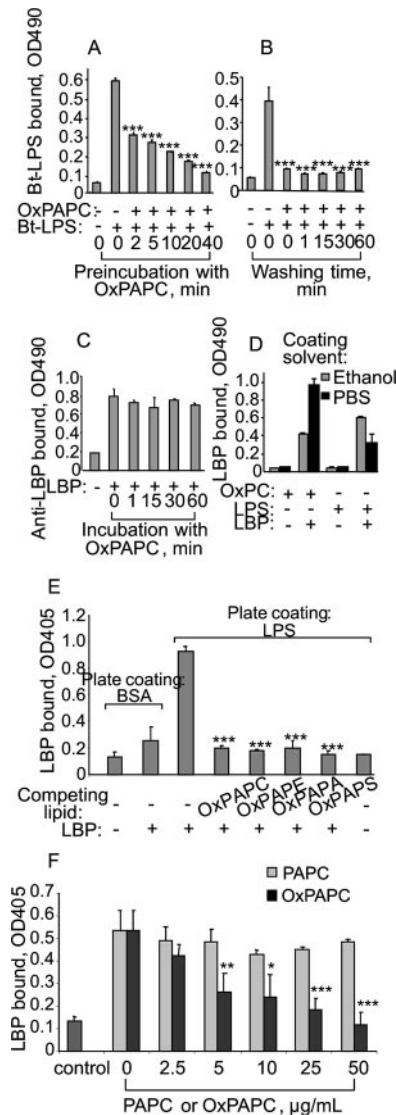


Figure 4. Binding of OxPLs to LBP prevents LBP/LPS interactions. A, Time kinetics of LBP inactivation by OxPAPC. B and C, Washing of OxPAPC-pretreated LBP for up to 1 hour does not restore binding of LPS. D, LBP binds to OxPAPC and LPS immobilized in 96-well dish. E, OxPLs resuspended in buffer compete for binding of LBP to dishes coated with LPS. F, Oxidation is a prerequisite for the ability of OxPAPC to prevent LPS/LBP binding.

(Figure 5B). The complex formed by OxPAPS and sCD14 apparently was noncovalent as indicated by the lack of sCD14 bandshift in denaturing SDS-gels (supplemental Figure VB), previously shown to separate covalent adducts of oxidized lipids with proteins from intact proteins.²⁴ These data suggest that OxPLs formed noncovalent complexes with sCD14 characterized by slow dissociation rate that were stable during the time of gel-electrophoresis (about 3 hours).

The experiments on OxPL/sCD14 binding (Figure 5A and 5B; supplemental Figure VA) were performed in artificial buffer containing no other lipids or proteins. To test whether an OxPL/sCD14 complex can be formed in the presence of the high physiological concentrations of lipids and proteins observed in normal plasma, we incubated human plasma with several classes of oxidized and native PLs, separated proteins

in native gels, and detected endogenous sCD14 by Western blotting. In contrast to recombinant sCD14, sCD14 present in human plasma migrated as several isoforms that all were detected by 3 different monoclonal antibodies to CD14 (not shown), and could be removed from plasma by immune adsorption using well-characterized anti-CD14 mAb, MEM-18 (supplemental Figure VC). OxPAPE and OxPAPS, but not OxPAPC or unoxidized PLs, induced shifts in mobility of sCD14 (Figure 5C). The interaction of OxPLs with sCD14 was specific because OxPAPS did not change general pattern of plasma proteins migration in nondenaturing gel (supplemental Figure VD). Similar bandshift of sCD14 was observed in murine plasma collected a few minutes after i.v. injection of OxPAPS (Figure 5D). These data show that binding of OxPLs to sCD14 is characterized by high selectivity as compared to other proteins, and rapidly occurs within the physiological milieu of human and mouse plasma.

The results presented in Figure 5A through 5D demonstrate formation of a complex between OxPLs and sCD14; however, they do not answer whether this binding prevents interaction of sCD14 with LPS. To elucidate this question, we performed competitive assay and tested whether OxPLs inhibit bandshift induced by LPS. Indeed, several classes of OxPLs inhibited formation of a band characteristic of LPS/sCD14 complex suggesting that OxPAPS and LPS compete for binding to the same or adjacent site(s), and cannot bind to the same sCD14 molecule simultaneously (Figure 5E).

Discussion

In this work we compared, under standard experimental conditions, the antiendotoxin activities of several OxPLs. The major conclusion is that oxidized products generated from different classes and molecular species of PUFA-PLs produce qualitatively similar inhibition of the effects of LPS, thus pointing to variable oxidized *sn*-2 residues as the key determinant of the antiendotoxin activity. Such “relaxed” structural specificity suggests that multiple antiendotoxin species formed *in vivo* from different classes, and molecular species of PUFA-PLs may reach total concentrations sufficient to inhibit TLR4.

In this work we addressed a hypothesis that OxPLs are weak agonists for TLR4 that inhibit action of a strong agonist, LPS, but at the same time induce low-grade activation of this receptor. We did not observe induction of TLR4 downstream genes related to acute inflammation (E-selectin, TNF α , IL-1 α , IL-1 β , COX-2, IL-6) by OxPLs that were tested in several systems ranging from natural cell populations (whole blood) to individual primary cell types (HUVECs, blood monocytes, fibroblasts) or highly artificial HEK cells transfected with TLR4 and MD-2. Previously, we showed that OxPLs inhibit LPS-induced upregulation of VCAM-1 and ICAM-1, which are key molecules mediating extravasation of leukocytes.⁹ In addition, it has been shown that OxPL-induced synthesis of IL-8 is independent of TLR4.²⁵ Altogether, the data imply that OxPLs are not canonical agonists of TLR4 inducing the same set of inflammatory genes as LPS or agonistic lipid A species. Rather, the results substantiate the role for OxPLs as potential antagonists of LPS in acute inflammation. However, these data do not rule out that some

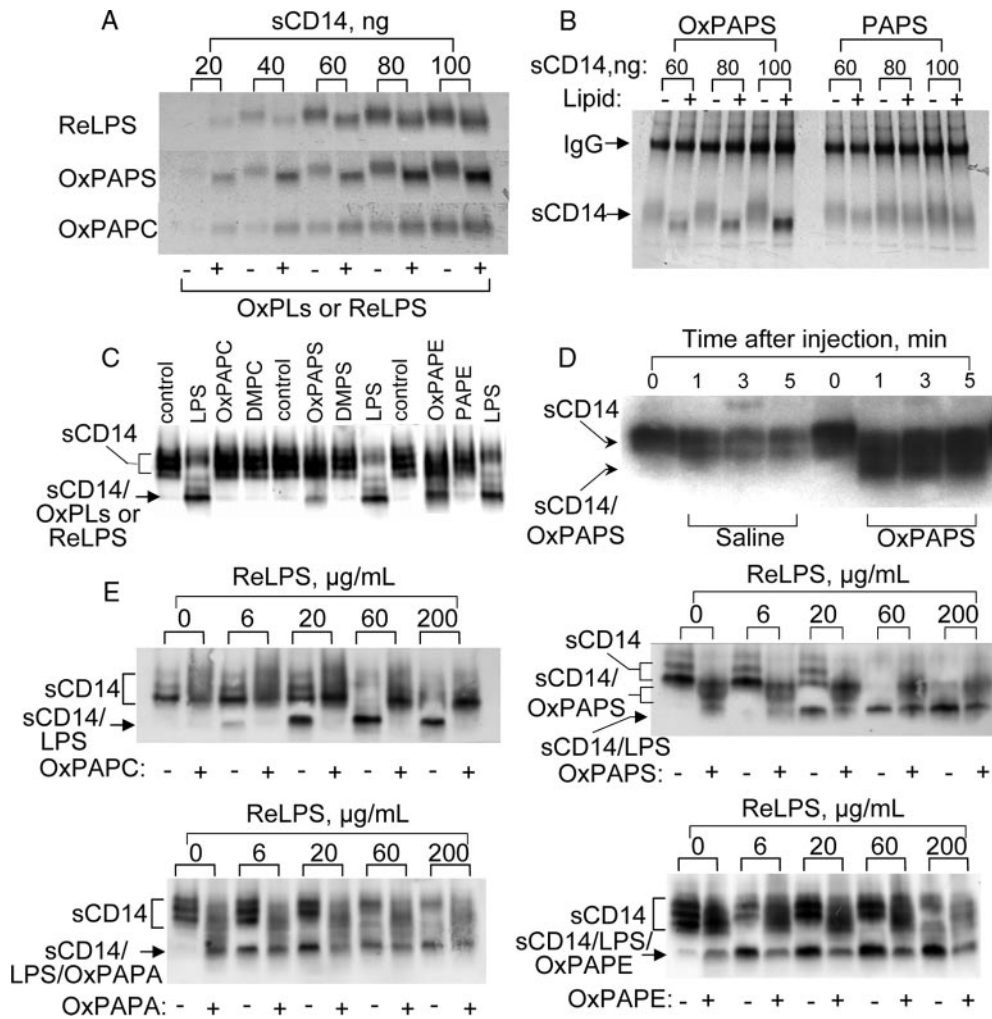


Figure 5. Binding of OxPLs and LPS to sCD14 is mutually exclusive. A and B, Recombinant sCD14 or nonimmune IgG were preincubated with ReLPS or phospholipids, separated by nonreducing PAGE and stained with silver. C through E, Results of nonreducing PAGE and Western blotting with anti-CD14 of human plasma mixed with phospholipids (C), plasma of mice injected with OxPAPS (D), or human plasma preincubated with OxPLs and ReLPS (E).

other genes may be regulated by OxPLs via TLR4 acting in combination with other receptors or signaling adaptors.

An important finding of this study is that OxPLs inhibit on the same cell type both extracellular (ie, present in blood plasma and extracellular fluid) and cell-associated steps in LPS recognition or signaling. According to our data, OxPLs inhibit in endothelial cells activity of both soluble factors (LBP and sCD14) and cell-associated components of TLR4 cascade. This model suggests that the maximal inhibition of LPS effects by OxPLs is achieved as a result of multiple “hits” at different levels. The multi-hit model reconciles previous conflicting views on intravascular extracellular targets of TLR4 inhibition by OxPLs.²⁶

Finally, we characterized for the first time the mechanism of inhibition of LBP and sCD14 by OxPLs. Our data support the view that OxPLs bind to LBP and sCD14, and that the binding of OxPLs and LPS is mutually exclusive. Binding of OxPLs prevents interaction of these proteins with LPS, thus suppressing activation of TLR4. In other words, OxPLs are not LPS “scavengers” that bind LPS and mask it from receptors, but they act analogously to receptor antagonists.

The data from several laboratories show that OxPLs accumulate under a variety of inflammatory conditions, including acute lung inflammation^{11,27} and atherosclerosis.⁵⁻⁷ Concentrations of oxidized species reach intracellularly, locally within tissues, or in circulation micromolar concentrations^{7,28,29} comparable to those inhibiting TLR4 in vitro. These data support the notion that TLR4 antagonism by OxPLs may represent relevant negative feedback in acute inflammation. According to this model, OxPLs generated as a by-product of oxidative burst inhibit activation of TLRs and shut down inflammatory reactions. However, uncontrolled accumulation of OxPLs is likely to induce proinflammatory effects and thus to promote chronic inflammation. Further studies are required to characterize the factors determining the balance of pro- and antiinflammatory effects of OxPLs.

Acknowledgments

The authors thank Mario Hilpert for technical assistance.

Sources of Funding

The work was supported by the grants from Fonds zur Förderung wissenschaftlicher Forschung (P18232-B11 and P20801-B11 to

V.N.B.), Österreichischer Forschungsförderungsgesellschaft (project 815445 to V.N.B.), and Austrian National Bank (project 12532ONB to O.V.O.).

Disclosures

None.

References

- Fukai M, Hayashi T, Yokota R, Shimamura T, Suzuki T, Taniguchi M, Matsushita M, Furukawa H, Todo S. Lipid peroxidation during ischemia depends on ischemia time in warm ischemia and reperfusion of rat liver. *Free Radic Biol Med*. 2005;38:1372–1381.
- Tyurina YY, Tyurin VA, Epperly MW, Greenberger JS, Kagan VE. Oxidative lipidomics of gamma-irradiation-induced intestinal injury. *Free Radic Biol Med*. 2008;44:299–314.
- Ando K, Beppu M, Kikugawa K. Evidence for accumulation of lipid hydroperoxides during the aging of human red blood cells in the circulation. *Biol Pharm Bull*. 1995;18:659–663.
- Matsura T, Togawa A, Kai M, Nishida T, Nakada J, Ishibe Y, Kojo S, Yamamoto Y, Yamada K. The presence of oxidized phosphatidylserine on Fas-mediated apoptotic cell surface. *Biochim Biophys Acta*. 2005;1736:181–188.
- Gniwotta C, Morrow JD, Roberts LJ, Kuhn H. Prostaglandin F₂-like compounds, F₂-isoprostanes, are present in increased amounts in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 1997;17:3236–3241.
- Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugiu B, Fox PL, Hoff HF, Salomon RG, Hazen SL. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J Biol Chem*. 2002;277:38503–38516.
- Watson AD, Leitinger N, Navab M, Faull KF, Horkko S, Witztum JL, Palinski W, Schwenke D, Salomon RG, Sha W, Subbanagounder G, Fogelman AM, Berliner JA. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J Biol Chem*. 1997;272:13597–13607.
- Berliner JA, Gharavi NM. Endothelial cell regulation by phospholipid oxidation products. *Free Radic Biol Med*. 2008;45:119–123.
- Bochkov VN, Kadl A, Huber J, Gruber F, Binder BR, Leitinger N. Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. *Nature*. 2002;419:77–81.
- Jyrkkanen HK, Kansanen E, Inkala M, Kivela AM, Hurttala H, Heimonen SE, Goldsteins G, Jauhiainen S, Tiainen S, Makkonen H, Oskolkova O, Afonyushkin T, Koistinaho J, Yamamoto M, Bochkov VN, Yla-Herttuala S, Levonen AL. Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. *Circ Res*. 2008;103:e1–e9.
- Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van LG, Ermolaeva M, Veldhuizen R, Leung YH, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JS, Slutsky AS, Akira S, Hultqvist M, Holmdahl R, Nicholls J, Jiang C, Binder CJ, Penninger JM. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. 2008;133:235–249.
- Miller YL, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, Witztum JL. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *J Biol Chem*. 2003;278:1561–1568.
- Walton KA, Cole AL, Yeh M, Subbanagounder G, Krutzik SR, Modlin RL, Lucas RM, Nakai J, Smart EJ, Vora DK, Berliner JA. Specific phospholipid oxidation products inhibit ligand activation of toll-like receptors 4 and 2. *Arterioscler Thromb Vasc Biol*. 2003;23:1197–1203.
- Walton KA, Gugiu BG, Thomas M, Basseri RJ, Eliav DR, Salomon RG, Berliner JA. A role for neutral sphingomyelinase activation in the inhibition of LPS action by phospholipid oxidation products. *J Lipid Res*. 2006;47:1967–1974.
- Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. *J Biol Chem*. 2008;283:24748–24759.
- Hawkins LD, Ishizaka ST, McGuinness P, Zhang H, Gavin W, DeCosta B, Meng Z, Yang H, Mullarkey M, Young DW, Yang H, Rossignol DP, Nault A, Rose J, Przetak M, Chow JC, Gusovsky F. A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity. *J Pharmacol Exp Ther*. 2002;300:655–661.
- Kitchens RL, Thompson PA, Munford RS, O'Keefe GE. Acute inflammation and infection maintain circulating phospholipid levels and enhance lipopolysaccharide binding to plasma lipoproteins. *J Lipid Res*. 2003;44:2339–2348.
- Murch O, Collin M, Hinds CJ, Thiemermann C. Lipoproteins in inflammation and sepsis. I. Basic science. *Intensive Care Med*. 2007;33:13–24.
- Wurfel MM, Wright SD. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. *J Immunol*. 1997;158:3925–3934.
- Gordon BR, Parker TS, Levine DM, Feuerbach F, Saal SD, Sloan BJ, Chu C, Stenzel KH, Parrillo JE, Rubin AL. Neutralization of endotoxin by a phospholipid emulsion in healthy volunteers. *J Infect Dis*. 2005;191:1515–1522.
- Akashi S, Saitoh S, Wakabayashi Y, Kikuchi T, Takamura N, Nagai Y, Kusumoto Y, Fukase K, Kusumoto S, Adachi Y, Kosugi A, Miyake K. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med*. 2003;198:1035–1042.
- Scott MG, Vreugdenhil AC, Buurman WA, Hancock RE, Gold MR. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J Immunol*. 2000;164:549–553.
- Hailman E, Lichtenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med*. 1994;179:269–277.
- Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor. *J Biol Chem*. 2001;276:24473–24481.
- Erridge C, Webb DJ, Spickett CM. Toll-like receptor 4 signalling is neither sufficient nor required for oxidised phospholipid mediated induction of interleukin-8 expression. *Atherosclerosis*. 2007;193:77–85.
- Mackman N. How do oxidized phospholipids inhibit LPS signaling? *Arterioscler Thromb Vasc Biol*. 2003;23:1133–1136.
- Yoshimi N, Ikura Y, Sugama Y, Kayo S, Ohsawa M, Yamamoto S, Inoue Y, Hirata K, Itabe H, Yoshikawa J, Ueda M. Oxidized phosphatidylcholine in alveolar macrophages in idiopathic interstitial pneumonias. *Lung*. 2005;183:109–121.
- Gruber F, Oskolkova O, Leitner A, Mildner M, Mlitz V, Lengauer B, Kadl A, Mrass P, Kronke G, Binder BR, Bochkov VN, Leitinger N, Tschachler E. Photooxidation generates biologically active phospholipids that induce heme oxygenase-1 in skin cells. *J Biol Chem*. 2007;282:16934–16941.
- Podrez EA, Byzova TV, Febbraio M, Salomon RG, Ma Y, Valiyaveetil M, Poliakov E, Sun M, Finton PJ, Curtis BR, Chen J, Zhang R, Silverstein RL, Hazen SL. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nat Med*. 2007;13:1086–1095.