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A Novel Family of Atherogenic Oxidized Phospholipids Promotes Macrophage Foam Cell Formation via the Scavenger Receptor CD36 and Is Enriched in Atherosclerotic Lesions*

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The macrophage scavenger receptor CD36 plays an important role in binding and uptake of oxidized forms of low-density lipoprotein (LDL), foam cell formation, and lesion development during atherosclerosis. The structural basis of CD36-lipoprotein ligand recognition is an area of intense interest. In a companion article we reported the characterization of a structurally conserved family of oxidized choline glycerophospholipids (oxPC_{CD36}) that serve as novel high affinity ligands for cells stably transfected with CD36, mediating recognition of multiple oxidized forms of LDL (Podrez, E. A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., Sun, M., Finton, P., Shan, L., Gugiu, B., Fox, P. L., Hoff, H. F., Salomon, R. G., and Hazen, S. L. (July 8, 2002) *J. Biol. Chem.* 277, 10.1074/jbc.M203318200). Here we use macrophages from wild-type and CD36 null mice to demonstrate that CD36 is the major receptor on macrophages mediating recognition of oxPC_{CD36} species when presented (+/− plasma) in pure form, within PC bilayers in small unilamellar vesicles, and within liposomes generated from lipid extracts of native LDL. We also show that oxPC_{CD36} promote CD36-dependent recognition when present at only a few molecules per particle, resulting in macrophage binding, uptake, metabolism, cholesterol accumulation, and foam cell formation. Finally, using high performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS), we demonstrate that oxPC_{CD36} are generated *in vivo* and are enriched in atherosclerotic lesions. Collectively, our data suggest that formation of this novel family of oxidized phospholipids participates in CD36-mediated recognition of oxidized lipoproteins and foam cell formation *in vivo*.

CD36 is a multifunctional cellular receptor with broad ligand specificity (1, 2). It is expressed in a number of cells including microvascular endothelial cells, platelets, adipocytes, striated muscles, macrophages, and some vascular smooth muscle cells (1, 2). CD36 regulates cellular adhesion and angiogenesis, serving as a receptor for thrombospondin; it also serves as a scavenger receptor in macrophages, mediating uptake of apoptotic cells and modified lipoproteins, and participates in carbohydrate and lipid metabolism, modulating insulin resistance and long chain fatty acid transport (3–8). CD36 has recently been implicated in a variety of pathologic conditions, including atherosclerosis, diabetes, and cardiomyopathy. Perhaps the most compelling data on the role of CD36 in atherosclerosis are from studies of CD36-deficient mice, which show a 70–80% reduction in aortic lesion size (9). *In vitro* experiments demonstrate that macrophages from CD36-deficient mice take up different forms of oxidized LDL[1] poorly and are resistant to foam cell formation, providing a mechanism for the atheroprotection observed in CD36 null mice (9, 10). Given the potential clinical significance of this receptor, it is important to know the nature of the ligand(s) in oxidized LDL (oxLDL) that are recognized by CD36 and support foam cell formation *in vivo*.

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[1] The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; BHT, butylated hydroxytoluene; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyllindocarbocyanine perchlorate; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; DTPA, diethylenetriamine pentaacetic acid; 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-dodecendioic acid and 5-keto-6-octendioic acid esters of 2-lysoPC; HPLC, high performance liquid chromatography; LC/ESI/MS/MS, HPLC with on-line electrospray ionization tandem mass spectrometry; MPM, mouse peritoneal macrophage; MPO, myeloperoxidase; MRM, multiple reaction monitoring; NO_2^-, nitrite; lyso-PC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; NO_2-LDL, LDL modified by the MPO-H_2O_2-NO_2^- system; NO_2-PAPC, PAPC vesicles modified by the MPO-H_2O_2-NO_2^- system; OV-PC and ON-PC, the 5-oxovaleric acid and 9-oxononanoic acid esters of 2-lysoPC; oxPC_{CD36}, oxidized phosphatidylcholine species that bind with high affinity to CD36; PAPC, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine; PBS, phosphate-buffered saline; POPC, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine; PLPC, 1-hexadecanoyl-2-octadecadi-9′,12′-enoyl-*sn*-glycero-3-phosphocholine; WHHL, Watanabe heritable hyperlipidemic rabbits; WT, wild type.

The scavenger receptor functions of CD36 toward oxLDL may, in theory, result from recognition of the lipid moiety, apoB-100 or apoB-100 modified by oxidized lipids. Oxidized lipids were first suggested to participate in macrophage recognition of oxLDL based on studies using liposomes generated from lipid extracts of LDL oxidized by free Cu^{2+} (Cu^{2+}-oxLDL) and mouse peritoneal macrophages (MPM) (11). A role for CD36 as the macrophage receptor responsible for recognition of oxidized lipids in Cu^{2+}-oxLDL has subsequently been shown (10, 12). Oxidized phospholipids covalently linked to apolipoprotein B-100 (apoB) in extensively oxidized LDL (*e.g.* Cu^{2+}-oxLDL) have been suggested to serve as ligands for CD36, based on competition studies using reconstituted apoB-100 from Cu^{2+}-oxLDL and studies employing monoclonal antibodies to phospholipid-protein adducts (12, 13).

We recently employed a combination of mass spectrometry and both analytical and synthetic chemistry to isolate and structurally define a novel family of oxidized choline glycerophospholipids (oxPC) that, in an unbound state, serve as high affinity ligands for CD36 (14). We further demonstrated their formation during the oxidization of LDL by multiple distinct pathways and their participation in CD36-mediated recognition of different forms of oxidized LDL (14). The highly conserved nature of the critical structural elements required for oxidized phospholipids to serve as ligands for CD36 were defined, namely, a phospholipid with a truncated *sn*-2 acyl group that incorporates a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl ($oxPC_{CD36}$) (Scheme 1) (14). Finally, in competition studies using cells transfected with CD36, we demonstrated that the binding site for $oxPC_{CD36}$ species (and oxLDL) on CD36 is spatially and functionally distinct from that of other known CD36 ligands (14). Collectively, these results suggested that formation of this novel family of oxidized phospholipids might play an important role in CD36-mediated recognition of oxidized lipoproteins, senescent or apoptotic cells, and foam cell formation. However, the physiological relevance of $oxPC_{CD36}$ has not yet been established. Namely, their formation *in vivo*, their ability to promote cholesterol loading and foam cell formation via CD36, and the levels of $oxPC_{CD36}$ required to promote CD36-mediated recognition by macrophages have not yet been explored.

In this study we demonstrate that $oxPC_{CD36}$ inserted into small unilamellar phospholipid vesicles or liposomes containing neutral lipids derived from LDL can function as ligands for CD36 on macrophages, resulting in the internalization of the entire particle and subsequent cholesterol and cholesteryl ester loading. Specificity of $oxPC_{CD36}$-mediated foam cell formation in macrophages is confirmed using MPM from wild-type and CD36 null mice. Finally, a potential atherogenic role for $oxPC_{CD36}$ species *in vivo* is supported by our demonstration that $oxPC_{CD36}$ are both enriched in atherosclerotic lesions and capable of promoting scavenger receptor recognition when present at only a few molecules per particle.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture media and additives were purchased from Invitrogen. $Na^{125}I$ 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 Radiolabel Chemicals, Inc. (St. Louis, MO), and [^{3}H]cholesteryl linoleate was from PerkinElmer Life Sciences. 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), phosphatidylserine, and 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were obtained from Sigma 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 (15). All buffers were treated with Chelex-100 resin (Bio-Rad) and supplemented with diethylenetriaminepentaacetic acid (DTPA) to remove trace levels of transition metal ions that might catalyze lipid oxidation during incubations. LDL was labeled with $Na^{125}I$ to a specific activity between 100 and 250 dpm/ng of protein, as described (16). Incorporation of [^{14}C]oleate into cholesteryl esters by cells following incubation with the indicated lipoproteins (50 μg/ml) were determined as described (15).

Synthesis of Phospholipids—Total syntheses and purification of the γ-hydroxy-α,β-unsaturated aldehydic phospholipids ligands for CD36 were performed as described elsewhere (17, 18). The structures of all synthetic lipids were confirmed by multinuclear NMR and high resolution mass spectrometry prior to use (17–19). Synthetic lipids were routinely analyzed by HPLC with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) as described (14). 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 composed of PLPC, POPC, or PAPC with varying mol % of specific oxidized phospholipids were prepared in argon-sparged sodium phosphate buffer by extrusion (10×) through a 0.1-μm polycarbonate filter using an Avanti Mini-Extruder Set (Avanti Polar Lipids) at 37 °C. For direct binding experiments, [^{3}H]DPPC (25 μCi/mg of phospholipids) or 1 mol % of the fluorescent dye DiI was added to phospholipids.

Cells—Thioglycollate-elicited MPM from wild-type (C57BL/6) and CD36 null mice (6) were isolated and cultured as described (15). CD36 null mice were back-crossed at least six generations on a C57BL/6 background. Foam cell formation was assessed following incubation of MPM with liposomes and quantified histologically as described (20). Briefly, liposomes were formed from native LDL lipids that were extracted according to the method of Bligh and Dyer (21), dried under the nitrogen, reconstituted in argon-sparged PBS at 37 °C in sealed reaction vials (under argon atmosphere) at a final concentration of 0.5 mg phospholipids/ml in the presence *versus* absence of the indicated oxPC species. Liposomes were generated by brief sonication and subsequent extrusion (10 passages) through an 0.1-μm polycarbonate filter under an argon atmosphere. Thioglycollate-elicited MPM were incubated for 48 h in RPMI 1640 containing 1% bovine serum albumin and butylated hydroxytoluene (BHT, 20 μM) in the presence of the 50 μg/ml liposomes. Cytochalasin D (0.5 μg/ml) was included in the medium to suppress nonspecific phagocytosis. Medium containing the appropriate modified liposomes was exchanged every 24 h of incubation. Cells were fixed with 4% formaldehyde, stained with Oil Red-O to detect intracellular neutral lipids, and counter-stained with hematoxylin. Cells containing more then 10 lipid droplets were scored as "foam cells" (20). At least 10 fields and 500 cells/condition were counted.

Phospholipid Separation and Mass Spectrometric Analysis—Lipids were initially extracted three times by the method of Bligh and Dyer (21) from tissue homogenates (generated using a ratio of 1 g of tissue:10 ml of 50 mM sodium phosphate (pH 7.0) supplemented with DTPA (2 mM) and BHT (100 μM) under argon atmosphere). The combined extracts were dried rapidly under nitrogen, resuspended in methanol:H_2O (98:2, v:v), a known amount of dimyristoylphosphatidylcholine internal standard was added, and then neutral lipids in the lipid extracts were removed by passage through a C18 minicolumn (Supelclean LC-18 SPE tubes, 3 ml; Supelco Inc., Bellefonte, PA). The recovered polar phospholipids were dried under nitrogen and stored in the dark under an argon atmosphere at −80 °C until analysis within 24 h. Quantification of the various oxPC species was performed using LC/ESI/MS/MS as described recently (14). Briefly, lipids were maintained under inert atmosphere (argon or nitrogen) at all times. Mass spectrometric analyses were performed on a Quatro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, UK) equipped with an electrospray ionization probe and interfaced with an HP 1100 HPLC (Hewlett-Packard, Wilmington, DE). Lipids were quantified on a Prodigy ODS-18 column (250 × 2 mm, 5 μm, Phenomenex, Torrance, CA) at a flow rate of 0.2 ml/min. Mass spectrometric analyses were performed on-line using electrospray ionization tandem mass spectrometry 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 phosphopholipids 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.* $[MH]^+ \rightarrow m/z$ 184). A discontinuous gradient

was used by mixing solvent A (0.2% formic acid in HPLC-grade water) with solvent B (0.2% formic acid in HPLC-grade methanol), as follows: initial equilibration of column at 85% solvent B; increasing from 85 to 88% solvent B from 0–12 min; increasing to 100% solvent B from 12 to 14 min; and then isocratic elution with solvent B from 14 to 20 min. 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 described earlier (14). OV-PC and ND-PC were quantified similarly but also by 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) as described (14). Calibration curves constructed with a fixed amount of dimyristoylphosphatidylcholine and varying mol % of each synthetic oxidized PC species were used to correct for the differences in ionization response factors observed among the different lipids. In additional preliminary studies the quantification methods employed were validated independently for each analyte by demonstrating results identical to those obtained by the method of standard additions. In independent studies the overall recovery 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.

Tissue Storage and Processing—The thoracic aorta from Watanabe heritable hyperlipidemic (WHHL) rabbits was isolated, rinsed in argon-sparged PBS supplemented with 100 μM BHT and 100 μM DTPA, submerged in the same buffer, covered in argon, flash-frozen in liquid nitrogen, and then stored at −80 °C until analysis. Aortas relatively free of lipid lesions were obtained from WHHL rabbits age 10–12 weeks, whereas aortas with confluent lesions were recovered from WHHL rabbits >6 months old. Frozen sections of aortas were pulverized with a stainless steel mortar and pestle under liquid nitrogen, the powder transferred to glass threaded test tubes equipped with PTFE-lined caps, and then lipids were extracted by the method of Bligh and Dyer (21) under argon in the presence of BHT (100 μM). At least nine samples of aortic tissues (atherosclerosis-laden and normal) were dissected from thoracic and abdominal aortas from three animals in each group for analyses. Quantification of lipids was then performed by LC/ESI/MS/MS as described above.

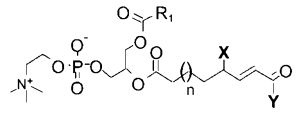
Fluorescence Microscopy—Cells were cultured in Lab-Tek II® glass chamber slides (Nalge Nunc Int., Naperville, IL), incubated in the appropriate medium in the presence of DiI-labeled PAPC vesicles containing 1–25 mol % of the indicated specific oxidized phospholipid for 2 h at 4 °C (for binding studies) or for 1 h at 37 °C (for uptake studies). A forty-fold excess of unlabeled POPC vesicles was included to inhibit nonspecific binding and uptake. Cells were washed three times with PBS, then fixed in 4% formaldehyde in PBS (for 1 h), washed in PBS, and mounted in Vectashield® mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescent preparations were viewed with a Leica fluorescent microscope with a 50× objective. Images were acquired using the Magnafire imaging system (Optronics, Goleta, CA) and further analyzed using Adobe Photoshop 5.5.

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

CD36 Is the Major Receptor on Macrophages That Accounts for the Binding and Uptake of oxPC$_{CD36}$—We have recently characterized oxidized choline glycerophospholipids with an *sn*-2 acyl group that incorporates a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl as high affinity ligands for the scavenger receptor CD36 (14). Their structures, chemical names and abbreviations are shown in Scheme 1. Several receptors are present on macrophages that can recognize phospholipid ligands, including CD36, SR-BI, SR-PSOX, and others (22–24). To define both the contribution of CD36 on macrophages in the recognition of oxPC$_{CD36}$ and the specificity of these oxPC species for CD36, we examined the binding of oxPC$_{CD36}$ to MPM from mice homozygous for a null mutation in CD36 (CD36-KO) or from wild-type (WT) mice possessing congenic backgrounds (6). Small unilamellar vesicles were made containing PAPC as a carrier lipid, synthetic oxPC$_{CD36}$ species, and trace levels of the fluorescent lipid DiI. The binding (4 °C, Fig. 1*a*) and uptake (37 °C, Fig. 1*b*) of the phospholipid vesicles to MPM was then examined using fluorescence microscopy. Vesicles made of only

a



b

PAPC series n=1	X	Y	PLPC series n=5	X	Y
HOdiA-PC	OH	OH	HDdiA-PC	OH	OH
KOdiA-PC	=O	OH	KDdiA-PC	=O	OH
HOOA-PC	OH	H	HODA-PC	OH	H
KOOA-PC	=O	H	KODA-PC	=O	H

SCHEME 1. *a*, the core structural motifs conserved among the various isolated oxidized PC species that support CD36 binding activity are shown (adapted from Ref. 14). Products generated from oxidized PAPC and PLPC are similar except for the chain length of the truncated oxidized fatty acid esterified to the *sn*-2 position of lysophosphatidylcholine. R_1, fatty acid such as palmitate or oleate. *n*, chain length (number of methylene groups). *b*, the structures, chemical names, and abbreviations of identified phospholipid species with CD36 binding activity derived from oxidized PAPC and PLPC are shown.

PAPC (and fluorescent-label tracer) failed to bind specifically to CD36 on MPM. Only weak (and comparable) diffuse staining was detected for both WT and CD36-KO MPM. In contrast, vesicles containing either PAPC oxidized by the myeloperoxidase (MPO)-H_2O_2-NO_2^- system or those containing one of the identified PC species that serve as a CD36 ligand (*e.g.* data for KOOA-PC shown) were readily bound and taken up by WT macrophages (Fig. 1). In contrast, binding and uptake of these vesicles by CD36-KO MPM was negligible.

Addition of oxPC$_{CD36}$ to Cholesterol-laden Particles Promotes CD36-dependent Accumulation of Cholesterol and Foam Cell Formation in Macrophages—To directly assess the functional consequences of oxPC$_{CD36}$-dependent acquisition of CD36 recognition and uptake, we tested whether addition of these ligands to cholesterol-containing vesicles or liposomes facilitates cholesterol accumulation and foam cell formation in macrophages. OxPC$_{CD36}$ were incorporated into either phospholipid:cholesterol (80:20 mol:mol) vesicles (Fig. 2, *a–c*) or liposomes derived from lipid extracts of native LDL (Fig. 2*d*) and then incubated with WT and CD36-KO MPM. For these studies, trace levels (≪1 mol %) of different radioactive lipids were incorporated into the phospholipid:cholesterol vesicles to monitor uptake from distinct portions of the particles (*e.g.* [^{3}H]DPPC in Fig 2*a*; [^{14}C]cholesterol in Fig. 2*b*; [^{3}H]cholesteryl linoleate in Fig. 2*c*). Incorporation of oxPC$_{CD36}$ into the phospholipid:cholesterol vesicles resulted in significant CD36-dependent uptake of the particles by MPM (Fig. 2, *a–c*). Qualitatively similar results were observed regardless of which radiolabeled tracer was used, consistent with holoparticle uptake via CD36. Significant increases in cellular free cholesterol content, as detected by [^{14}C]cholesterol accumulation, was observed in WT but not CD36-KO macrophages after a 2-h incubation with vesicles containing oxPC$_{CD36}$ (Fig. 2*b*). Moreover, CD36-dependent accumulation of unesterified [^{3}H]cholesterol was observed in WT MPM when vesicles were labeled with trace amounts of [^{3}H]cholesteryl linoleate (Fig. 2*c*), indicating that lipid vesicles were internalized and cholesterol esters hydrolyzed intracellularly. Similar increases in CD36-specific uptake (37 °C) of oxPC$_{CD36}$-containing particles were observed at lower mol % of oxPC$_{CD36}$ species (data not shown). In parallel experiments, we compared the ability of LDL modified by the MPO-H_2O_2-NO_2^- system (NO_2-LDL, 50 μg/ml) to stimulate cholesterol esterification in WT MPM in the absence or presence of

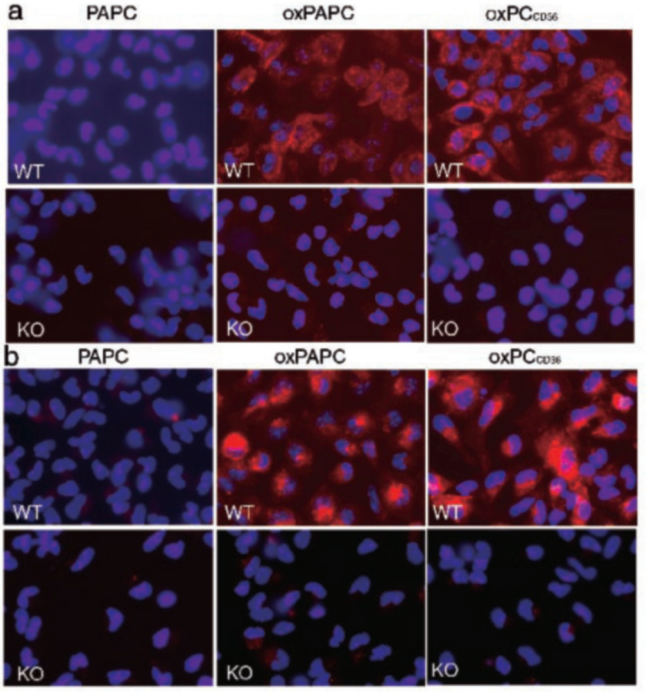


FIG. 1. **CD36 is the major receptor on macrophages that accounts for the binding and uptake of oxPC$_{CD36}$.** Thioglycollate-elicited MPM from WT and CD36 null mice (*KO*) were cultured for 24 h in RPMI 1640 containing 10% fetal bovine serum. Cells were then incubated with DiI-labeled (1 mol %) small unilamellar vesicles to assess either binding (*a*) or uptake (*b*) of vesicles to cells as described under "Experimental Procedures." Binding studies were performed in RPMI 1640 containing 5% lipoprotein-deficient serum, 20 μM HEPES for 2 h at 4 °C. Uptake studies were performed in RPMI 1640 containing 5% lipoprotein-deficient serum for 1 h at 37 °C. Small unilamellar vesicles composed of 1-palmitoyl,2-oleoyl PC (200 μg/ml) were added to the media in all studies to block nonspecific binding and uptake of labeled phospholipid vesicles. Vesicles were composed of 1 mol % diI and PAPC only (*PAPC*), PAPC with the addition of 49 mol % of PAPC oxidized by the MPO-H_2O_2-NO_2^- system in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) at 37 °C for 20 h (*oxPAPC*), or PAPC with the addition of 49 mol % synthetic KOOA-PC (*oxPC$_{CD36}$*) as described under "Experimental Procedures." DiI-labeled vesicles were added to cells at final concentrations of 5 μg/ml for binding studies or 10 μg/ml for uptake studies.

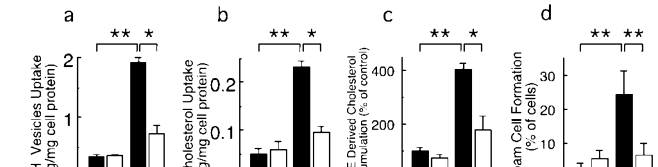


FIG. 2. **Addition of oxPC$_{CD36}$ to cholesterol containing vesicles or liposomes confers CD36-dependent binding, uptake, and metabolism on the particles, leading to accumulation of cholesterol and foam cell formation in macrophages.** *a–c*, the uptake of differently labeled vesicles by WT (*solid bars*) and CD36-KO (*open bars*) MPM are shown. Vesicles containing 40 mol % of PAPC, 40 mol % of HDdiA-PC, 20 mol % of cholesterol, and trace amounts of [^{3}H] DPPC (*a*), [^{14}C]cholesterol (*b*), or [^{3}H]cholesteryl linoleate (*c*) were generated. *a* and *b*, uptake of vesicles by MPM was determined as in Fig 1. *c*, [^{3}H]cholesteryl linoleate-labeled vesicles were incubated with MPM as in *panel a*, and then unbound vesicles were washed from the cells, cellular lipids were extracted, and radioactivity in the cellular unesterified cholesterol pool was quantified. *d*, liposomes composed of lipid extracts from native LDL with the addition of either 5 mol % HDdiA-PC (+) or 5 mol % PAPC (−) were generated. Following incubation with WT and CD36-KO MPM for 2 days, foam cell formation was quantified as described under "Experimental Procedures." *, $p < 0.005$; **, $p < 0.0005$.

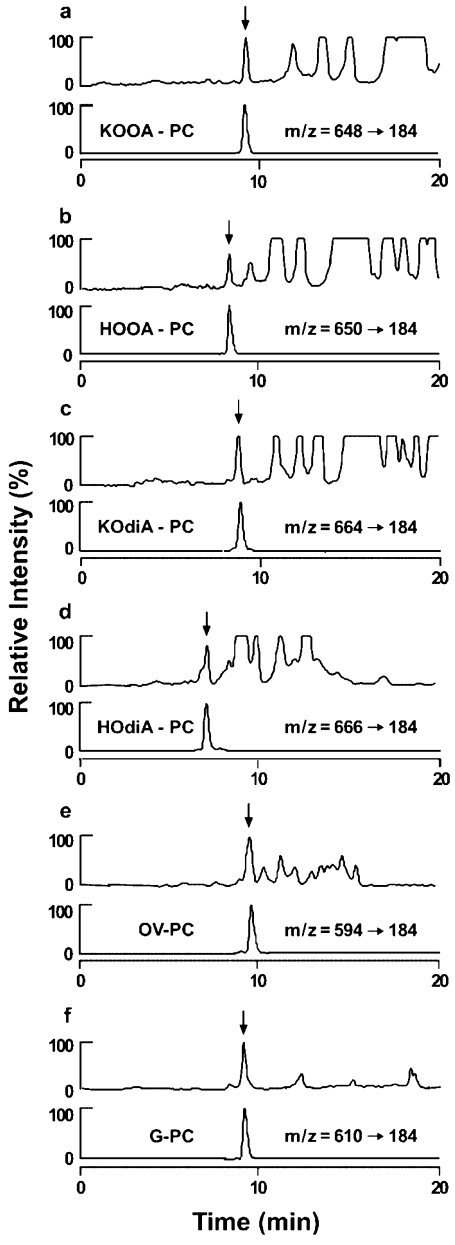


FIG. 3. **Exemplary LC/ESI/MS/MS data illustrating *in vivo* detection of oxPAPC species.** Oxidized PC species were extracted from aortas from WHHL rabbits with confluent lesions. Lipids were fractionated on an analytical reverse phase HPLC column and quantified using mass spectrometric analyses as described under "Experimental Procedures." The ion current tracing of oxPC extracted from aortic tissue obtained using LC/ESI/MS/MS with MRM mode is shown. The *upper tracing* in each *panel* represent MRM analyses at specified parent → daughter ion transitions for the indicated oxPC species within lipid extracts from atherosclerotic lesions. The *lower tracing* in each *panel* illustrate ion current tracing of indicated synthetic oxPC standards analyzed under identical LC/ESI/MS/MS conditions.

individual synthetic oxPC$_{CD36}$ species (20 μM). Although NO_2-LDL markedly stimulated [^{14}C]oleate incorporation into cellular cholesterol ester pools in WT MPM in the absence of competitors, as previously reported (15), addition of the oxPC$_{CD36}$ species, but not the precursor lipids (PAPC or PLPC), inhibited [^{14}C]oleate incorporation to a level comparable with that observed following exposure to native LDL (data not shown). Finally, oxPC$_{CD36}$-dependent formation of foam cells was observed in WT but not CD36-KO MPM following a 48-h incubation with liposomes generated from lipid extracts of native LDL lipids and 5 mol % of oxPC$_{CD36}$ (Fig. 2*d*).

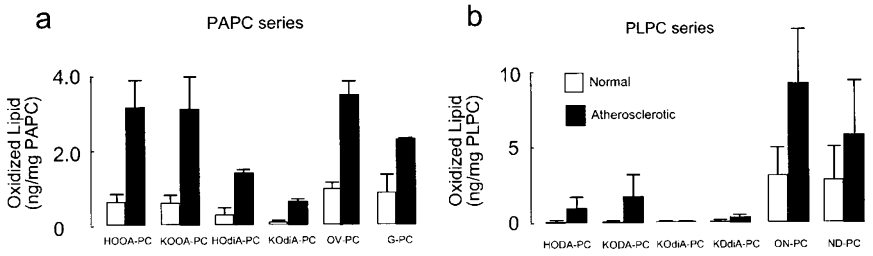


FIG. 4. **$OxPC_{CD36}$ are enriched in atherosclerotic lesions.** The contents of the indicated oxidized PC species in normal and atherosclerotic rabbit aortas were determined by LC/ESI/MS/MS analysis as described under "Experimental Procedures." Data are expressed as mean ± S.D.

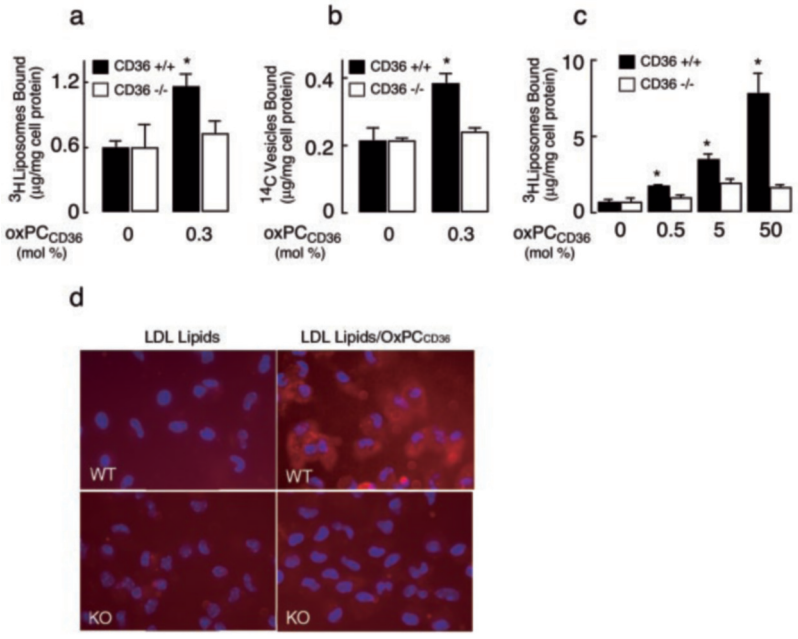


FIG. 5. **Trace levels of $oxPC_{CD36}$ are sufficient to confer CD36-dependent recognition by macrophages.** Liposomes derived from lipid extracts of native LDL (*a*) and PAPC vesicles (*b*) were prepared in the absence and presence of 0.3 mol % $oxPC_{CD36}$ (total) and trace levels of high specific activity (25 μCi/mg total phospholipid) [^{3}H]cholesterol (*a*) or [^{14}C]DPPC (*b*). The $oxPC_{CD36}$ used for liposome and vesicle preparation were composed of a mixture of all eight identified $oxPC_{CD36}$ species (Scheme 1) in the proportions observed within NO_2-LDL preparations (14). The binding of liposomes and vesicles to macrophages was then determined using WT (*solid bars*) and CD36-KO (*open bars*) MPM. *c*, the binding of liposomes to WT and CD36-KO MPM was performed using methods similar to those described for *panel a* except for the use of liposomes containing only a single species of $oxPC_{CD36}$ (HDdiA-PC) at the indicated mol %. *d*, liposomes were generated from lipid extracts of native LDL supplemented with 1 mol % diI and either no addition (*LDL Lipids*) or 1 mol % (total) $oxPC_{CD36}$ mixture as in *panels a* and *b*. Binding to WT and CD36-KO MPM was then monitored by fluorescence microscopy.

$OxPC_{CD36}$ Species Are Enriched in Atherosclerotic Lesions—To determine whether $oxPC_{CD36}$ species are formed *in vivo*, normal and atherosclerotic aortas were isolated from WHHL rabbits, and the levels of multiple distinct specific oxidized phospholipids were determined using LC/ESI/MS/MS analyses as described under "Experimental Procedures." Each of the oxPC species monitored was present *in vivo*. Identification of each species was based upon the detection of ions with mass-to-charge (*m/z*) ratio identical to that of the parent lipid, which following collision-induced dissociation, subsequently also gave rise to an anticipated characteristic daughter ion, all at the retention time of the appropriate authentic synthetic oxPC species. Exemplary LC/ESI/MS/MS data confirming *in vivo* detection of each oxPAPC species in atherosclerotic rabbit aortas are presented in Fig. 3. For illustrative purposes, the *upper tracing* of each *panel* of Fig. 3 demonstrates LC/ESI/MS/MS analysis in the positive ion mode using MRM between the transition from the protonated parent ion to the characteristic daughter ion generated by loss of the polar head group (*i.e.* $[M+H]^+ \rightarrow 184$). The *lower tracing* of each *panel* shows independent ion current tracings for similar MRM analyses of the appropriate synthetic lipid standard.

Significant increases in the content of each of the CD36 ligands derived from oxPAPC (HOOA-PC, KOOA-PC, HOdiA-PC, KOdiA-PC) and oxPLPC (HODA-PC, KODA-PC, HDdiA-PC, and KDdiA-PC) (see Scheme 1 for structures) were noted in the diseased vessels (Fig. 4). Interestingly, levels of $oxPC_{CD36}$ species derived from PAPC oxidation were comparable with that observed for the 5-oxovaleric acid and glutaric acid esters of 2-lysoPC (OV-PC and G-PC, Fig. 4*a*), bioactive phospholipids initially described for their ability to promote monocyte adhesion to endothelial cells (19). We recently showed that OV-PC and G-PC are formed during further oxidation of PAPC-derived $oxPC_{CD36}$ (14). Atherosclerotic tissue levels of $oxPC_{CD36}$ species formed during PLPC oxidation (HODA-PC, KODA-PC, HDdiA-PC, and KDdiA-PC) were also increased over that of normal aorta but at lower levels than observed for the more highly oxidized ON-PC and ND-PC, the 9-oxononanoic acid and nonanedioic acid monoesters of 2-lysoPC (Fig. 4*b*). ON-PC and ND-PC are formed by further oxidation of the PLPC-derived $oxPC_{CD36}$ and are the PLPC-derived analogs of OV-PC and G-PC (14).

Trace Levels of $oxPC_{CD36}$ within Particles Are Sufficient to Confer CD36-dependent Recognition by Macrophages—The demonstrated enrichment of $oxPC_{CD36}$ and their oxidation products (OV-PC, G-PC, ON-PC, and ND-PC) in atherosclerotic lesions strongly suggests that these species may participate in recognition of oxidized LDL and cellular membranes *in vivo*. To further assess the potential pathophysiological relevance of $oxPC_{CD36}$ formation *in vivo*, we examined the levels of $oxPC_{CD36}$ required to confer detectable increases in CD36-specific recognition of particles using MPM from WT and CD36-KO mice. Small unilamellar vesicles (composed of PAPC) and liposomes (generated from lipid extracts of native LDL) were prepared with tracer levels (<0.1 mol %) of radiolabel (either [^{3}H]cholesterol ester or [^{14}C]DPPC) and low mol % (relative to phospholipid content) $oxPC_{CD36}$. Remarkably, whether presented within a bilayer of a vesicle or the surface of a liposome, significant ($p < 0.01$) and reproducible increases in CD36-specific binding of $oxPC_{CD36}$-containing particles were observed at only 0.3 mol % of $oxPC_{CD36}$ species (Fig. 5, *a* and *b*). When $oxPC_{CD36}$ levels were varied within liposomes, dose-dependent increases in CD36-specific binding were observed, whether monitored using a

radiolabel tracer (Fig. 5*c*, [^{3}H]cholesterol) or fluorophore (DiI). Fig. 5*d* illustrates CD36-specific binding of DiI-labeled liposomes to WT, but not to CD36-KO MPM, in the presence, but not the absence, of 1 mol % $oxPC_{CD36}$.

DISCUSSION

Numerous lines of evidence support a physiological role for $oxPC_{CD36}$ species. First, demonstration of their enrichment in atherosclerotic lesions by LC/ESI/MS/MS analyses (Fig. 4) confirms their formation *in vivo*. Second, addition of $oxPC_{CD36}$ to cholesterol-containing particles was shown to confer macrophage CD36-dependent binding, uptake, and metabolism of cholesterol esters with a resultant accumulation of cholesterol and foam cell formation (Fig. 2). Third, a mechanistic role for CD36 as the major scavenger receptor recognizing this novel family of oxidized choline glycerophospholipids is confirmed through studies employing WT and CD36 null mice. Fourth, further support of a physiological role of $oxPC_{CD36}$ is the demonstration that incorporation of only trace levels of $oxPC_{CD36}$ (as low as 0.3 mol %) conferred CD36-dependent binding to macrophages (Fig. 5). A native LDL particle possesses ~700 molecules of phospholipid, composed mostly of PAPC and PLPC (25). Thus, 0.3 mol % would correspond to only a few molecules of $oxPC_{CD36}$ when extrapolated to an LDL particle. The levels of $oxPC_{CD36}$ observed within LDL oxidized by multiple distinct oxidation systems *in vitro* are in excess of this level (1–5 mol %) when CD36-dependent lipoprotein recognition is observed (14). Recognition of $oxPC_{CD36}$ also occurred regardless of whether the species were incorporated within the bilayer structure of a small unilamellar vesicle or embedded within a liposome derived from lipid extracts of native LDL, presumably at the hydrophobic/hydrophilic interface at the particle surface. Further, binding to $oxPC_{CD36}$-containing particles to CD36 increased with increasing mol % of ligand within the surface of a particle (Fig. 5*c*), consistent with enhancement of binding through mutivalent (*i.e.* multiple receptor-ligand) interactions. The MPO-H_2O_2-NO_2^- system was used as a reproducible mechanism for oxidizing lipids. The physiological relevance of this pathway for initiating lipid oxidation *in vivo* is supported by recent studies with leukocytes isolated from individuals with MPO deficiency (26) and by the recent demonstration that MPO serves as a strong and independent predictor of coronary artery disease risk (27).

It is interesting to note that small increases in oxPC-dependent vesicle uptake were also observed in CD36 null mice (Fig. 2). These results suggest that the incorporation of $oxPC_{CD36}$ species may also promote particle recognition by alternative scavenger receptors on MPM, but to a lesser extent than CD36. We speculate that the homologous scavenger receptor B1 is a likely candidate. It belongs to the same family of cell surface receptors as CD36, is expressed in macrophages (23), and recognizes modified lipoproteins similarly to CD36 (28). However, as our data demonstrate, the role of other receptors in recognizing $oxPC_{CD36}$ is minor as compared with the role of CD36.

LC/ESI/MS/MS analyses of various oxPC species in fresh vascular tissues were performed using conditions developed to minimize intrapreparative oxidation of lipids and preserve recovery of each analyte monitored (based upon control studies using trace levels of individual synthetic standards and deuterated precursors, as described in Ref. 14). When analyzed using these methods, the content of $oxPC_{CD36}$ species observed *in vivo* was comparable with those observed for the more terminally oxidized end products of PAPC, OV-PC, and G-PC (Fig. 4). The $oxPC_{CD36}$ species identified are thus relatively abundant in vascular tissues. Moreover, like OV-PC and G-PC, which possess proinflammatory activities (19, 29), $oxPC_{CD36}$ species may possess biological activities extending beyond serving as ligands for CD36. In addition, by virtue of their capacity to carry cargo within oxLDL into CD36-expressing macrophages, $oxPC_{CD36}$ may indirectly promote biological activities such as PPARγ activation (30–32) or oxysterol-induced cytotoxicity (33) and apoptosis (34). Finally, it is tempting to speculate that individuals with increased circulating levels of $oxPC_{CD36}$ in plasma lipoproteins are at an increased risk for atherosclerotic lesion development and progression. The demonstration of structurally defined oxidized PC species that serve as apparent endogenous ligands for the macrophage scavenger receptor CD36 provides new opportunities for probing the physiological and pathophysiological activities of this complex and multifaceted receptor, as well as for assessing the role of oxidation in atherogenesis.

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