Phosphocholine as a pattern recognition ligand for CD36[1,S]

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Abstract **We have previously shown that CD36 recognizes oxidation products of phospholipids on oxidized LDL (OxLDL) such as 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC). The current study was designed to examine whether the phosphocholine (PC) headgroup in POVPC constitutes an obligatory binding target for CD36. To examine the contribution of PC in the binding of POVPC to CD36, we used well-defined synthetic oxidized phospholipids (OxPLs) cross-linked to BSA or to a hexapeptide. The OxPL adducts were then tested for their ability to bind to CD36-transfected cells and for their ability to inhibit OxLDL binding to CD36. Both POVPC-BSA and POVPC-peptide adducts were high-affinity ligands for CD36 and potent inhibitors of OxLDL binding. Enzymatic removal of the entire PC moiety of the POVPC-peptide, or of the choline headgroup alone, as well as substitution of the choline headgroup by ethanolamine abrogated the inhibitory activity of POVPC. Interestingly, PC by itself or cross-linked to BSA did not show any intrinsic competition activity. In conclusion, our data demonstrate that the PC headgroup of OxPL alone is sufficient for binding to CD36, but only if presented in the correct conformation as in OxPL of OxLDL or as in POVPC-peptide adducts.—** Boullier, A., P. Friedman, R. Harkewicz, K. Hartvigsen, S. R. Green, F. Almazan, E. A. Dennis, D. Steinberg, J. L. Witztum, and O. Quehenberger. **Phosphocholine as a pattern recognition ligand for CD36.** ***J. Lipid Res.*** **2005.** 46: **969–976.**

Supplementary key words scavenger receptor • oxidized phospholipids • atherosclerosis

Mounting evidence implicates oxidized low density lipoprotein (OxLDL) in the pathogenesis of atherosclerosis. The oxidative modifications are presumed to occur after the entry of plasma LDL into the intima and are catalyzed by the cellular constituents of the arterial wall. Although clearance of the highly cytotoxic OxLDL may protect the surrounding tissue from damage, the unregulated uptake of OxLDL by macrophages within the arterial wall leads to the formation of lipid-laden foam cells and the development of the fatty streak, the hallmark of early atherosclerotic lesions. The recognition and uptake of OxLDL by macrophages is mediated by specific cell surface scavenger receptors, including CD36, a class B scavenger receptor (1). CD36 is a heavily glycosylated protein with broad ligand specificity, a characteristic of pattern recognition receptors. It binds long-chain fatty acids (2), anionic phospholipids (3), β-amyloid (4), advanced glycation end products (5), and OxLDL (1). It also functions in the recognition and removal of *Plasmodium falciparum*-infected erythrocytes (6) and apoptotic cells (7). It serves as the primary receptor for platelet adhesion to collagen (8) and may mediate the antiangiogenic activity of thrombospondin (9).

Recent data derived from CD36-deficient mice support an important role of CD36 in foam cell formation and atherogenesis. A significant decrease in binding and degradation of OxLDL was observed in macrophages from null mice compared with those from control mice (10). Similarly, macrophages from CD36-deficient patients were less capable of binding and degrading OxLDL and accumulated less cholesteryl ester than macrophages from control subjects (11). The relative contribution of scavenger receptors in the uptake of OxLDL was further addressed in mice lacking both scavenger receptor A and CD36 (12). Binding and uptake studies demonstrated that scavenger receptor A and CD36 were the principal macrophage receptors responsible for the binding of OxLDL and the accumulation of cholesteryl ester derived from modified lipoproteins. Consistent with a principal pathogenic role of scavenger receptors in atherogenesis, the targeted disrup-

Abbreviations: Ac-TGTKGY, Ac-threonine-glycine-threonine-lysine-glycine-tyrosine; apoE, apolipoprotein E; KLH, keyhole limpet hemocyanin; OxLDL, oxidized low density lipoprotein; OxPL, oxidized phospholipid; PAMP, pathogen-associated molecular pattern; PC, phosphocholine; PLC, phospholipase C; PLD, phospholipase D; POVG, 1-palmitoyl-2-(5′-oxovaleroyl)-glycerol; POVPA, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphatidic acid; POVPC, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine; POVPE, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphoethanolamine.

[1] Trudy Forte served as Guest Editor for this manuscript.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional table and two figures.

Manuscript received 16 December 2004 and in revised form 27 January 2005.

Published, JLR Papers in Press, February 1, 2005.
DOI 10.1194/jlr.M400496-JLR200

tion of the CD36 gene markedly reduced lesion formation (10). Collectively, these data demonstrate that CD36 is importantly involved in the uptake of OxLDL by macrophages and in atherosclerotic lesion development.

In previous studies, we (13) and subsequently others (14, 15) have shown that the binding of OxLDL to CD36 is mediated in part by oxidized phospholipids (OxPLs). However, the specific epitopes on such OxPLs that trigger the binding of these lipids have not been fully defined. Natural autoantibodies against OxLDL have been isolated from hypercholesterolemic apolipoprotein E (apoE)-deficient mice (16). One of them, designated EO6, has been shown to specifically recognize the phosphocholine (PC) headgroup of PC-containing OxPLs, such as that found in 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) (17). The observation that EO6 inhibited the uptake of OxLDL by macrophages suggested that the PC group of OxPLs might be a specific binding motif on OxLDL recognized by macrophage scavenger receptors (13). The present study was designed to examine the involvement of the PC headgroup in the binding of POVPC to CD36 using well-defined synthetic lipids.

METHODS

Materials

COS-7 cells were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium with 4.5 g/l glucose was from Bio-Whittaker (Walkersville, MD); fetal bovine serum was from Gemini Bioproducts, Inc. (Calabasas, CA). Penicillin-streptomycin, L-glutamine, and trypsin-EDTA were from Irvine Scientific (Santa Ana, CA). FuGene6 was purchased from Roche Applied Science (Indianapolis, IN). Anti-human CD36 antibody (clone FA6-152) was purchased from Immunotech (Marseille, France). $Na^{125}I$ (2,000 Ci/mmol) was from ICN (Costa Mesa, CA), and Iodogen was from Pierce (Rockford, IL). PC-keyhole limpet hemocyanin (KLH) and PC-BSA were from Bioresearch Technologies, Inc. (Novato, CA). All of the phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Schiff's Reagent (for detection of aldehydes by thin-layer chromatography), Sigma Spray Reagent Molybdenum Blue (for detection of phosphates by thin-layer chromatography), BSA, PBS, phospholipase C (PLC) from *Bacillus cereus*, phospholipase D (PLD) from *Streptomyces species* and *Streptomyces chromofuscus*, ethanolamine, methyl sulfide, sodium cyanoborohydride, and sodium borohydride were purchased from Sigma-Aldrich (St. Louis, MO). Solvents and preparative as well as analytical thin-layer chromatography plates were purchased from Omnisolv, EMD Chemicals (Gibbstown, NJ). The peptide Ac-threonine-glycine-threonine-lysine-glycine-tyrosine (Ac-TGTKGY) was purchased from Synpep (Dublin, CA). Bio-Gel P-2 gel fine (45–90 μm) was from Bio-Rad (Hercules, CA).

Transfection of COS-7 cells with CD36

COS-7 cells were transfected with CD36 DNA as previously described (13). COS-7 cells were maintained in glucose-containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). For transfection, cells were grown on a six-well plate to ~70–80% confluence and transfected with a mixture of 2 μg of human CD36 DNA subcloned into pSG5 (Stratagene) and 6 μl of FuGene6 (Roche). After 24 h, the cells were harvested with EDTA and plated on a 24-well plate at 0.2×10^6 cells/well. Nontransfected COS-7 cells were used as a con-

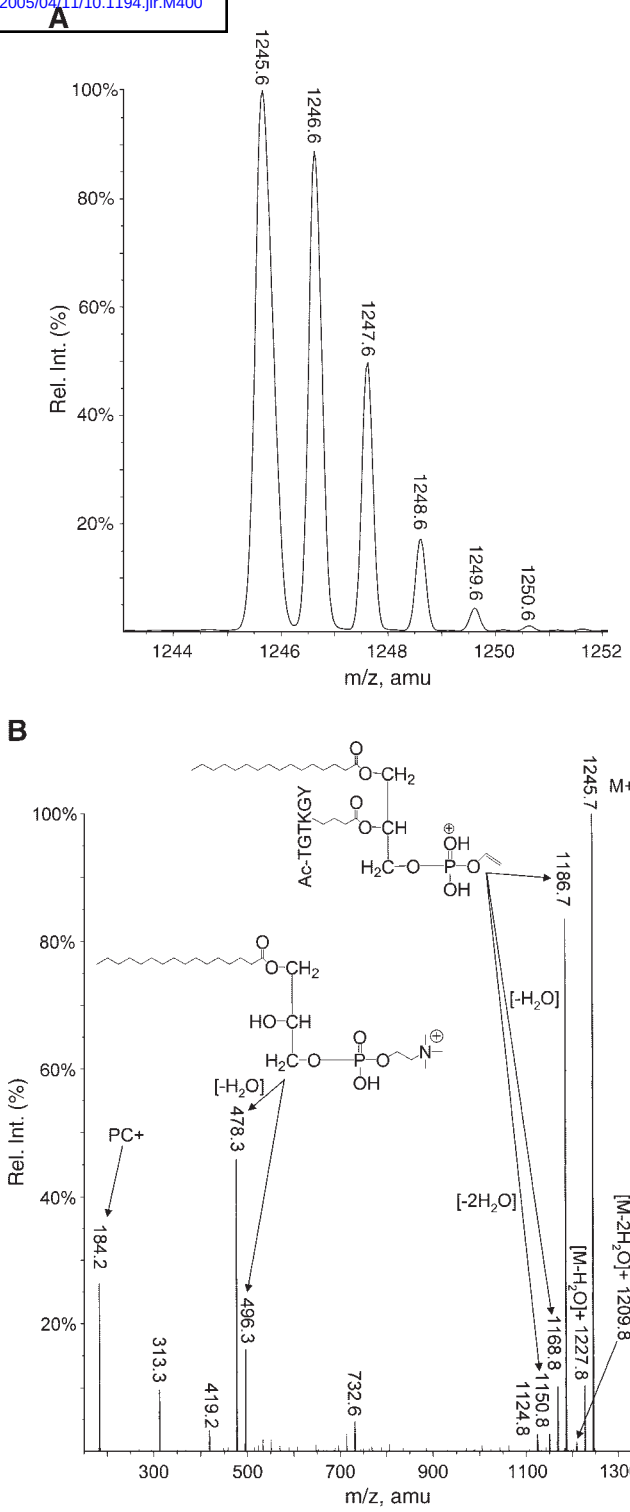

Fig. 1. Mass spectral analysis of the singly charged 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC)-peptide [M^+]. A: LC-enhanced resolution mass spectrum of POVPC-peptide. Note the 1 amu mass difference between adjacent isotopic peaks, indicating the 1^+ charge state. B: LC-MS/MS collision-induced fragmentation of the POVPC-peptide parent [M^+]. All of the fragments were assigned to the POVPC-peptide parent, and the proposed molecular structures of two fragments are shown. The molecular structures of all other fragments are shown in supplementary Fig. I. Rel. Int., relative intensity.

trol. The various cellular assays were performed 48 h after transfection. Cell surface expression of CD36 was determined by flow cytometry using the anti-human CD36 antibody, clone FA6-152.

Lipoprotein preparation and modification

LDL (density = 1.019–1.063 g/ml) was isolated from normolipidemic human plasma by ultracentrifugation (18). After dialysis against PBS containing 0.3 mM EDTA, the protein concentration was determined (19). Native LDL was iodinated by the method of Salacinski et al. (20) and dialyzed extensively against PBS to remove free iodine. The specific activity of the LDL was ~250 cpm/ng protein. Oxidized LDL was prepared by incubating LDL at 100 μg/ml with 10 μM $CuSO_4$ for 18 h at 37°C. The degree of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (21). Butylated hydroxytoluene (20 μM) and EDTA (0.1 mM) were added to prevent further oxidation. The unlabeled OxLDL was concentrated to ~1 mg/ml, sterile-filtered, and stored at 4°C.

Synthesis of lipid-peptide adducts

POVPC was synthesized as described previously (22). The synthetic aldehyde-containing lipids were reacted with the Ac-TGTKGY peptide at an equimolar ratio in 0.1 M ammonium carbonate buffer, pH 7.9, at 37°C. The Schiff base formed between the ε-amine of the lysine and the aldehyde was reduced by the addition of sodium cyanoborohydride, and the reaction mixture was incubated overnight at 37°C. The product was purified by HPLC using an isocratic mobile phase (60:20:20 isopropanol-acetonitrile-water) containing 0.2% formic acid. Two peaks were observed. One, which did not contain the adduct by mass spectrometry and presumably representing unreacted peptide, did not compete for the binding of OxLDL to CD36 (data not shown). The second contained phosphate and competed effectively with OxLDL, and the presence of the expected adduct was confirmed by mass spectrometry using a 4000 QTRAP (Applied Biosystems, Foster City, CA) run in full-scan mode (mass range 400–1,500 amu) or enhanced resolution mode. The ion source of the mass spectrometer was run in positive electrospray mode. Two separate LC-enhanced resolution mass spectra were obtained, one corresponding to the singly charged ion (POVPC-peptide M^+ ion) at a monoisotopic *m/z* of 1,245.6 (**Fig. 1A**), the other corresponding to the doubly charged ion [POVPC-peptide $(M+H)^{2+}$ ion] at *m/z* 623.4 (see supplementary Fig. I). Integration of the area under each isotopic peak in both spectra agrees with the calculated isotopic ratio for the POVPC-peptide ($C_{58}H_{102}N_{80}O_{19}P$) (see supplementary Table I). An LC-MS/MS analysis was also conducted to further confirm the presence of POVPC-peptide. Figure 1B shows the MS/MS enhanced product ion spectrum for the parent ion of 1,245.6 amu. Each of the fragments was identified and assigned to the parent POVPC-peptide molecule. As an example, the proposed molecular structures of two fragments are shown in Fig. 1B. The molecular structures of all other identified fragments are shown in supplementary Fig. II.

POVPC-AcTGTKGY (2 μmol) was further treated with PLD from *Streptomyces species* (100 units) in borate buffer at pH 8.0 to yield 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphatidic acid (POVPA)-AcTGTKGY or with PLC (100 units) in sodium acetate buffer at pH 6.5 to yield 1-palmitoyl-2-(5′-oxovaleroyl)-glycerol (POVG)-AcTGTKGY (**Fig. 2**). 1-Palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphoethanolamine (POVPE)-AcTGTKGY was obtained by incubating POVPC-AcTGTKGY (2 μmol) with ethanolamine (3 M final concentration) and 500 units of PLD from *Streptomyces chromofuscus* in 0.1 M sodium acetate, pH 5.6, with 30 mM $CaCl_2$ under constant stirring. All compounds used in ligand binding studies were purified by thin-layer chromatography, and their purity was confirmed by ultraviolet spectroscopy (to detect the tyrosine of the peptide), by molybdate spray (to detect the phosphate in the headgroup), and by ninhydrin (to detect the primary amine of the ethanolamine headgroup).

POVPC-BSA was synthesized and purified as previously described (17). The BSA adduct (32 μmol POVPC/μmol BSA) was iodinated by the method of Salacinski et al. (20) followed by extensive dialysis against PBS to remove free iodine. The specific activity of the adduct was ~3,000 cpm/ng BSA. The POVPC-peptide adduct was also iodinated at the tyrosine residue as described above. The free iodine was removed by chromatography through a desalting column (Bio-Gel P-2 gel; Bio-Rad). The specific activity of the purified POVPC-peptide was ~1,000 cpm/pmol POVPC.

Ligand binding analysis

Competition of OxLDL binding. The ligand binding assays were carried out 48 h after transfection of the COS-7 cells with CD36. The cells were placed on ice for 10 min, washed twice with cold PBS, and incubated for 2 h at 4°C with 5 μg/ml ^{125}I-OxLDL in the presence of various concentrations of the different competitors. After the incubation, the cells were washed twice with ice-cold PBS containing 0.1% BSA, twice with PBS, and then lysed by

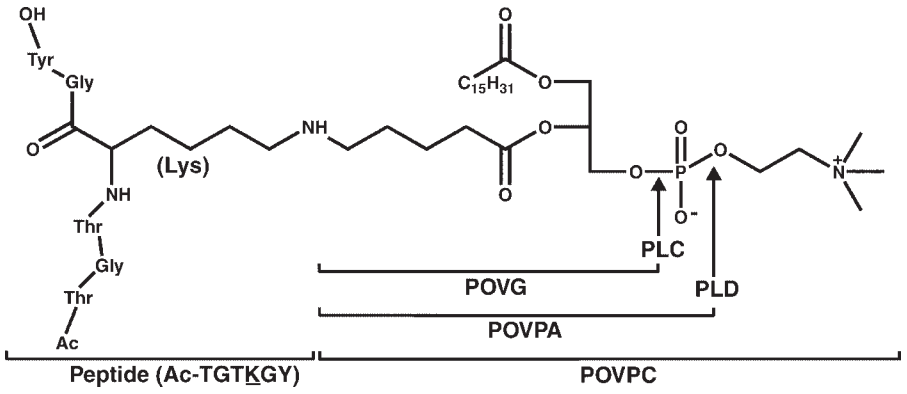

Fig. 2. Structures of peptide adducts of oxidized phospholipids. The ε-amino group of the lysine of the peptide was used to conjugate the peptide to POVPC. Arrows indicate the established cleavage points of the phospholipases used. Ac-TGTKGY, Ac-threonine-glycine-threonine-lysine-glycine-tyrosine; PLC, phospholipase C; PLD, phospholipase D; POVG, 1-palmitoyl-2-(5′-oxovaleroyl)-glycerol; POVPA, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphatidic acid; POVPC, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine.

the addition of 0.2 N NaOH. Aliquots were taken to measure the protein content by the method of Lowry et al. (19) and the cell-associated radioactivity using a γ spectrometer. All assays were done in triplicate.

Binding of ^{125}I-POVPC adducts. The direct binding of ^{125}I-POVPC-BSA was estimated by incubating the transfected cells with various concentrations of ^{125}I-POVPC-BSA. The binding of POVPC-peptide was examined by inhibition binding analysis. Briefly, the transfected cells were incubated for 2 h at 4°C with trace amounts of ^{125}I-POVPC-peptide in the presence of increasing concentrations of unlabeled POVPC-peptide as well as other OxPL peptides. Binding isotherms were generated and binding data were determined using Prism4 software (GraphPad Software, San Diego, CA).

RESULTS

To examine the potential contribution of the PC moiety in the binding of OxPLs to CD36, we first characterized the binding of the POVPC-BSA adduct to CD36. As shown in **Fig. 3A**, the CD36-transfected cells bound ^{125}I-POVPC-BSA with high affinity and in a saturable manner, characteristic of specific binding. Scatchard analysis of the equilibrium binding data revealed a binding affinity of 0.19 ± 0.02 μM for POVPC-BSA, expressed in moles of BSA. Under the conditions of synthesis used, ~32 mol of POVPC was covalently bound per mole of BSA (23). On that basis, the affinity for POVPC presented on BSA was 6.5 ± 0.5 μM. Consistent with our previous data (13), POVPC-BSA proved to be an effective competitor and dose-dependently inhibited the binding of OxLDL to the CD36-transfected cells (Fig. 3B). These data suggest that POVPC and similar structures may constitute ligands on OxLDL that are recognized by scavenger receptors, including CD36. It should be noted here that the *sn*-2 aldehyde of POVPC was used to covalently link it to the amino groups of lysines of BSA via a reduced Schiff base adduct. Thus, the POVPC-BSA adduct did not contain any reactive carbonyl groups in the *sn*-2 position that could be potential ligands for CD36 (14). This suggests the possibility that the PC group itself, as presented in POVPC, was the essential ligand.

Because several moles of POVPC are covalently bound per mole of BSA, the OxPL-BSA adduct represents a multivalent ligand for CD36 in which individual epitopes may contribute to the binding either by interacting with multiple binding sites on CD36 or by receptor cross-linking, or both. To study the binding of POVPC as a monovalent ligand for CD36, we substituted a short peptide of six amino acid residues (Ac-TGTKGY), randomly chosen from the sequence of apoB, for BSA. This peptide contained only one available lysine residue to form the Schiff base, and the N terminus was blocked by acetylation so that each mole of peptide contained only a single mole of lipid. The structure of the POVPC-peptide was confirmed by mass spectroscopic analysis (Fig. 1). Compared with the POVPC-BSA adduct, the POVPC-peptide adduct appeared to be an even more potent inhibitor of OxLDL binding to the CD36-transfected cells (**Fig. 4**). However, it is important to note that the concentration of inhibitors in both cases was expressed in terms of the concentration of POVPC (nanomoles of POVPC per milliliter), presented either as a BSA conjugate or as a hexapeptide conjugate. These results imply that some fraction of the POVPC molecules on the multiligand POVPC-BSA adduct are unavailable for binding to the receptors because of conformational or steric constraints.

To test more rigorously the role of the PC group of POVPC-peptide as a binding epitope for CD36, we sequentially hydrolyzed the functional group at the *sn*-3 position with PLD or PLC (Fig. 2). The PLD selectively hydrolyzed the choline group to yield POVPA-peptide, whereas the PLC selectively hydrolyzed the entire PC group in the *sn*-3 position to produce POVG-peptide (Fig. 2). In contrast to the intact POVPC-peptide, neither of these enzymatic hydrolysis products was an effective inhibitor for OxLDL

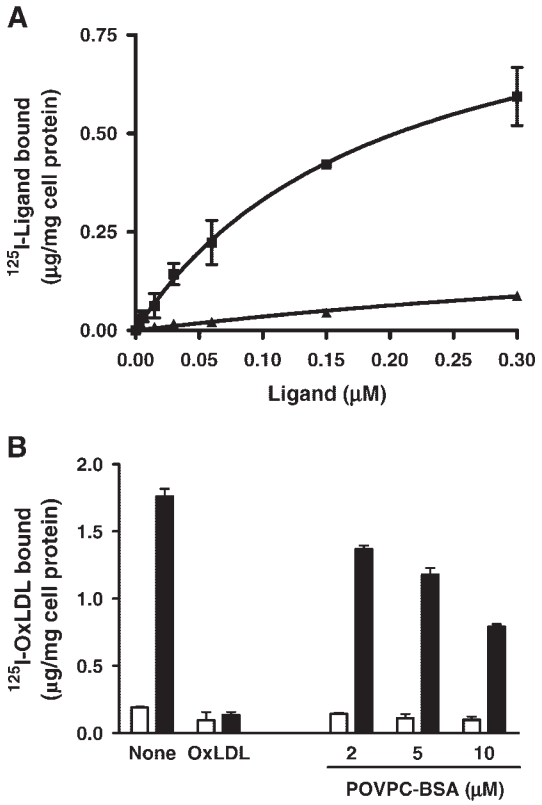

Fig. 3. Binding of POVPC-BSA to CD36. A: Direct binding of ^{125}I-POVPC-BSA to CD36. CD36-transfected cells were incubated with ^{125}I-POVPC-BSA (closed squares) or ^{125}I-BSA (closed triangles) at the indicated protein concentrations for 2 h at 4°C. The values represent means ± SD (n = 3) of two independent experiments. The binding affinity of POVPC-BSA was determined by Scatchard analysis of the binding data (dissociation constant = 0.19 ± 0.02 μM). On average, 32 mol of POVPC was covalently attached per mole of BSA. On that basis, the affinity for POVPC presented on BSA was 6.5 ± 0.5 μM. B: Inhibition of ^{125}I-oxidized low density lipoprotein (OxLDL) binding to CD36 by the POVPC-BSA adduct. CD36-transfected cells (closed bars) and control cells (open bars) were incubated with 5 μg/ml ^{125}I-OxLDL in the presence of the indicated concentrations of the POVPC-BSA adduct. As a positive control, a 30-fold excess of unlabeled OxLDL was used as a competitor. After 2 h of incubation at 4°C, ^{125}I-OxLDL binding was determined. The values represent means ± SD of triplicate determinations derived from three representative experiments.

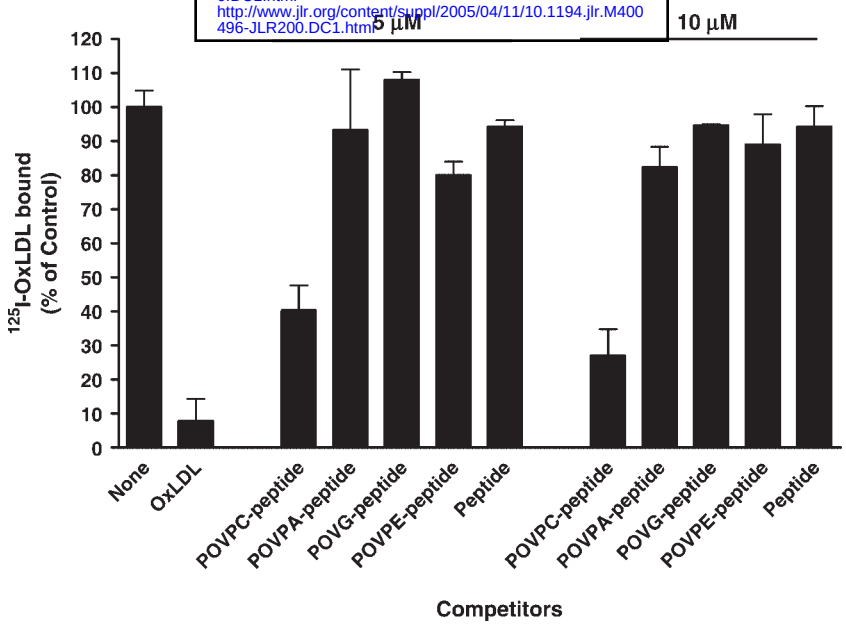

Fig. 4. Inhibition of ^{125}I-OxLDL binding to CD36-transfected cells by enzymatically modified products of the POVPC-peptide. CD36-transfected cells were incubated with 5 μg/ml ^{125}I-OxLDL in the presence of the indicated concentrations of the various competitors. After 2 h of incubation at 4°C, OxLDL binding was determined. Data are presented as percentage binding of ^{125}I-OxLDL, 100% being the value of the binding without any competitor. Each value represents the mean ± SD of triplicate determinations from two separate experiments. POVPE, 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphoethanolamine.

binding (Fig. 4). Similarly, the POVPE-peptide, in which the choline group of POVPC was replaced by ethanolamine, had no inhibitory effect on OxLDL binding to CD36.

The important role of the PC moiety as a binding motif for CD36 was further established by direct binding analysis. The ^{125}I-POVPC-peptide bound to the transfected cells with an affinity of 4.81 ± 1.25 μM, which was similar to the binding affinity of the POVPC-BSA adduct (**Fig. 5**). Removal or substitution of the headgroup as in POVPA, POVG, and POVPE abrogated the binding of these lipid-peptide adducts to CD36, and no inhibition of ^{125}I-POVPC-peptide binding was observed (data not shown). These data suggest that the PC headgroup is essential for the binding of POVPC to CD36 and imply a critical involvement of the choline group for the recognition of OxPL by CD36.

To examine whether PC by itself is recognized by CD36 or whether a specific conformational presentation is required for recognition, we examined the ability of PC to compete for the binding of OxLDL to CD36 when presented either as a free salt or covalently bound to KLH (PC-KLH) or BSA (PC-BSA). As shown in **Fig. 6**, neither free PC in solution (Fig. 6A) nor cross-linked to KLH or BSA (Fig. 6B) inhibited OxLDL binding. The failure of PC by itself to compete suggests that a specific conformational presentation of the headgroup is required for recognition by CD36.

DISCUSSION

The role of CD36 as the principal scavenger receptor responsible for the uptake of OxLDL by macrophages and foam cell formation has been solidly established (1, 11, 12). The present study was undertaken to identify and characterize the oxidation epitopes that may mediate the recognition and removal of modified lipoproteins by CD36. Of particular interest are the oxidation products derived from phosphatidylcholine, including POVPC. In a previous study, we made the observation that the POVPC-BSA adduct competed for the binding of OxLDL to CD36-transfected cells (13). We concluded from this study that OxPLs, present either in the lipid phase or covalently attached to apoB, constitute major binding epitopes and mediate the recognition of OxLDL by CD36. We have now extended these initial observations and identified the PC headgroup of oxidized phosphatidylcholine as an obligatory binding target for CD36.

During the oxidative modification of LDL, a variety of oxidized molecules are generated, including OxPLs (24). There is now a large body of evidence that such OxPLs have proinflammatory and proatherogenic properties and that their accumulation in the vessel wall contributes significantly to the pathological consequences of developing atherosclerotic lesions (24–30). Several bioactive lipids, including POVPC, have been demonstrated in lesion areas where they may regulate inflammatory functions of endothelial cells, such as the expression of adhesion proteins and chemokines (24, 31–33). In addition to the effects on the cellular constituents of the vessel wall, these oxidized lipids have also been implicated in the adaptive and innate immune response. We have previously cloned a series of natural autoantibodies from hypercholesterolemic mice directed against neoself antigens formed dur-

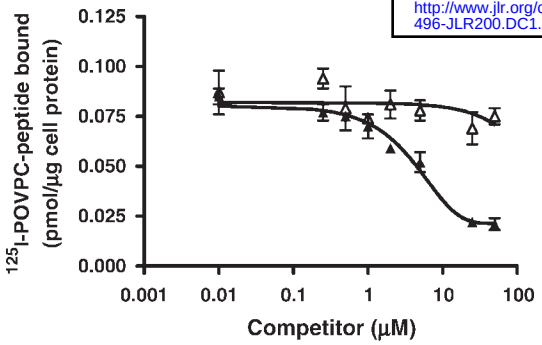

Fig. 5. Competition of ^{125}I-POVPC-peptide binding to CD36. CD36-transfected cells were incubated with trace amounts of ^{125}I-POVPC-peptide for 2 h at 4°C in the presence of the unlabeled of POVPC-peptide (closed triangles) and the unconjugated peptide (open triangles) at the indicated concentrations. Binding analysis of ^{125}I-POVPC-peptide revealed a binding affinity of 4.81 ± 1.25 μM. Results represent means ± SD (n = 3) of two separate experiments.

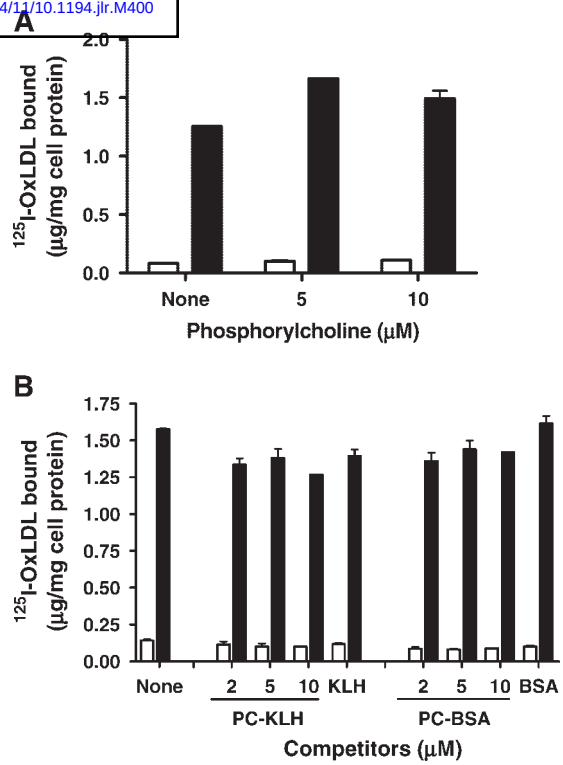

Fig. 6. Competition of ^{125}I-OxLDL binding to CD36 by free phosphocholine (PC) and PC-protein adducts. Control cells (open bars) and CD36-transfected cells (closed bars) were incubated with 5 μg/ml ^{125}I-OxLDL for 2 h at 4°C in the absence or presence of PC presented as a salt in solution (A) or as protein adducts with keyhole limpet hemocyanin (KLH) or BSA (B). KLH and BSA were used as negative controls at 10 μM. The values represent means ± SD of triplicate determinations.

ing oxidation of LDL (16). One of these natural antibodies, EO6, binds to the PC headgroup of OxPLs on OxLDL but not the PC headgroup of unoxidized phosphatidylcholine. In turn, EO6 blocks the uptake of OxLDL by macrophages (17, 22, 34).

These OxPLs also serve as ligands for CD36 and may mediate, at least in part, the recognition and removal of OxLDL by macrophages. Our data clearly implicate the PC headgroup of modified phosphatidylcholine in mediating the interaction with CD36. As was the case with the natural antibody EO6, CD36 also binds the PC headgroup only when other parts of the phospholipid have been modified by oxidation. Podrez et al. (14) have suggested that modifications of the *sn*-2 fatty acid may also contribute to the binding to CD36 and may represent a separate class of ligands on some OxPLs for CD36. To rule out any binding contribution of such oxidized polyunsaturated fatty acids in our studies and to reconstitute a ligand as it may be present on apoB of OxLDL, we conjugated the aldehyde in the *sn*-2 position of POVPC to BSA or to a lysine-containing peptide with subsequent reduction of the Schiff base to a secondary amine. Therefore, the binding of OxPLs to CD36 could only be attributed to the PC residue of the OxPL that became exposed and accessible for CD36 binding as a consequence of conformational changes associated with oxidative events. It is important to note that our data directly address the issue of the role of the PC headgroup as a ligand. Our data do not exclude the possibility that oxidized moieties in the *sn-2* side chain of POVPC could also coordinate to other receptor binding sites of CD36 and, thus, could also be involved in the binding of the isolated POVPC lipid.

In support of a critical role of PC in the binding of OxPLs and OxLDL to CD36, the hydrolysis products of the POVPC-peptide adduct after treatment with PLD or PLC to remove choline or PC, respectively, were no longer competitors for the OxLDL ligand. Similarly, the substitution of the PC headgroup by phosphoethanolamine abrogated the inhibitory effect of POVPC-peptide. Furthermore, the PC group by itself when covalently bound to protein (KLH or BSA) did not compete for the binding of OxLDL to CD36, suggesting that the lipid environment and structural elements of the oxidized phosphatidylcholine molecule were necessary to maintain the appropriate conformation and presentation of PC to be recognized as a ligand.

CD36 developed as part of the innate immune system, and the scavenger function of CD36 is evolutionarily conserved (35, 36). Aside from its function as a receptor for OxLDL, CD36 is thought to play an important role in the recognition and removal of cells undergoing programmed cell death and of pathogens during infection, in cooperation with the phosphatidylserine receptor or the αvβ3 integrin (7, 37). The ligands for CD36 on apoptotic cells and pathogens are not well characterized, but the initial recognition of microbes as they enter the body is based on the presentation of common structural patterns. PC is known to be recognized by the innate immune system, and this may provide host defenses against microbial infection, such as by *Streptococcus pneumoniae*, in which PC is a dominant epitope of the cell wall polysaccharide. Furthermore, PC of OxPLs are also present on the outer leaflet of apoptotic cells (38, 39), and the PC of certain bacteria, of apoptotic cells, and of OxLDL all share molecular mimicry (34, 40). Thus, the PC moieties presented in these

contexts represent pathogen-associated molecular patterns (PAMPs), which are recognition ligands for pattern recognition innate immune receptors such as CD36. Consistent with this, we have previously shown that binding of both OxLDL and apoptotic cells to CD36-transfected cells and macrophages is mediated through a common epitope with structural similarities to POVPC (13, 39). Based on the data derived from EO6, which strongly blocked that binding, we propose that the PC moiety represents such a common PAMP.

In summary, our data strongly implicate a particular conformational presentation of PC as a PAMP, which is recognized by several facets of innate immunity, including natural antibodies, C-reactive protein (41), and pattern recognition receptors such as CD36 and likely scavenger receptor class B type I (42). In this context, the exposure of PC as a result of the oxidation of phospholipids present on OxLDL mediates the recognition of OxLDL by innate immune responses. The application of these findings with regard to the recognition of microbial pathogens and apoptotic cells by macrophage scavenger receptors is currently under study.

This work was supported by National Institutes of Health Grants HL-56989 (Specialized Center of Research in Molecular Medicine and Atherosclerosis), HL-57505, HL-69464, GM-20501, and GM-069338 and by Tobacco-Related Disease Research Program Grant 10KT-0220. The authors thank Jennifer Pattison for her technical assistance in the isolation of the lipoproteins.

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