Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36

Eugene A. Podrez¹, Eugenia Batyreva², Zhongzhou Shen¹, Renliang Zhang¹,⁴, Yijun Deng², Mingjiang Sun², Paula J. Finton¹, Lian Shan¹,³, Bogdan Gugiu², Henry F. Hoff¹,³, Robert G. Salomon² and Stanley L. Hazen¹,³,⁴

¹Department of Cell Biology and ⁴Department of Cardiovascular Medicine, Cleveland Clinic Foundation, Cleveland, OH
²Department of Chemistry, Case Western Reserve University, Cleveland, OH
³Department of Chemistry, Cleveland State University, Cleveland, OH
⁴Center for Cardiovascular Diagnostics and Prevention, Preventive Cardiology Section, Cleveland Clinic Foundation, Cleveland, OH

Address correspondence to: Stanley L. Hazen, Cleveland Clinic Foundation, Lerner Research Institute, Department of Cell Biology, 9500 Euclid Ave., NC-10, Cleveland, OH 44195, Tel: 216/445-9763; Fax: 216/444-9404
email: hazens@ccf.org; home page URL: http://www.lerner.ccf.org/ri/pi/hazens.html

Abbreviations: acLDL, acetylated LDL; apo, apolipoprotein; BHT, butylated hydroxytoluene; CE, cholesteryl ester; Cu²⁺oxLDL, Cu²⁺-oxidized LDL; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DMEM, Dulbecco's Modified Eagle's Medium; DMPC, 1,2 ditetradecanoyl-sn-glycero-3-phosphocholine; DTPA, diethylenetriamine pentaacetic acid; ELS, evaporative light scattering; GGOx, glucose-glucose oxidase; FCS, fetal calf serum; G-PC and ND-PC, the glutaric and nonanedioic monoesters of 2-lysoPC; HDdiA-PC and HOdiA-PC, the 9-hydroxy-10-dodecenedioic acid and 5-hydroxy-8-oxy-6-octenedioic acid esters of 2-lysoPC; HODA-PC and HOOA-PC, the 9-hydroxy-12-oxo-10-dodecanoic acid and 5-hydroxy-8-oxy-6-octenoic acid esters of 2-lysoPC; H₂O₂, hydrogen peroxide; KODA-PC and KOOA-PC, the 9-keto-12-oxo-10-dodecanoic acid and 5-keto-8-oxy-6-octenoic acid esters of 2-lysoPC; KDdiA-PC and KOdiA-PC, the 9-keto-10-dodecenedioic acid and 5-keto-octene-6-octenoic acid esters of 2-lysoPC; LC/MS/MS, HPLC with on-line electrospray ionization tandem mass spectrometry; LDL, low density lipoprotein; LPDS, lipoprotein deficient serum; MPM, mouse peritoneal macrophage; MPO, myeloperoxidase; MRM, multiple reaction monitoring; NO₂⁻, nitrite; Lyso-PC, 1-palmitoyl-sn-glycero-3-phosphocholine; NO₂-LDL, LDL modified by the MPO-H₂O₂-NO₂⁻ system; NO₂-PAPC, PAPC vesicles modified by the MPO-H₂O₂-NO₂⁻ system; NMR, nuclear magnetic resonance; OV-PC and ON-PC, the 5-oxovaleric acid and 9-oxononanoic acid esters of 2-lysoPC; oxPCCD36, oxidized phosphatidyl choline species that bind with high affinity to CD36; PAPC, 1-hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-sn-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-octadec-9'-enoyl-sn-glycero-3-phosphocholine; PLPC, 1-hexadecanoyl-2-octadecadi-9',12'-enoyl-sn-glycero-3-phosphocholine; PS, phosphatidyl serine; RNS, reactive nitrogen species, SR-AI, scavenger receptor class A type I; TSP-1, thrombospondin-1.

Running Title: Identification of oxidized lipid ligands for CD36

Key Words: CD36, scavenger receptor, oxidized phospholipid, atherosclerosis, myeloperoxidase
Abstract

The macrophage scavenger receptor CD36 plays an important role in the uptake of oxidized forms of LDL and contributes to lesion development in murine models of atherosclerosis. However, the structural basis of CD36 lipoprotein ligand recognition is unknown. We now identify a novel class of oxidized phospholipids that serve as high affinity ligands for CD36 and mediate recognition of oxidized forms of LDL by CD36 on macrophages. Small unilamellar vesicles of homogeneous phosphatidylcholine (PC) molecular species were oxidized by the myeloperoxidase (MPO)-H2O2-NO2− system, and products were separated by sequential HPLC with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). In parallel, fractions were tested for their ability to bind to CD36. Four major structurally-related phospholipids with CD36 binding activity were identified from oxidized 1-hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-sn-glycero-3-phosphocholine (PAPC), and four corresponding structural analogs with CD36 binding activity were identified from oxidized 1-hexadecanoyl-2-octadecadi-9',12'-enoyl-sn-glycero-3-phosphocholine (PLPC). Each was then synthetically prepared, its structure confirmed by multinuclear NMR and high resolution mass spectrometry, and shown to possess identical CD36 binding activity and LC/ESI/MS/MS characteristics in both native and derivatized forms. Based upon the structures of the active compounds identified, and structure-function studies with a variety of synthetic analogs, we conclude that the structural characteristics required for high affinity binding of oxidized PC species to CD36 are a phospholipid with an sn-2 acyl group that incorporates a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl (oxPC_{CD36}). LC/ESI/MS/MS studies demonstrate that oxPC_{CD36} are formed during LDL oxidation by multiple distinct pathways. We conclude that formation of this novel class of oxidized PC species contributes to CD36-mediated recognition of lipoproteins oxidized by MPO and other biologically relevant mechanisms. Further, the present results offer structural insights into the molecular patterns recognized by the scavenger receptor CD36 and provide a platform for the development of potential therapeutic inhibitory agents.
INTRODUCTION

CD36 is a heavily glycosylated, single chain, integral plasma membrane protein that belongs to an evolutionarily conserved family of proteins that serve as scavenger and lipid receptors (1,2). It is expressed on the surface of adipocytes, microvascular endothelial cells, macrophages, platelets, and specialized epithelial cells (1,2). CD36 functions \textit{in vivo} in scavenger recognition of oxidized lipoproteins and senescent or apoptotic cells, fatty acid transport, cell-matrix interactions, and anti-angiogenic actions (3-5). Its deficiency in humans has been correlated with alterations in myocardial fatty acid uptake, hypertrophic cardiac myopathy and insulin resistance (6-8). Recent studies have focused attention on CD36 as a participant in the atherosclerotic process because of its ability to recognize oxidized forms of LDL (oxLDL) (3,4,9-11). CD36 mediates lipid accumulation and macrophage foam cell formation \textit{in vitro} and \textit{in vivo} (3,12,13). It is heavily expressed in lipid rich atheroma and CD36 knockout mice demonstrate a dramatic decrease in lesion progression (9). In addition, uptake of oxLDL through CD36 plays a role in differentiation of monocytes and in the induction of nuclear receptors such as the peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$), a receptor that participates in lipid and carbohydrate metabolism (12,14-16).

We recently described a pathway for oxidative modification of LDL by the myeloperoxidase (MPO)-H$_2$O$_2$-NO$_2^-$ system of monocytes (17). LDL modified by MPO-generated reactive nitrogen species (NO$_2$-LDL) is avidly taken up and degraded by macrophages \textit{in vitro}, leading to cholesterol deposition and foam cell formation. The macrophage scavenger receptor CD36 is responsible for recognition of NO$_2$-LDL and is essential for foam cell formation in this model (10). The pathway appears physiologically plausible for several reasons. First, a number of studies demonstrated that enzymatically active MPO accumulates in subendothelial space, leading to MPO-dependent nitration and chlorination of targets (18-22). Second, recent genetics and clinical studies further suggest a role for MPO in development of atherosclerosis in human subjects. A cross sectional analysis of nearly 100 individuals with MPO deficiency showed that MPO deficient subjects have a reduced rate of cardiovascular disease (23). Similarly, decreased prevalence of atherosclerosis was recently reported for subjects containing a single nucleotide polymorphism in the promoter region of the MPO gene that results in decreased expression in reporter constructs \textit{in vitro} (24). Third, we have demonstrated that MPO-generated reactive nitrogen species convert LDL into a form recognized by CD36 at
Identification of oxidized lipid ligands for CD36

pathophysiologically concentrations of nitrite and in the presence of serum constituents, in contrast to LDL oxidation by free transition metal ions (e.g. Cu$^{2+}$) (10). Moreover, studies examining peroxidation of endogenous plasma lipids by activated leukocytes isolated from normal and MPO-deficient subjects strongly supports a role for the MPO-H$_2$O$_2$ system of human leukocytes as a physiological mechanism for initiating lipid peroxidation in vivo (25). Finally, a recent clinical study identified MPO levels in blood and leukocytes as strong independent predictors of coronary artery disease in angiographically defined subjects (26).

Although the scavenger receptor functions of CD36 are well documented, the exact molecular structure(s) of the ligand(s) recognized by CD36 remain unknown. Oxidized lipids were first suggested to participate in recognition of oxLDL by mouse peritoneal macrophages (MPM) based on studies using liposomes generated from lipid extracts of LDL that was extensively oxidized by Cu$^{2+}$ (Cu$^{2+}$-oxLDL) (27). A role for CD36 as the receptor responsible for recognition of oxidized lipids extracted from Cu$^{2+}$-oxLDL was subsequently shown (10,11). Oxidized phospholipids covalently linked to apolipoprotein B-100 (apoB) in extensively oxidized LDL (e.g. Cu$^{2+}$-oxLDL) have also been suggested to serve as ligands for CD36 based upon indirect competition studies using a monoclonal antibody to oxidized PAPC-protein adducts and either reconstituted apoB from Cu$^{2+}$-oxLDL or adducts of BSA with the aldehydic phospholipid 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (OV-PC), an oxidation product of PAPC (11,28,29). We have shown that the lipid portion of LDL and PAPC vesicles that are mildly oxidized by the MPO-H$_2$O$_2$-NO$_2^-$ system serve as ligands for CD36 using stable transfected cells and mouse peritoneal macrophages (MPM) from wild type vs. CD36 knockout mice (10). Conversion of LDL into a ligand for CD36 is a very early event during LDL oxidation in this system, occurring before substantial modification of apoB, as monitored by loss of free lysine residues and alteration in relative electrophoretic mobility (10).

While studies thus far clearly support the notion that oxidized phospholipids play a major role in the binding of oxLDL forms (including NO$_2$-LDL) to CD36, particularly early in the oxidation process, the precise nature of the lipid ligand or ligands within the lipid phase of oxLDL species have not yet been identified. We now report the first systematic study aimed at directly identifying the structures of specific oxidized phospholipids that serve as high affinity ligands for the scavenger receptor CD36.
EXPERIMENTAL PROCEDURES

Materials

Tissue culture media and additives were purchased from Life Technologies (Gaithersburg, MD). Na$^{[125]}$I and $[^{14}C]$oleate were supplied by ICN Pharmaceutical, Inc. (Costa Mesa, CA). $[^{14}C]$cholesterol, $[^{14}C]$PAPC and $[^{3}H]$ DPPC were from American Radiolabeled Chemicals, Inc., (St. Louis, MO), and $[^{3}H]$cholesteryl linoleate was from Du Pont NEN (Boston, MA). C57BL/6 mice (16-20 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, NY). 1-Hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-sn-glycero-3-phosphocholine (PAPC), 1-hexadecanoyl-2-octadec-9'-enoyl-sn-glycero-3-phosphocholine (POPC) and 1-hexadecanoyl-2-octadec-9',12'-dienoyl-sn-glycero-3-phosphocholine (PLPC), phoshatidyl serine (PS) and 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-CD36 monoclonal antibody, FA6-152, was purchased from Immunotech (Westbrook, ME). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Methods

General procedures. Human myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) and LDL were isolated and quantified as described (17). All buffers were treated with Chelex-100 resin (Bio-Rad, Hercules, CA) and supplemented with diethylenetriaminepentaacetic acid (DTPA) to remove trace levels of transition metal ions that might catalyze LDL oxidation during incubations. LDL was labeled with Na$^{[125]}$I to a specific activity between 100 and 250 dpm/ng protein, as described (30). Extraction of cellular lipids and thin-layer chromatography separation of radiolabeled cholesterol esters were performed as described (17). Incorporation of $[^{14}C]$oleate into cholesteryl esters by cells following incubation with the indicated lipoproteins (50 µg/ml), were determined as described (17).

Synthesis of phospholipids. Total syntheses of the $\gamma$-hydroxy-\(\alpha,\beta\)-unsaturated aldehydic phospholipids, the 9-hydroxy-12-oxo-10-dodecenoic acid and 5-hydroxy-8-oxo-6-octenoic acid esters of 2-lysoPC (HODA-PC and HOOA-PC, respectively) (31), the $\gamma$-keto-\(\alpha,\beta\)-unsaturated aldehydic phospholipids, the 9-keto-12-oxo-10-dodecenoic acid and 5-keto-8-oxo-6-octenoic acid esters of 2-lysoPC (KODA-PC and KOOA-PC, respectively), as well as the analogous carboxylic phospholipids, the 9-hydroxy-10-dodecenedioic acid and 5-hydroxy-8-oxo-6-octenedioic acid esters of 2-lysoPC (HDdiA-PC and HOdiA-PC, respectively), and the 9-keto-10-dodecenedioic acid and 5-keto-6-
octendioic acid esters of 2-lysoPC (KDdiA-PC and KODiA-PC, respectively) were performed as
described elsewhere (32). Saturated aldehydic phospholipids, the 5-oxovaleric acid and 9-oxononanoic
acid esters of 2-lysoPC (OV-PC and ON-PC, respectively) were synthesized from stable phospholipid
precursors containing a dimethylacetal-protected carbonyl that was deprotected in the presence of
catalytic amounts of acidic ion-exchange resin Amberlyst-15 (29). Their carboxylic analogs, the glutaric
and nonanedioic monoesters of 2-lysoPC (G-PC, ND-PC, respectively), were prepared by coupling 2-
lyso-PC with the corresponding acid anhydride (29,32). Purification was achieved by flash silica
column chromatography or HPLC, as described elsewhere (29,32). The structures of all synthetic lipids
were confirmed by multinuclear NMR and high resolution mass spectrometry prior to use (29,31,32).
Synthetic lipids were routinely analyzed by HPLC with on-line electrospray ionization tandem mass
spectrometry. If lipids were found to be less than 98% pure, they were re-isolated prior to use.

Vesicle preparation and modification. Stock solutions (2 mg/ml) of small unilamellar vesicles
comprised of PLPC, POPC or PAPC with varying mol % of specific oxidized phospholipids were
prepared in argon-sparged sodium phosphate buffer by extrusion (ten times) through a 0.1 µm
polycarbonate filter using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL) at
37°C. For direct binding experiments, [3H]DPPC (25 µCi/mg of phospholipids) or 1 mol % of the
fluorescent dye DiI was added to phospholipids. CD36 ligands were isolated from PAPC or PLPC
vesicles (0.2 mg lipid/ml) following incubation with MPO (30 nM), an H2O2–generating system
(constant flux of 0.80 µM/min) comprised of glucose (100 µM) and glucose oxidase (100 ng/ml), and
NaNO2 (0.5 mM) at 37°C for 20 h. Reactions were stopped by the addition of BHT (50 µM) and
catalase (300 nM) and stored under argon atmosphere at -80°C.

Lipoprotein modification. LDL modified by MPO-generated nitrating intermediates (NO2-LDL)
was formed by incubating LDL (0.2 mg protein/ml) at 37°C in 50 mM sodium phosphate, pH 7.0, 100
µM DTPA, 30 nM MPO, 100 µg/ml glucose, 20 ng/ml glucose oxidase and 0.5 mM NaNO2 for 8 h.
Oxidation reactions were terminated by addition of 40 µM BHT and 300 nM catalase to the reaction
mixture. LDL acetylation was performed as described earlier (17). Oxidation of LDL (0.2 mg
protein/ml) by copper was performed by dialysis vs. 5 µM CuSO4 in PBS for 24 h at 37°C. Oxidation
was terminated by addition of BHT (40 µM) and DTPA (100 µM) and dialysis against PBS containing
DTPA (100 µM). Oxidation of LDL by ceruloplasmin-bound copper was performed as described (33).

**Cells.** Thioglycollate-elicited mouse peritoneal macrophages (MPM) from wild-type (C57BL/6) were isolated and cultured as described (17). Human foreskin fibroblasts were cultured as described previously (17). CHO cells expressing mouse scavenger receptor class A type I (CHO-mSR-AI) and control vector-transfected parental LDL receptor-negative CHO cells were a generous gift from Dr. M. Krieger (MIT, Boston, MA) (34). Experiments with CHO-mSR-AI were performed on confluent cell monolayers in HAM’s F-12 medium containing 3% lipoprotein deficient fetal calf serum, BHT (20 µM), DTPA (100µM), and catalase (300 nM). 293 cells (embryonic kidney epithelial cells transformed with adenovirus) were obtained from ATCC (Rockville, MD) and maintained in DMEM with 5% FCS. CD36 expressing 293 cells were a generous gift from Dr. W. Frazier (Washington University, St. Louis, MO) (35). 293 transfected cells were grown in the presence of G418 (500 µg/ml), clones were isolated, and expression of CD36 confirmed by FACS analysis using the monoclonal antibody FA6-152. Experiments with 293 cells and CHO cells were performed on confluent cell monolayers in the appropriate culturing media containing 200 µg/ml LDL, BHT (20 µM), DTPA (100 µM), and catalase (300 nM).

**Phospholipid separation, CD36 competition assay and mass spectrometric analysis.** Lipids were maintained under inert atmosphere (argon or nitrogen) at all times. Lipids from either oxidized PAPC or PLPC vesicles, or from NO\_2-LDL, were extracted three times sequentially by the method of Bligh and Dyer (36) immediately after adding an equal volume of saturated NaCl solution (to enhance lipid extraction). The combined chloroform extracts were evaporated under nitrogen, and lipids were then resuspended in methanol (at approximately 200 µg/0.1 mL), filtered through an Acrodisc CR PTFE filter and applied on a reverse-phase column (Luna C18, 250x10 mm, 5µm, Phenomenex, Torrence, CA, USA). Lipids were resolved at a flow rate of 3 mL/min using a ternary (acetonitrile/methanol/H\_2O) gradient (Gradient I, below) generated by a Waters 600 E Multisolvent delivery system HPLC (Waters, Milford, MA, USA), and monitored using an evaporative light scattering detector (Sedex 55, Sedere, Alfortville, France). Gradient I:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>12</th>
<th>20</th>
<th>30</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile (%)</td>
<td>65</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>
Identification of oxidized lipid ligands for CD36

MeOH (%)  20  20  23  30  30  
H2O (%)  15  10  7  0  0

The ability of lipids within collected fractions to block \(^{125}\text{I}\)-NO\(_2\)LDL binding to CD36 was then examined as follows. Lipids were rapidly extracted into chloroform, dried under N\(_2\), resuspended in 20 µl PBS with 10% ethanol and further diluted (final ethanol concentration ≤ 0.5%) in DMEM containing \(^{125}\text{I}\)-NO\(_2\)LDL (5 µg/ml), 5% FCS, 200 µg/ml LDL, BHT (20 µM), DTPA (100 µM), and catalase (300 nM) (10). The resulting mixture was incubated with CD36-transfected 293 cells for 3 h at 4°C, and then unbound \(^{125}\text{I}\)-NO\(_2\)LDL removed by washing with ice cold PBS. The amount of bound \(^{125}\text{I}\)-NO\(_2\)LDL was then determined. CD36 independent binding of \(^{125}\text{I}\)-NO\(_2\)LDL was assessed in vector-transfected 293 cells and subtracted from binding to CD36-transfected 293 cells as a background. The binding to vector-transfected cells was typically less than 10-20 % of that to CD36 transfected cells. Each measurement was performed in triplicate and % of inhibition was calculated as follows: 100 x (binding in the absence of competitor - binding in the presence of competitor / binding in the absence of competitor).

Further fractionation and isolation of bioactive lipids was performed on combined lipid extracts from three separations that were dried under N\(_2\), resuspended in chloroform (300 µl) supplemented with BHT, and maintained under argon atmosphere. An aliquot of the fraction (2/3rds) was removed, evaporated under nitrogen and resuspended in HPLC buffer (methanol/water; 85/15; v/v) immediately prior to injection on reverse phase HPLC column. The remaining lipids were derivatized as described below (see Phospholipid derivatization section).

Mass spectrometric analyses were performed on a Quatro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, U.K.) equipped with an electrospray ionization (ESI) probe and interfaced with an HP 1100 HPLC (Hewlett-Packard, Wilmington, DE). Lipids (both free and following derivatization) were resolved on a Luna C18 250 x 4.6 mm, 5µm column (Phenomenex, Torrance, CA) at a flow rate of 0.8 ml/min. A discontinuous gradient (Gradient II) was used by mixing solvent A (methanol (MeOH):H\(_2\)O, 85:15, v:v) with solvent B (MeOH), as follows: isocratic elution with solvent A from 0-7 min; increasing to 88% solvent B from 7-10 min; increasing to 91% solvent B from 10-34 min; and then increasing to 94% solvent B from 34-52 min. The column effluent was split such that 45 µl/min was introduced to the mass spectrometer and 755 µl/min was collected and
analyzed for biological activity. In some cases, biological activity was also determined using the same gradient following injection of authentic standards. Mass spectrometric analyses were performed online using electrospray ionization tandem mass spectrometry (ESI/MS/MS) in the positive ion mode with multiple reaction monitoring (MRM) mode (cone potential 60 eV / collision energy 20-25 eV). The MRM transitions used to detect the oxidized phospholipids present in each fraction were the mass to charge ratio (m/z) for the molecular cation [MH]+ and the daughter ion m/z 184, the phosphocholine group (i.e. [MH]+ → m/z 184). Oxime derivatives of phospholipids were monitored at m/z [MH+29]+ → m/z 184.

Quantification of the various oxidized PC species was performed using LC/ESI/MS/MS in positive ion mode using MRM. Formic acid (0.1%) was included in the mobile phases. Distinct oxidized phospholipid species were identified by using m/z for protonated parent ion → daughter ion transitions specific for each individual phospholipid and their retention times, as illustrated in Figs. 2 and 3. OV-PC and ND-PC were quantified similarly but by also monitoring at the m/z for the transition between the hemiacetal formed with methanol for each analyte and the loss of polar head group (m/z 184).

Lipids were initially extracted three times by the method of Bligh and Dyer (36) from lipoproteins in the presence of BHT. The combined extracts were rapidly dried under nitrogen, resuspended in methanol:H2O (98:2, v:v), and then neutral lipids in the lipid extracts were removed by passage through a 18C minicolumn (Supelclean LC-18 SPE tubes, 3 ml; Supelco Inc., Bellefonte, PA). A known amount of dimyristyl phosphatidyl choline (DMPC) was added to the polar lipid fraction as an internal standard, and the lipids were dried under nitrogen and stored under an argon atmosphere at −80 °C until analysis within 24 h. Calibration curves were constructed with a fixed amount of DMPC and varying mol% of each synthetic oxidized PC species and used to correct for the differences in ionization response factors observed amongst the different lipids. In additional preliminary studies the quantification methods employed were independently validated for each analyte by demonstrating identical results to those obtained by the method of standard additions.

A key methodological issue was to develop techniques for quantification of PC species that prevented (and monitored for) any significant intrapreparative oxidation from occurring and artificially forming oxPC species. To achieve this it was necessary to manipulate lipids in the presence of BHT.
and under an argon or nitrogen atmosphere (in a glove box) whenever possible. An additional methodological problem was the desire to separate neutral lipids before analysis by LC/ESI/MS/MS while avoiding binding of lipids of interest to minicolumns (aminopropyl, silica, or C18 examined) since significant levels of artificial oxidation always occurred (in our hands) under these conditions, precluding meaningful quantitative results. This apparently occurred because of trace levels of contaminant transition metal ions present on the minicolumns. To avoid this problem, all aqueous solvents were chelex-100 rinsed prior to use. Further, all columns and filters were pre-rinsed with DTPA-containing solvents (at neutral pH) and polar lipids were collected as a flow-through from the 18C minicolumn, as described above. Independent studies with trace levels of synthetic oxPC demonstrated that under the conditions employed, polar lipids, such as each of the synthetic oxPC species quantified, were recovered in near quantitative yield within the column flow-through, while neutral lipids were retained on the disposable minicolumns. The prevention of intrapreparative oxidation during sample handling was confirmed by frequent spiking of samples with either parent lipid (PAPC or PLPC) or uniformly deuterated PAPC, monitoring at the appropriate m/z for parent → daughter transitions of products by MRM, and demonstrating either no significant difference in quantification of analyte (when spiking with the precursor PAPC or PLPC), or no significant detectable levels of deuterated analogs of the oxPC species (when spiking with deuterated PAPC). Moreover, in independent studies the overall recoveries of each synthetic lipid (when analyzed at the trace levels observed in biological samples with POPC carrier) was confirmed to be in excess of 80% under the conditions employed.

**Phospholipid derivatization.** Methoxylamine derivatives of HOOA-PC, HODA-PC, KODA-PC and KOOA-PC were prepared by resuspending 100 µg of standard in 1% methoxylamine-hydrochloride in 500 µl of 10 mM PBS (pH = 4.0) and incubating 1 h at 37 °C. Methoxylamine derivatives of the ketoacids, KOdiA-PC and KDdiA-PC, were prepared using 20% methoxylamine–HCl in the same buffer. After derivatization, lipids were extracted by the method of Bligh and Dyer (36), dried under N₂ and analyzed by LC/ESI/MS/MS with MRM. Biologically active fractions (equivalent to 300 µg of oxPAPC or oxPLPC) were dried under N₂ and incubated with 1% methoxylamine-HCl (for peaks II and III) or with 20% methoxylamine–HCl (peak I) as described for standards. After derivatization, lipids were extracted by the method of Bligh and Dyer, dried under N₂
and analyzed by LC/ESI/MS/MS with MRM in pure methanol. Biologically active fractions were analyzed under identical conditions. Pentafluorobenzyl ester derivatives of carboxylic acids were produced by resuspending dried lipids from HPLC fractions (or 100 µg of standard) in 200 µl of 10% pentafluorobenzyl bromide in dry acetonitrile and 200 µl of 20% solution of N,N-diisopropylethylamine in dry acetonitrile under an argon blanket. Pentafluorobenzyl ester derivatives were dried under anhydrous argon, resuspended in dry methanol, and analyzed by LC/ESI/MS/MS with MRM in pure (anhydrous) methanol. Biologically active fractions were analyzed under identical conditions. Lipids derivatizations with NaBH₄, NaCNBH₃ and dinitrophenylhydrazone were performed as described (37).

Statistics. Data represent the mean ± S.D. of the indicated number of samples. Statistical analyses were made using a paired Student’s t test. For all hypotheses, the significance level was 0.05.
RESULTS

Identification of oxidized phospholipids possessing CD36 binding activity. Our long-term goal was to define oxidized phospholipid species within oxLDL preparations that promote CD36 recognition. However, because of the complex lipid composition of LDL and the subsequent generation of a vast array of lipid oxidation products, initial structural characterization efforts focused on studies using defined model systems with homogeneous synthetic phospholipids as targets. The two most abundant molecular species of choline glycerophospholipids present in LDL, which incidentally possess readily oxidizable fatty acid constituents at their sn-2 position, were used (i.e. PAPC, 1-palmitoyl-2-arachidonyl sn-glycero-3-phosphocholine; and PLPC, 1-palmitoyl-2-linoleoyl sn-glycero-3-phosphocholine). To isolate the specific oxidized lipids within PAPC vesicles modified by the MPO-H$_2$O$_2$-NO$_2^-$ system (NO$_2$-PAPC vesicles) that serve as ligands for CD36, lipids were extracted (N.B. > 95% of the CD36 binding activity was extractable into the organic phase using the Bligh and Dyer procedure (36); data not shown), and then fractionated by reverse phase HPLC. Lipids within each fraction were then tested for their ability to inhibit binding of [¹²⁵I]NO$_2$-LDL to 293 cells stably transfected with human CD36. Three major peaks of CD36 binding activity (Peaks I, II and III) were observed (Fig.1a, upper panel). CD36 binding activity co-eluted with only minor lipid components, as detected by evaporative light scattering (Fig.1a, lower panel), indicating that only a small proportion of PAPC oxidation products could serve as ligands for CD36. Interestingly, there was no inhibitory activity in either the initial fractions that contain the vast majority of the polar oxidized lipids, including those that contain no phospholipid backbone (i.e. sn-2 cleavage products like 4-hydroxy-2-nonenal, 4-HNE), or in the latter fractions containing less oxidized and parent phospholipids. No significant CD36 binding activity was observed in fractions that co-eluted with synthetic standards of 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (OV-PC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (G-PC), oxidized phospholipids derived from PAPC (29) (Fig. 1a, lower panel, retention times b and a, respectively).

In a parallel set of experiments, lipids from PLPC vesicles modified by the MPO-H$_2$O$_2$-NO$_2^-$ system (NO$_2$-PLPC vesicles) were extracted into organic solvents and then the products fractionated by reverse phase HPLC. Fractions were then similarly analyzed for CD36 binding activity. Again, three peaks of CD36 binding activity were observed (Fig.1b, upper panel). No significant CD36
binding activity was detected in fractions that eluted at the retention times of the synthetic standards of ON-PC (1-palmitoyl-2-(9-oxonanonyl)-sn-glycero-3-phosphocholine) and ND-PC (1-palmitoyl-2-(8-carboxyoctanoyl)-sn-glycero-3-phosphocholine) (Fig. 1b, lower panel, retention times d and c, respectively), the structural analogs of the PAPC-derived bioactive oxidation products OV-PC and G-PC.

Identification of CD36 ligands in peaks I, II and III derived from NO$_2$-PAPC and NO$_2$-PLPC. Each fraction possessing CD36 binding activity was further subjected to sequential reverse phase HPLC separation with on-line electrospray ionization tandem mass-spectrometry (LC/ESI/MS/MS) using a distinct solvent system and elution gradient from that initially employed. The column eluent was split (45µl/min to the mass detector and the remainder to a fraction collector), and the capacity of lipids within the fractions to inhibit binding of [$^{125}$I]NO$_2$-LDL to CD36-transfected 293 cells was determined.

Molecular structures for the major biologically active constituents in Peaks I, II, and III derived from lipids in NO$_2$-PAPC and NO$_2$-PLPC vesicles were determined by a combination of tandem mass spectrometry (in both positive and negative ion modes) of active fractions, and unambiguous total syntheses by methods either recently described (31) or that will be reported elsewhere (32). The identities of the isolated oxidation products and synthetic standards were confirmed by derivatization with multiple agents (NaBH$_4$, NaBD$_4$, and NaCNBH$_3$ for reducible groups; methyl hydroxyl amine and dinitrophenyhydrazone for reactive carbonyl moieties; pentaflourobenzyl bromide for carboxyl groups), and subsequent tandem MS analysis in both positive ion and negative ion modes (data not shown).

The structures of the oxidized PC species possessing CD36 binding activity (oxPC$_{CD36}$) are shown in Fig. 1c. Of note, the structures identified for Peaks I, II and III isolated from oxidized PLPC species were the structural analogs of the respective lipids identified in Peaks I, II and III isolated from oxidized PAPC. They possessed identical functional groups and differed only by the chain length of the truncated fatty acid tethered to the sn-2 position (Fig. 1c). The core structural motif conserved amongst the various isolated oxidized PC species that support CD36-binding activity is shown in Fig. 1d. Peak II isolated from NO$_2$-PAPC and NO$_2$-PLPC vesicles represented the major overall fraction with CD36 binding activity observed during initial HPLC separation (Fig. 1a, 1b). The biologically
active lipid identified in each was a PC species with a truncated sn-2 fatty acid constituent possessing \( \alpha,\beta \)-unsaturated \( \gamma \)-hydroxy aldehyde moieties, analogous to 4-HNE. The 5-hydroxy-8-oxo-6-octenoic acid and 9-hydroxy-12-oxo-10-dodecenoic acid esters of 2-lysoPC (HOOA-PC and HODA-PC, respectively, Fig. 1c, Table 1) are likely generated during oxidative fragmentation of the polyunsaturated fatty acid by reactions that alternately liberate aliphatic cleavage products with \( \alpha,\beta \)-unsaturated \( \gamma \)-hydroxy aldehyde moieties, as in formation of 4-HNE (38). The active compounds in Peak III recovered from NO\(_2\)-PAPC and NO\(_2\)-PLPC vesicles are analogous to HOOA-PC and HODA-PC, but incorporate a \( \gamma \)-keto group instead of a \( \gamma \)-hydroxy group (KOOA-PC and KODA-PC, respectively). Peak I isolated from NO\(_2\)-PAPC and NO\(_2\)-PLPC vesicles each contained two constituents with CD36-binding activity (Fig. 1c). These represented the carboxylic acid analogs of the aldehydic PC species identified in Peaks II and III. The \( \alpha,\beta \)-unsaturated \( \gamma \)-keto carboxylic acid-containing PC species isolated from NO\(_2\)-PAPC and NO\(_2\)-PLPC vesicles, KOdiA-PC and KDdiA-PC, respectively (Fig. 1c, Table 1), accounted for the vast majority of CD36 binding activity in Peak I derived from PAPC and PLPC oxidized by the MPO-H\(_2\)O\(_2\)-NO\(_2\)- system.

Synthetic lipid standards were prepared for each oxPC species and their structures confirmed by high resolution mass spectrometry and multinuclear NMR (31,32). The identities of the oxidized lipids in fractions exhibiting CD36 binding activity were then confirmed by demonstrating: 1) that their chromatographic and mass-spectrometric characteristics are identical to those of the totally synthetic standards; 2) that derivatization of the isolated fractions resulted in loss of the appropriate m/z of the parent (non-derivatized) lipids as monitored by LC/ESI/MS/MS; 3) that the chromatographic and mass-spectrometric characteristics of the derivatized lipids within active fractions are identical to those of the derivatized synthetic standards; and 4) that the synthetic standards possess CD36 binding activity.

Data illustrating this approach for Peaks I, II and III (panels a,b and c, respectively) derived from NO\(_2\)-PAPC and NO\(_2\)-PLPC vesicles is shown in Figs. 2 and 3, respectively. For example, Fig. 2b represents a compilation of studies that identify the compound in Peak II from NO\(_2\)-PAPC vesicles possessing CD36 binding activity as HOOA-PC. Peak II from oxidized PAPC (Fig. 1a) was re-chromatographed on a second reverse phase HPLC column with a distinct solvent system. Column eluent was split and analyzed on-line with a mass detector (discussed below) or diverted to a fraction
Identification of oxidized lipid ligands for CD36

Lipids within each fraction were rapidly extracted, dried under N₂, reconstituted in phosphate buffered saline (PBS) in vesicles and tested for their ability to inhibit [¹²⁵I]NO₂-LDL binding to CD36 transfected cells (Fig. 2b, top tracing). One major (retention time ~19 min) and one minor (retention time ~8 min) peak of CD36 binding activity were found. The major peak of activity had an elution time identical to that of authentic synthetic HOOA-PC. The full scan positive ion mass spectrum of the fraction containing the peak of CD36 binding activity (retention time ~19 min) is illustrated (Fig.2b, inset to upper tracing). Only two major ions are observed with m/z 650 and m/z 672, consistent with the protonated [M+H]⁺ and sodiated [M+Na]⁺ forms of HOOA-PC. The middle panel of Fig. 2b demonstrates parallel LC/ESI/MS/MS analysis in the positive ion mode using multiple reaction monitoring (MRM) between the transition from the protonated parent ion [M+H]⁺ to the characteristic daughter ion generated by loss of the polar head group (m/z 650 → 184). The retention time observed (retention time ~19 min) was identical to that of synthetic HOOA-PC standard (data not shown). Derivatization of the lipids in Peak II with methyl hydroxylamine and subsequent LC/ESI/MS/MS analysis using positive ion MRM revealed loss of the parent lipid (observed while monitoring m/z 650 → 184; data not shown) and generation of two new ions while monitoring the transition m/z 679 → 184, which corresponds to the transition between the anticipated protonated parent ion of HOOA-PC methoxime [M+H]⁺ and a characteristic daughter ion. The retention times of the ions monitored (Fig.2b, lower trace) were identical to that of the syn and anti isomers of synthetic HOOA-PC methoxime standard. Moreover, parallel LC/MS (and tandem MS, data not shown) analyses demonstrated identical positive (and negative) ion mass spectra for lipids eluting concurrent with the retention times of synthetic syn and anti HOOA-PC methoximes, with m/z 679 and m/z 701, consistent with the protonated [M+H]⁺ and sodiated [M+Na]⁺ forms of HOOA-PC methoxime isomers, respectively (Fig. 2b, lower trace, inset). Finally, the minor peak of CD36 binding activity observed in Fig. 2b, upper panel (retention time ~8 min) was identified as HODiA-PC (by similar methods as outlined above). It was generated during handling of Peak II, owing to the ease of further oxidation of HOOA-PC, and its formation was avoided by manipulation of lipids at all times under an argon atmosphere. Similar studies were performed for each lipid oxidation product identified in Fig. 1c. Only data for the most abundant component in Peak I isolated from extracts of NO₂-PAPC and NO₂-
Identification of oxidized lipid ligands for CD36

PLPC vesicles, the \( \alpha,\beta \)-unsaturated \( \gamma \)-keto carboxylic acid analogs (KODiA-PC and KDdiA-PC), are shown.

Oxidation products of PC possessing sn-2 esterified \( \gamma \)-hydroxy(oxo)\( -\alpha,\beta \)-unsaturated carbonyl-containing fatty acid specifically bind to CD36. To demonstrate CD36-specific binding activity and compare the potency of the various isolated oxidized PC species, several distinct types of experiments were performed. First, each of the indicated specific oxidized lipids were mixed with their parent lipid as carrier (either PAPC or PLPC, Fig. 4a and 4b, respectively) and small unilamellar vesicles were prepared to normalize the manner of presentation of the distinct oxPC species. Varying amounts of vesicles were then tested for their capacity to block CD36-specific recognition of \([^{125}\text{I}]\text{NO}_2\)-LDL binding to CD36 transfected cells. Whereas vesicles comprised exclusively of non-oxidized lipids (PAPC or PLPC) failed to compete with the CD36 ligand, vesicles containing the various indicated oxPC species effectively blocked CD36-specific binding to a fixed amount of its labeled lipoprotein ligand (Fig. 4a, 4b). The most potent CD36 ligands identified were the keto acid analogs (KODiA-PC and KDdiA-PC), followed by the keto aldehyde analogs (KOOA-PC and KODA-PC). Similar results were observed in control experiments in which CD36-transfected cells were first pre-incubated with the vesicles, washed, and then \([^{125}\text{I}]\text{NO}_2\)-LDL binding immediately determined (data not shown). Vesicles containing the various indicated oxPC\textsubscript{CD36} species, but not those comprised exclusively of non-oxidized lipids (PAPC or PLPC), also blocked \([^{125}\text{I}]\text{NO}_2\)-LDL binding to primary cultures of thioglycolate-elicited mouse peritoneal macrophages from wild type C57BL/6 mice, which express CD36 (data not shown).

PC species possessing sn-2 esterified \( \gamma \)-hydroxy(oxo)\( -\alpha,\beta \)-unsaturated, carbonyl-containing fatty acids are recognized by CD36 in direct binding assays. To more directly demonstrate CD36 binding activity in the isolated lipids, we performed a parallel series of studies using direct binding assays (rather than competition assays). Each specific oxPC species was synthetically prepared and then incorporated into small unilamellar vesicles comprised of unoxidized parent lipid (PAPC or PLPC) as carrier and a tracer level of \([^3\text{H}]\text{1,2-dipalmitoyl-sn-glycero-3-phosphocholine}\) (DPPC). Vesicles were then tested for their capacity to specifically bind to CD36 transfected cells or control vector-transfected 293 cells. Whereas vesicles comprised of PAPC or PLPC alone failed to demonstrate CD36-specific binding, vesicles containing of the putative CD36 ligands bound to CD36 transfected
Identification of oxidized lipid ligands for CD36

...cells at significantly greater levels than control cells (Fig. 4c). The nature of lipids that were used as carriers for oxPC<sub>CD36</sub> did not appear to be critical for demonstrating CD36-specific recognition since comparable results were observed using various lipid carriers, including PAPC, PLPC (Fig. 4c), POPC or PC-cholesterol mixtures (up to 50 mol% cholesterol; data not shown). The rank order of CD36 binding activity amongst the isolated oxidized PC species was consistent with their CD36 inhibitory capacity noted in competition assays. Addition of the CD36-blocking monoclonal antibody FA6, but not isotype control antibody, blocked binding of the [3H]DPPC-doped vesicles containing any one of the isolated oxidized PC species, further confirming the specificity of binding for CD36 (Fig. 4d, only data for KOOA-PC and KODA-PC shown). Of note, oxidized species possessing a terminal carboxylic acid moiety bound more effectively to CD36 than those with aldehyde groups (e.g. HOOA-PC vs. HODiA-PC; Fig. 4c), and those possessing γ-oxo (carbonyl) vs. hydroxyl moieties also had highest affinity for CD36 (Fig. 4c). Thus, the 4-HNE analogs HOOA-PC and HODA-PC (derived from PAPC and PLPC, respectively), which likely possess HNE-like chemical reactivity and can potentially form covalent adducts with proteins via formation of Schiff bases or Michael adducts (39), were the least potent CD36 ligands within this class of novel lipids. In contrast, the more extensively oxidized γ-hydroxy (oxo), carboxylic acid containing species (HODiA-PC, KODiA-PC, HDdiA-PC and KDdiA-PC), which by comparison are relatively unreactive electrophiles, bound to CD36 more effectively (Fig. 4c).

To further demonstrate an oxPC<sub>CD36</sub>-CD36 interaction, vesicles consisting of 100% KODiA-PC doped with tracer amounts of [14C]-labeled DPPC were prepared and then used in direct binding studies. High affinity binding to CD36-transfected 293 cells, but not to vector transfected cells, was observed, and the addition of the CD36-specific mAb FA6, but not isotype-specific non-immune control IgG, blocked binding of the [14C]-labeled KODiA-PC vesicles (data not shown).

Structure-function studies to further characterize the structural requirements of oxidized PC species for binding to CD36. Synthetic analogs of the isolated oxidized PC species, as well as other lipids reported to be ligands for CD36 (e.g. long chain fatty acids, PS, OV-PC) were similarly evaluated for their CD36 binding activity in the competition assay to further evaluate structure-function relationships critical for CD36 recognition of oxidized lipid species (Table 1, Fig. 5). Neither 4-HNE nor cholesterol esters possessing oxidized fatty acids with α,β-unsaturated γ-hydroxy functional
Identification of oxidized lipid ligands for CD36

groups (e.g. synthetic HOOA-cholesterol or HODA-cholesterol) served as ligands for CD36 (either free or incorporated into lipid vesicles/liposomes +/- BSA), as evidenced by their inability to effectively compete with $[^{125}]\text{I}NO_2$-LDL (Table 1). Other known ligands for CD36, including long chain fatty acids, the anionic phospholipid PS, and thrombospondin-1, all failed to significantly compete with $[^{125}]\text{I}NO_2$-LDL for CD36 recognition, whereas oxidized lipoproteins and oxidized lipid vesicles were effective competitors (Table 1 and Fig. 5a). This suggests that the binding site for oxPC species (and oxLDL) on CD36 is spatially and functionally distinct from that of either PS, thrombospondin-1 or long chain fatty acids. Consistent with this hypothesis, these ligands (i.e. thrombospondin-1, PS, free fatty acids) failed to block CD36-dependent recognition of PAPC vesicles containing oxPC$_{CD36}$ species (Fig. 5b, data for KOOA-PC shown).

To further explore structural features responsible for supporting CD36 binding activity in oxidized lipids, multiple synthetic PC species with distinct oxidized fatty acids at their sn-2 position (e.g. various hydroperoxides, hydroxides or the indicated structural analogs) were synthesized and examined – all failed to serve as CD36 ligands (Table 1). Significantly, OV-PC was considerably less effective in binding to CD36 than oxPC species possessing sn-2 esterified $\gamma$-hydroxy(oxo)-$\alpha,\beta$-unsaturated carbonyl-containing fatty acids (Table 1). None of the oxidized free fatty acids examined bound to CD36 (whether free or incorporated (5-50 mol%) in small unilamellar vesicles comprised of PAPC). Moreover, either repositioning of the $\gamma$-hydroxy moiety by one methylene group to the $\delta$-position (HOT-PC, Table 1), or its loss through dehydration forming a dienoyl-PC species (dienoyl-PC, Table 1), resulted in total elimination of CD36 recognition.

**OxPC$_{CD36}$ species are formed in lipoproteins oxidized by multiple distinct pathways and support oxLDL recognition by CD36.** The results thus far demonstrate that oxPC$_{CD36}$ are the major constituents within oxidized PAPC and PLPC vesicles that bind to CD36, and that oxPC$_{CD36}$-containing vesicles will both directly bind to CD36 as well as compete with various oxLDL for CD36 recognition. To directly test whether or not oxPC$_{CD36}$ promote oxLDL recognition by CD36, lipid extracts from NO$_2$-LDL were fractionated by sequential HPLC as described in Figs. 1 and 2. The major fractions supporting CD36 recognition co-migrated with the identified oxPC$_{CD36}$ derived from oxidation of PAPC and PLPC (not shown). Online ESI/MS/MS analyses as outlined for Figs. 2 and 3 confirmed the presence of oxPC$_{CD36}$ within these fractions. Subsequent quantification of various specific oxPC$_{CD36}$
species by LC/ESI/MS/MS analysis in native and oxidized forms of LDL revealed substantial increases in the content of the CD36 ligands (Fig. 6, data for native LDL, NO₂-LDL and Cu²⁺-oxLDL shown). All oxidation pathways examined resulted in significant increases in the specific lipid oxidation products monitored; however, the relative proportions of oxPC species formed by the various pathways demonstrated consistent differences. For example, regardless of what time point of oxidation was examined, HODA-PC and HOOA-PC were major products of LDL oxidation by MPO. In contrast, LDL oxidized by free and protein-bound transition metal ions (i.e. Cu²⁺- or ceruloplasmin-catalyzed) consistently demonstrated increased levels of the more highly oxidized and truncated oxidation products, OV-PC and ON-PC, relative to the CD36 ligands HODA-PC and HOOA-PC. OxPC_{CD36} species were implicated in mediating CD36-dependent binding in lipoproteins oxidized by pathways alternative to the MPO-H₂O₂-NO₂⁻ system since binding of either Cu²⁺- or ceruloplasmin-oxidized LDL to CD36-transfected 293 cells was blocked by PAPC vesicles containing each of the oxPC_{CD36} species shown in Fig. 1c, but not unoxidized PAPC (Fig. 7a, only data for HOOA-PC shown). Finally, the specificity of the oxPC_{CD36} species as ligands for the scavenger receptor CD36 vs. other lipoprotein receptors was evaluated. PAPC vesicles containing each specific oxPC_{CD36} species, but not unoxidized PAPC, were observed to effectively block binding of CD36 to its ligand, [¹²⁵I]NO₂⁻-LDL. In contrast, no inhibition in binding of either [¹²⁵I]LDL or [¹²⁵I]acetylated LDL to their corresponding receptors, the LDL receptor or the scavenger receptor class A type I (mSR-AI), respectively, was noted (Fig. 7b, only data for HOOA-PC shown).
DISCUSSION

The scavenger receptor CD36 is implicated in a diverse array of physiological processes including, but not limited to, roles in lipid metabolism, insulin resistance, matrix interactions, host defenses, and scavenger receptor recognition (1,4,8,40-42). This broad array of activities arises, in part, because of the multiple distinct ligands CD36 can interact with and the numerous downstream effectors CD36 impacts upon. In addition to its potential role in lipid accumulation and foam cell formation, the scavenger receptor functions of CD36 have been linked to recognition of senescent and apoptotic cells (43,44), and the delivery of ligands for the nuclear hormone transcription factor, peroxisome proliferator-activated receptor (PPAR)-γ (14,15,45,46). PPAR-γ has pleiotropic effects linked to regulation of lipid and carbohydrate metabolism (45,47-50). While studies thus far reported have shown that ligands on oxLDL for CD36 are contained within the lipid portion of the particle (10,11,51-53), the structural nature of the lipids that support CD36 binding has not been elucidated. Identification of the lipid ligand(s) has been difficult owing to the large number and functional complexity of products generated during oxidation of LDL and the daunting challenges of their isolation, structural and biochemical characterization and synthesis.

We now report the first systematic study aimed at identifying the structures of specific oxidized lipids that serve as ligands for the scavenger receptor CD36. The structures of the specific molecular species of choline glycerophospholipids identified were established using a combination of cell binding studies and multiple distinct chromatographic and mass spectrometric methods, in conjunction with: i) results of numerous derivatization strategies to ascertain functional groups on isolated products; ii) inference of structures of products that appeared plausible based upon known mechanisms of lipid oxidation and fragmentation; and iii) de novo synthesis of each lipid. The identities of the oxidation products and synthetic standards were then confirmed by demonstration that the synthetic species recapitulate all biological, chemical, mass spectrometric and chromatographic characteristics of the lipids isolated from oxPAPC and oxPLPC. Finally, LC/ESI/MS/MS analyses demonstrate the generation of these species in lipoproteins oxidized by multiple distinct pathways in vitro. The oxPC_{CD36} species identified herein thus represent a novel family of biologically active lipids that likely participate in numerous CD36 dependent processes.
In previous studies we reported that monocytes use MPO-generated reactive nitrogen species to convert LDL into a high uptake form for macrophages, NO$_2$-LDL (17). We then reported that the scavenger receptor CD36 (based upon studies with human and mouse macrophages, as well as CD36-transfected 293 cells) accounted for the majority of [¹²⁵I]NO$_2$-LDL binding to macrophages (10), and other oxLDL forms (9). This collection of data served as the impetus to further define the structural nature of the ligand(s) on oxLDL that specifically interact with CD36. We selected the competition assay for [¹²⁵I]NO$_2$-LDL – CD36 binding as our screening assay because multiple lines of evidence indicate the specificity of this interaction: (i) a 40-fold molar excess of non-labeled NO$_2$-LDL was an effective competitor (>80% inhibition in competition studies examining [¹²⁵I]NO$_2$-LDL binding, uptake, and degradation by both macrophages (human and mouse) and 293 transfected cells. In contrast, native and acetylated LDL both failed to significantly block macrophage recognition of [¹²⁵I]NO$_2$-LDL; (ii) [¹²⁵I]NO$_2$-LDL binding and degradation by macrophages (including human monocyte-derived macrophages, resident and elicited murine macrophages, and human monocytic-like cell lines THP-1 and U937) were saturable and specific; (iii) recognition of NO$_2$-LDL on both macrophages and CD36 transfected 293 cells was characterized by high affinity binding (Kd ≅ 20 nM); (iv) specificity of the recognition of [¹²⁵I]NO$_2$-LDL by CD36 was shown by the finding that the anti-CD36 blocking mAb, FA6, significantly inhibited binding of monocyte-derived and MPO-generated [¹²⁵I]NO$_2$-LDL to CD36-transfected cells, as well as to human monocyte-derived macrophages, whereas isotype-matched nonimmune antibody had no effect; and (v) binding (4°C), uptake (37°C), and degradation (37°C) of [¹²⁵I]NO$_2$-LDL by MPM recovered from mice homozygous for a null mutation in CD36 was 70-80% lower than that in MPM from wild-type mice possessing isogenetic backgrounds. The present findings extend these observations to the structural level of the presumptive ligand(s) on oxLDL, and suggest that oxPC$_{CD36}$ mediate CD36-specific binding. The MPO-H$_2$O$_2$-NO$_2^-$ system was used as a physiological and reproducible mechanism for oxidizing lipids. The relevance of this pathway for initiating lipid oxidation in vivo is supported by recent studies with leukocytes isolated from individuals with MPO deficiency (25), and by the recent demonstration that blood and leukocyte MPO levels correlate with CAD risk (26). It should also be noted that LC/ESI/MS/MS analyses (Fig 6.) demonstrate the generality of oxPC$_{CD36}$ formation by multiple distinct oxidation systems. Moreover, the present studies show that oxPC$_{CD36}$ are capable of blocking binding of
multiple distinct oxLDL forms to CD36 (Fig. 7). Despite the vast array of products formed following oxidation of even a single molecular species of precursor PC (i.e. PAPC or PLPC), only a small number manifest significant CD36 binding activity.

The structural motifs in oxPC species that support CD36 binding are highly conserved (Figs. 1d). The α,β unsaturated group generates a bi-planar core about which a trans configuration of polar oxygen atoms must be displayed (OH or C=O at γ-position, C=O or (C=O)OH at sn-2 fatty acid terminus). Binding is relatively independent of the long chain fatty acid bound at the sn-1 position (palmitate vs. oleate; data not shown). Incorporation of all of these elements in an amphipathic molecule such as PC was required since neither the free oxidized fatty acids nor their cholesterol ester analogs were recognized by CD36 (Table 1). Moreover, even modest alterations to the central required structural elements, such as the position (and presence) of the γ-OH (or oxo) group, abolished binding to the scavenger receptor. It should also be noted that recognition of oxPC<sub>CD36</sub> species was illustrated in distinct assay systems (competition and direct binding), including use of vesicles consisting of 100% synthetic oxPC<sub>CD36</sub> doped with radiolabel tracer (<< 0.1 mol % [<sup>14</sup>C]DPPC), as well as where the manner of lipid presentation was varied. These included incorporation of the specific lipid ligands into a small unilamellar lipid vesicle, doping of lipid ligands into mixed liposomes (+/- albumin), or addition of lipid ligands in pure form from a concentrated ethanolic stock. Finally, similar results were noted when CD36-transfected cells were first pre-incubated with vesicles, washed, and then [<sup>125</sup>I]NO<sub>2</sub>-LDL binding immediately determined. Thus, PC species possessing sn-2 γ-hydroxy(oxo)-α,β-unsaturated carbonyl-containing fatty acids appear to bind specifically to CD36.

One area that requires further study in the future is the potential contributions of alterations in lipid macromolecular or aggregate structure (i.e. mesomorphic form) induced by oxPC<sub>CD36</sub> species, and their contribution to CD36 recognition. Glycerophospholipids can adopt alternative polymorphic and mesomorphic forms (54). Phases and phase transitions of glycerophospholipids (54), and incorporation of more polar (and truncated) oxidized fatty acids as sn-2 constituents may alter lipid packing. We also have no data to address the issue of non-homogeneous dispersion of oxPC species within lipid mixtures (e.g. can a raft of oxPC species be formed within a PC bilayer?). There is little doubt that incorporation of trace amounts of oxPC species into a bilayer will result in alterations in membrane molecular dynamics in the immediate vicinity of the more polar oxPC species. However, as
noted above, numerous lines of evidence indicate that the receptor-lipid interaction monitored is specific and direct with the oxPC\textsubscript{CD36}, and not necessarily mediated by alterations in bulk lipid physical properties. These include studies showing that altering the manner of presentation of the oxPC\textsubscript{CD36} species to the receptor resulted in comparable results - whether the presumptive ligands were added in pure form from an ethanolic stock, or doped as a minor mole % into bulk lipid in a small unilamellar vesicle (+/- addition of protein). Further, the nature of the lipids used as carriers for oxPC\textsubscript{CD36} did not appear critical for CD36-specific recognition since comparable results were observed using various lipid carriers including PAPC, PLPC, POPC or PC-cholesterol mixtures (up to 50 mol% cholesterol). Finally, as noted above, only minimal alterations in structure to the core conserved structural motif that supports CD36 binding results in complete loss of CD36 binding activity (Table 1). Modest structural changes to the sn-2 fatty acid constituent that ablate CD36 recognition, such as addition of a γ-methylene group (HOT-PC, Table 1), or dehydration of the γ-OH moiety (dienoyl-PC , Table 1), would not likely induce significant alterations in the bulk packing (mesomorphic state) of diradyl glycerophospholipids (54).

It should also be noted that other ligands for CD36 and biologically active oxidized phospholipids in oxidized LDL may exist beyond the novel oxPC\textsubscript{CD36} species identified herein. For example, a recent study identified an oxidatively fragmented alkyl-ether choline glycerophospholipid, hexadecyl azelaoyl phosphocholine, in Cu\textsuperscript{2+}-oxLDL that is taken up by cells via a CD36 dependent pathway (46). Following more extensive oxidation of LDL, protein modification may become significant, including formation of protein-lipid adducts that could participate in CD36 recognition (11). For instance, BSA extensively modified by OV-PC competes for the binding of oxLDL to CD36 and is recognized by a monoclonal antibody to oxidized PAPC (EO6) that inhibits binding of oxLDL to CD36 (11). Recent characterization of EO6 suggests that it recognizes epitopes similar to the Schiff base adduct formed between OV-PC and lysine residues, as well as the aldol condensation product of OV-PC, but not monomeric lipid (55). In the current study we found that free OV-PC was a poor ligand for CD36 (Table 1). We did not directly assess whether a complex formed between BSA and OV-PC might serve as a ligand, although OV-PC incubated for 3h in culture medium containing 10% LPDS failed to inhibit CD36-mediated binding of oxLDL.
The formation of α,β-unsaturated γ-hydroxy aldehydes, such as 4-HNE, as truncated fatty acid products generated during transition metal ion-catalyzed oxidation reactions is well documented (39,56,57). Recent studies examining products formed during oxidation of free polyunsaturated fatty acids demonstrated that HOOA (5-hydroxy-8-oxo-6-octenoic acid) and HODA (9-hydroxy-12-oxo-10 dodecenoic acid) are co-produced in yields similar to 4-HNE during free radical oxidation of arachidonic and linoleic acids, respectively (38,58). Based upon this observation, it was postulated that by analogy to the formation of 4-HNE, HOOA-PC and HODA-PC might be formed during free radical oxidation of PAPC and PLPC, respectively (59). Antibodies raised against proteins modified by sn-2 α,β-unsaturated, γ-hydroxy-carbonyl-containing PCs were generated and used to demonstrate the presence of similar epitopes within LDL oxidized in vitro and in plasma proteins recovered from individuals with atherosclerosis (59). The present studies directly demonstrate that HOOA-PC and HODA-PC are formed within LDL exposed to multiple distinct oxidation pathways (Fig. 6). The present studies also significantly extend the number of species identified within this family and establish these unique oxPC species as ligands for the scavenger receptor CD36. Finally, while some of the oxPC_{CD36} species identified likely share chemical reactivities similar to 4-HNE (e.g. HOOA-PC and HODA-PC), such as the ability to generate Schiff bases and Michael adducts, this property is not critical to CD36 recognition. Binding studies performed in the presence vs. absence of LPDS or BSA yielded comparable results. Moreover, the specific oxPC_{CD36} species that serve as the more potent CD36 ligands are relatively unreactive chemically (i.e. the hydroxy acids HOdiA-PC and HDdiA-PC, and the keto acids KDdiA-PC and KOdiA-PC). Taken together, the present studies suggest that a high affinity, reversible interaction serves as the recognition event between CD36 and oxPC_{CD36} species. They also exclude a significant role for irreversible covalent modification of nucleophilic residues on CD36 by oxPC_{CD36} species.

One of the methodological challenges of the present study was the ease with which many of the oxPC_{CD36} species were further oxidized during sample handling, even while using methods aimed at excluding redox-active transition metal ions and inclusion of free radical scavengers such as BHT at all stages of analysis. For example, exposure of trace levels of synthetic oxPC_{CD36} species to conventional isolation procedures reported for analysis of other known oxPAPC species found in vivo (e.g. OV-PC, G-PC), resulted in substantial conversion of the oxPC_{CD36} species into more advanced (and truncated)
oxidation products including OV-PC and G-PC. Similarly, we observed that oxPC\textsubscript{CD36} derived from PLPC readily generated ON-PC and ND-PC, the structural analogs of OV-PC and G-PC. Our finding that OV-PC, G-PC, ON-PC and ND-PC are readily generated from certain \(\alpha,\beta\)-unsaturated, \(\gamma\)-hydroxy(oxo)carbonyl-containing PC indicates these more truncated phospholipids may be remnants of some oxPC\textsubscript{CD36} species. LC/ESI/MS/MS analyses of various oxPC species were performed using conditions developed to minimize intrapreparative oxidation of lipids and preserve near quantitative recovery of each analyte monitored (based upon control studies using trace levels of individual synthetic standards). When analyzed using these methods, the contents of oxPC\textsubscript{CD36} species observed were comparable to that detected for the more terminally oxidized end products of PAPC, OV-PC and G-PC (Fig. 6a). It remains to be determined whether the oxPC\textsubscript{CD36} species identified possess biological activities extending beyond serving as ligands for CD36.

The present studies suggest several potential strategies for blocking foam cell formation \textit{in vivo}. These include preventing formation of oxPC\textsubscript{CD36} through use of antioxidant strategies or enzyme (i.e. MPO) inhibitors, as well as development of selective receptor antagonists (i.e. to the CD36 “oxLDL binding site”). CD36 is a multiligand receptor with functionally distinct binding domains for its ligands (60-63). The competition studies presented in Fig. 5 suggest the feasibility of developing CD36 receptor antagonists that specifically block recognition of oxPC\textsubscript{CD36} species at the CD36 binding site for oxLDL without significant impact on CD36-dependent recognition of ligands such as fatty acids, thrombospondin-1 and PS. The structural insights into the molecular patterns recognized by the “oxLDL” site of CD36 identified herein may provide a platform for the rational development of such potential therapeutic inhibitory agents.
ACKNOWLEDGMENTS

We thank Dr. M. Krieger (MIT, Boston, MA) and Dr. W. Frazier (Washington University, St.
Louis, MO) for generously providing CHO cells expressing mSR-AI and 293 cells expressing human
CD36, respectively. This work was supported by National Institutes of Health grants HL62526,
HL70621 and HL61878 (to SLH), GM21249 (to RGS), HL53315 (to HFH and RGS), as well as the
Scientist Development Grant from American Heart Association (to EAP). RZ and LS are supported
by Fellowships from the American Heart Association. Z.S. was a recipient of a Jane Coffin Childs
Memorial Fund for Medical Research Fellowship.
REFERENCES


Identification of oxidized lipid ligands for CD36


Identification of oxidized lipid ligands for CD36


FIGURE LEGENDS

Figure 1. HPLC isolation of CD36 ligands generated during oxidation of (a) 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) and (b) 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) by the MPO-H₂O₂-NO₂⁻ system. Small unilamellar vesicles comprised of either PAPC (a) or PLPC (b) were exposed to the MPO-H₂O₂-NO₂⁻ system. Lipids were extracted and then fractionated by preparative reverse phase HPLC and monitored by evaporative light scattering (bottom panels) as described under “Methods.” In parallel, fractions were collected, lipids extracted, dried under nitrogen, reconstituted in PBS, and analyzed for their ability to compete for the binding of [¹²⁵I]NO₂-LDL to CD36-transfected 293 cells as described under “Methods.” Three main fractions contained significant levels of oxidized lipids with CD36 binding activity (designated Peaks I, II and III) following HPLC fractionation. Peaks I, II and III derived from oxPAPC (a), and Peaks I, II and III derived from oxPLPC (b), served as the source of lipids for subsequent isolation and structural characterization studies outlined below. Retention times of some known oxidized phospholipids derived from PAPC and PLPC are indicated: (a) G-PC (glutaric monoester of 2-lysoPC); (b) OV-PC (5-oxovaleric acid ester of 2-lysoPC); (c) ND-PC (9-carboxynonanoic ester of 2-lysoPC); (d) ON-PC (9-oxononanoic acid ester of 2-lysoPC). (c) The structures and nomenclature of oxidized phospholipids with CD36 binding activity isolated from oxidized PAPC and PLPC are shown. (d) The core structural motifs (boxed area) conserved amongst the various isolated oxidized PC species that support CD36-binding activity. Note that the products identified in Peaks I, II and III recovered following fractionation of oxPAPC and oxPLPC are similar except for the chain length (n = 1, PAPC; n = 5, PLPC) of the truncated oxidized fatty acid esterified to the sn-2 position.

Figure 2. Identification of CD36 ligands in Peaks I, II, III recovered from initial reverse phase HPLC fractionation of PAPC oxidized by the MPO-H₂O₂-NO₂⁻ system. Lipids within Peak I (Panel a), Peak II (Panel b) and Peak III (Panel c) recovered from preparative HPLC of oxPAPC species (Fig. 1a) were fractionated on a second analytical reverse phase HPLC column as described in “Methods.” (Upper tracings of panels a, b and c) Fractions (2 min) for each were collected and assayed for their ability to bind to CD36, as monitored by competition assay using [¹²⁵I]NO₂-LDL and CD36 transfected 293 cells. (Inserts to the upper tracings of panels a, b and c) The full scan mass
Identification of oxidized lipid ligands for CD36

The spectrum of ions present within the fraction containing the majority of CD36 binding activity for each is presented. The retention times and names of the ultimate CD36 ligands identified within each peak are indicated on the top tracing of each panel. **(Middle tracings of panels a, b and c)** The ion current tracing obtained by simultaneous analysis of a portion of diverted column eluent using on-line positive ion electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) with multiple reaction monitoring (MRM) mode is shown. The specified ion transitions correspond to the m/z of the appropriate parent and characteristic daughter ions of the indicated CD36 ligand. **(Lower tracings of panels a, b and c)** In a parallel experiment, lipids within the major fraction containing CD36 binding activity recovered off of the second reverse phase HPLC column (top tracing for each panel) were derivatized to their corresponding oxime by reaction with methyl hydroxylamine. Repeat analysis by LC/ESI/MS/MS in MRM mode using transitions described for native lipids (middle tracing of each panel) revealed the loss of the previously monitored ions (data not shown). However, monitoring at the appropriate transitions for m/z of the anticipated methoxime derivative and a characteristic daughter ion of each of the CD36 ligands revealed the new appearance of ions with identical retention times to that noted for synthetic oxime-derivatives of each of the indicated CD36 ligands (bottom ion current tracings for each panel). **(Insets to lower tracing for panels a, b and c)** The full scan positive ion mass spectrum of the oxime derivative for each indicated CD36 ligand was also obtained and is illustrated. Where syn and anti isomers of methoxime derivatives were resolvable (panels b and c), the full scan positive ion mass spectrum of each was identical, and only the mass spectrum for the early eluting isomer is shown.

**Figure 3. Identification of CD36 ligands in Peaks I, II, III recovered from initial reverse phase HPLC fractionation of PLPC oxidized by the MPO-H$_2$O$_2$-NO$_2^-$ system.** Lipids within Peak I (Panel a), Peak II (Panel b) and Peak III (Panel c) recovered from preparative HPLC of oxPLPC species (Fig. 1b) were fractionated on a second analytical reverse phase HPLC column as described in “Methods.” **(Upper tracings of panels a, b and c)** Fractions (2 min) for each were collected and assayed for their ability to bind to CD36, as monitored by a competition assay using $[^{125}\text{I}]$NO$_2$-LDL and CD36 transfected 293 cells. **(Insets to the upper tracings of panels a, b and c)** The full scan mass spectrum of ions present within the fraction containing the majority of CD36 binding activity is presented. The retention times and names of the ultimate CD36 ligands identified within each peak are
indicated on the top tracing of each panel. (Middle tracings of panels a, b and c) The ion current tracing obtained by simultaneous analysis of a portion of diverted column eluent using on-line positive ion electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) with multiple reaction monitoring (MRM) mode is shown. The specified ion transitions correspond to the m/z of the appropriate parent and characteristic daughter ions of the indicated CD36 ligand. (Lower tracings of panels a, b and c) In a parallel experiment, lipids within the major fraction containing CD36 binding activity recovered off of the second reverse phase HPLC column (top tracing for each panel) were derivatized to their corresponding oxime by reaction with methyl hydroxylamine. Repeat analysis by LC/ESI/MS/MS in MRM mode using transitions described for native lipids (middle tracing of each panel) revealed the loss of the previously monitored ions (data not shown). However, monitoring at the appropriate transitions for m/z of the anticipated methoxime derivative and a characteristic daughter ion of each of the CD36 ligands revealed the new appearance of ions with identical retention times to that noted for synthetic oxime-derivatives of each of the indicated CD36 ligands (bottom ion current tracings for each panel). (Insets to lower tracing for panels a, b and c) The full scan positive ion mass spectrum of the oxime derivative for each indicated CD36 ligand was also obtained and is illustrated. Where syn and anti isomers of methoxime derivatives were resolvable (panels b and c), the full scan positive ion mass spectrum of each was identical, and only the mass spectrum for the early eluting isomer is shown.

**Figure 4. Synthetic phospholipids with sn-2 esterified oxidized fatty acids possessing γ-hydroxy(oxo)-α,β-unsaturated carbonyl groups have CD36 binding activity.** (a) Small unilamellar vesicles consisting of PAPC alone, or a 50:50 mol:mol mixture of PAPC and the indicated synthetic oxidized phospholipid, were prepared as described under “Methods.” [125I]-NO₂LDL (5 µg apoprotein/ml) was then incubated with CD36-expressing 293 cells for 3 h at 4°C in the presence of increasing concentrations of the indicated phospholipid vesicles as competitors. Cells were then washed to remove unbound vesicles with ice cold PBS, solubilized with 0.1 M NaOH, and the amount of bound [125I]-NO₂LDL quantified. (b) Similar studies were performed using small unilamellar vesicles consisting of PLPC alone, or a 50:50 mol:mol mixture of PLPC and the indicated synthetic oxidized phospholipid. Data represent the mean ± S.D. of triplicate determinations of a representative experiment performed at least three times. (c) Small unilamellar vesicles consisting of unoxidized parent...
phospholipid alone (PAPC or PLPC), or a 50:50 mol:mol mixture of parent phospholipid and the indicated synthetic oxidized phospholipid, were prepared with tracer levels of high specific activity \[^3H\]DPPC (25 µCi/mg vesicles), as described under “Methods.” 293 cells stably transfected with CD36 or vector-transfected cells (0.25-0.3 mg cell protein/well) were incubated with 0.25 ml of 10 µg/ml of phospholipid vesicles (10 µg/mg cell protein) in DMEM for 2 h at 4°C in the presence of 40 fold excess of unlabeled POPC vesicles to block nonspecific binding. Unbound vesicles were then washed from cells with ice cold PBS, the cells solubilized with 0.1 M NaOH and bound radioactivity quantified. Data are expressed as a difference between binding to CD36 transfected cells and vector-transfected cells. The binding to vector-transfected cells was 10-20 % of that to CD36 transfected cells. Each experimental point represents the mean ± S.D. for triplicate determinations of a representative experiment performed at least three times. 

(d) Binding of \[^3H\]DPPC-labeled vesicles to CD36-transfected cells in the presence of the indicated competitors was performed as in panel c. Where indicated, the anti-CD36 blocking monoclonal antibody FA6 and isotype-matched non-immune IgG (20 µg/ml each) were included. Data are expressed as difference between binding to CD36 transfected cells and vector-transfected cells. Each experimental point represents the mean ± S.D. for triplicate determinations of a representative experiment performed at least three times.

**Figure 5. OxPC\(_{CD36}\) species bind to scavenger receptor CD36 at a site functionally distinct from other CD36 ligands.** (a) \[^{125}I\]NO\(_2\)-LDL (5 µg/ml) was incubated with 293 cells stably transfected with CD36 for 2 h at 4°C in DMEM in the presence of indicated competitors. Cells were then washed from unbound lipoproteins with ice cold PBS, solubilized with 0.1 M NaOH and bound radioactivity quantified. Concentrations of the competitors were 200 µg/ml for lipoproteins and phospholipids, 100 µM for fatty acids, 100 µg/ml for proteins and 40 µg/ml for oxPAPC (PAPC modified by 8h incubation with the MPO-H\(_2\)O\(_2\)-NO\(_2\) system). (b) Binding of \[^3H\]DPPC-labeled vesicles (10 µg/ml), instead of \[^{125}I\] NO\(_2\)-LDL, to CD36-transfected cells in the presence of the indicated competitors was performed as in Panel (a). Small unilamellar vesicles were prepared as described under “Methods” and consisted of a 50:50 mol:mol mixture of PAPC and synthetic KOOA-PC with tracer levels of high specific activity \[^3H\]DPPC (25 µCi/mg vesicles). Data are expressed as a percentage of binding vs. that observed in the absence of competitor. N.A. (No Addition), BSA (bovine serum albumin), PS (dipalmitoyl phosphatidyl serine), TSP-1 (thrombospondin-1). Each experimental
Identification of oxidized lipid ligands for CD36

Figure 6. Phospholipids with sn-2 esterified oxidized fatty acids possessing γ-hydroxy(oxo)-α,β-unsaturated carbonyl groups are generated in LDL oxidized by multiple distinct pathways. The contents of the indicated oxidized PC species were determined in native LDL and LDL oxidized by either the MPO-H₂O₂-NO₂⁻ system (NO₂-LDL) or Cu²⁺ (Cu²⁺-LDL) using LC/ESI/MS/MS as described under “Methods.” Data represent the mean ± S.D. of triplicate determinations.

Figure 7. OxPC_CD36 species specifically mediate binding of different forms of oxidized LDL to scavenger receptor CD36 vs. other lipoprotein receptors. (a) [¹²⁵I]LDL (0.2 mg/ml) was oxidized either by the MPO/H₂O₂/NO₂⁻ system (NO₂-LDL), copper sulfate (Cu⁺⁺ oxLDL) or ceruloplasmin-bound copper (Ceruloplasmin-oxLDL) as described under “Methods.” CD36-transfected 293 cells were then incubated with [¹²⁵I]-labeled oxidized lipoproteins (5 µg/ml) at 4°C in the absence (No Addition, N.A.) or presence (200 µg/ml) of the indicated competitors for 2 h in DMEM supplemented with 5% FCS and 20 µM HEPES. Cells were washed with ice cold PBS/BSA to remove unbound lipoproteins and bound lipoproteins were then quantified. Data are expressed as a percentage of binding vs. that observed in the absence of competitor (No Addition, N.A.). Each experimental point represents the mean ± S.D. of triplicate determinations of a representative experiment performed three times. (b) The effect of the indicated competitors on the binding of [¹²⁵I]NO₂-LDL to CD36-transfected 293 cells (NO₂-LDL/CD36), [¹²⁵I]LDL to human foreskin fibroblasts expressing the LDL receptor (LDL/LDLR) or [¹²⁵I] acetylated LDL to mSR-A-I-transfected CHO cells (acLDL/SR-A1) is shown. Cells were incubated in their corresponding media with 20 µM HEPES and 5 µg/ml of the indicated [¹²⁵I]-labeled lipoprotein in the absence or presence of 20 µg/ml of competitors for 2h at 4°C. Human foreskin fibroblasts were initially cultured for 12h in media with 10 % LPDS in order to increase LDL receptor expression. Unbound lipoproteins were removed by washing cells with ice cold PBS/BSA and remaining bound lipoproteins were then quantified. Competitors: No Addition (N.A.); small unilamellar vesicles comprised of 50:50, mol:mol PAPC and HOOA-PC (HOOA-PC); or un-oxidized PAPC (PAPC).
Legend to Table 1. Structural requirements for CD36 binding. The indicated lipids were analyzed for their ability to compete for the binding of $[^{125}\text{I}]\text{NO}_2$-LDL (5 µg/ml) to CD36-transfected 293 cells as described under “Methods.” Rank potencies of binding were determined by assessing the concentrations of synthetic lipid (presented as a 50:50, mol:mol, vesicle) required to block 50% of $[^{125}\text{I}]\text{NO}_2$-LDL binding (IC$_{50}$). For phospholipids only structures of fatty acids esterified at the sn-2 position of PC (palmitic acid at sn-1 position) are shown. Abbreviations: G-PC and ND-PC, the glutaric and nonanedioic monoesters of 2-lysoPC; HDdiA-PC and HOdiA-PC, the 9-hydroxy-10-dodecenedioic acid and 5-hydroxy-8-oxo-6-octenedioic acid esters of 2-lysoPC; HODA-PC and HOOA-PC, the 9-hydroxy-12-oxo-10-dodecenoic acid and 5-hydroxy-8-oxo-6-octenoic acid esters of 2-lysoPC; KODA-PC and KOOA-PC, the 9-keto-12-oxo-10-dodecenoic acid and 5-keto-8-oxo-6-octenoic acid esters of 2-lysoPC; KDdiA-PC and KODiA-PC, the 9-keto-10-dodecenedioic acid and 5-keto-6-octendioic acid esters of 2-lysoPC; OV-PC and OB-PC, the 5-oxovaleric acid and 4-oxobutyric acid esters of 2-lysoPC; PAPC, 1-hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-sn-glycero-3-phosphocholine; PLPC, 1-hexadecanoyl-2-octadecadi-9',12'-enoyl-sn-glycero-3-phosphocholine; HODA-CH and HOOA-CH, the 9-hydroxy-12-oxo-10-dodecenoic acid and 5-hydroxy-8-oxo-6-octenoic acid esters of cholesterol; dienoyl-PC, 1-palmitoyl-2-(13-Oxotridec-9E,11E-dienoyl)-sn-glycero-3-phosphatidyl choline; HOT-PC, 1-palmitoyl-2-(2E-5-Hydroxytridec-2-enediyl)-sn-glycero-3-phosphatidyl choline; 13-HODE-PC, 1-palmitoyl-2-(13(S)-hydroxy-(9Z,11E)-octadeca-9,11-dienoyl)-sn-glycero-3-phosphatidyl choline; 15-HETE-PC, 1-palmitoyl-2-(15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoyl)-sn-glycero-3-phosphatidyl choline; 15-HPETE-PC, 1-palmitoyl-2-(15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoyl)-sn-glycero-3-phosphatidyl choline.
Figure 1

(a) PAPC series
(b) PLPC series

<table>
<thead>
<tr>
<th>Peak</th>
<th>PAPC series</th>
<th>PLPC series</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HOdiA-PC</td>
<td>HDdiA-PC</td>
</tr>
<tr>
<td>I</td>
<td>KOdiA-PC</td>
<td>KDdiA-PC</td>
</tr>
<tr>
<td>II</td>
<td>HOOA-PC</td>
<td>HODA-PC</td>
</tr>
<tr>
<td>III</td>
<td>KOOA-PC</td>
<td>KODA-PC</td>
</tr>
</tbody>
</table>

X = OH or =O
Y = OH or H
Figure 3
Figure 4

(a) [125I]NO2-LDL Binding (% of control) vs. Phospholipid [µM]
(b) [125I]NO2-LDL Binding (% of control) vs. Phospholipid [µM]
(c) [3H]Vesicle Binding (ng/mg cell protein)
(d) [3H]Vesicle Binding (ng/mg cell protein)
Figure 5

(a) NO\textsubscript{2}-LDL

(b) KOOA-PC/PAPC Vesicles

<table>
<thead>
<tr>
<th>Competitors</th>
<th>NO\textsubscript{2}-LDL Bound (% of control)</th>
<th>[\textsuperscript{125}I]NO\textsubscript{2}-LDL Bound (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.A.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{2}-LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu\textsuperscript{2+} + oxLDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSP-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Competitors</th>
<th>KOOA-PC/PAPC Vesicles Bound (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>PAPC</td>
<td></td>
</tr>
<tr>
<td>P-LPC</td>
<td></td>
</tr>
<tr>
<td>oxPAPC</td>
<td></td>
</tr>
<tr>
<td>Oleate</td>
<td></td>
</tr>
<tr>
<td>Linolate</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td></td>
</tr>
<tr>
<td>TSP-1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

(a) [\(1^25\text{I}LDL\) Bound (% of control)]

Competitors

- NO$_2$-LDL
- Cu$^{++}$-ox-LDL
- Ceruloplasmin
- ox-LDL

(b) [\(1^25\text{I}LDL\) Bound (% of control)]

Competitors

- NO$_2$-LDL/CD36
- LD/LDLR
- acLDL/SR-AI
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$($\mu$M)</th>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAPC series</strong></td>
<td></td>
<td></td>
<td><strong>PLPC series</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPC</td>
<td><img src="PAPC.png" alt="Structure" /></td>
<td>&gt; 200</td>
<td>PLPC</td>
<td><img src="PLPC.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>15-HPETE-PC</td>
<td><img src="15-HPETE-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
<td>13-HODE-PC</td>
<td><img src="13-HODE-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>15-HETE-PC</td>
<td><img src="15-HETE-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
<td>13-HPODE-PC</td>
<td><img src="13-HPODE-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>HODiA-PC</td>
<td><img src="HODiA-PC.png" alt="Structure" /></td>
<td>15.5</td>
<td>HDdiA-PC</td>
<td><img src="HDdiA-PC.png" alt="Structure" /></td>
<td>14.5</td>
</tr>
<tr>
<td>KODiA-PC</td>
<td><img src="KODiA-PC.png" alt="Structure" /></td>
<td>3.9</td>
<td>KDdiA-PC</td>
<td><img src="KDdiA-PC.png" alt="Structure" /></td>
<td>2.0</td>
</tr>
<tr>
<td>HOOA-PC</td>
<td><img src="HOOA-PC.png" alt="Structure" /></td>
<td>46.8</td>
<td>HODA-PC</td>
<td><img src="HODA-PC.png" alt="Structure" /></td>
<td>75.4</td>
</tr>
<tr>
<td>KOOA-PC</td>
<td><img src="KOOA-PC.png" alt="Structure" /></td>
<td>6.2</td>
<td>KODA-PC</td>
<td><img src="KODA-PC.png" alt="Structure" /></td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Free Fatty Acids and Others**

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC$_{50}$($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic Acid</td>
<td><img src="Arachidonic_Acid.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td><img src="Linoleic_Acid.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td><img src="Oleic_Acid.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>4-HNE</td>
<td><img src="4-HNE.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>HOOA-CH</td>
<td><img src="HOOA-CH.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>OV-PC</td>
<td><img src="OV-PC.png" alt="Structure" /></td>
<td>138.3</td>
</tr>
<tr>
<td>OB-PC</td>
<td><img src="OB-PC.png" alt="Structure" /></td>
<td>82.9</td>
</tr>
<tr>
<td>HOT-PC</td>
<td><img src="HOT-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Dienoyl-PC</td>
<td><img src="Dienoyl-PC.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td>HODA-CH</td>
<td><img src="HODA-CH.png" alt="Structure" /></td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>
Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36
Eugene A. Podrez, Eugenia Batyreva, Zhongzhou Shen, Renliang Zhang, Yijung Deng, Mingjiang Sun, Paula J. Finton, Lian Shan, Bogdan Gugiu, Henry F. Hoff, Robert G. Salomon and Stanley L. Hazen

J. Biol. Chem. published online July 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203318200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts