Oxidized phospholipids, linked to apolipoprotein B of oxidized LDL, are ligands for macrophage scavenger receptors

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Abstract Previous studies have shown that macrophage receptors for oxidized LDL (OxLDL) recognize both the lipid and protein moieties, and that a monoclonal antibody against OxLDL, EO6, also recognizes both species. The present studies show directly that during LDL oxidation phospholipids become covalently attached to apolipoprotein B (apoB). After exhaustive extraction of lipids, apoB of native LDL contained 4 ± 3 moles of phosphorus/mole protein. In contrast, apoB of OxLDL contained approximately 75 moles of phosphorus/mole protein. Saponification of this apoB released phosphorus, choline, and saturated fatty acids in a molar ratio of 1.0:0.98:0.84. When LDL was reductively methylated prior to oxidation, the amount of phospholipid covalently bound was reduced by about 80%, indicating that the phospholipids attach at lysine epsilon amino groups. Progressive decreases in the phospholipid associated with apoB of OxLDL decreased the ability of the protein to compete for binding to macrophage scavenger receptors and decreased its reactivity with antibody EO6. We postulate that some oxidized phospholipids containing fatty acid aldehydes at the *sn*-2 position bind to lysine residues of apoB while others remain unreacted within the lipid phase. This would account for the interchangeability of lipid and apolipoprotein of OxLDL with respect to receptor binding and antibody recognition.—Gillotte, K. L., S. Hörkkö, J. L. Witztum, and D. Steinberg. **Oxidized phospholipids, linked to apolipoprotein B of oxidized LDL, are ligands for macrophage scavenger receptors.** ***J. Lipid Res.*** **2000.** 41: **824–833.**

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Oxidized LDL (OxLDL) is taken up much more rapidly by macrophages than native LDL and this may explain in part the generation of foam cells in the developing fatty streak lesion of atherosclerosis (1). There is now a good deal of evidence to support the view that oxidation of LDL is a significant element in the pathogenesis of atherosclerosis, including the demonstration in several different animal models that antioxidants inhibit lesion progression (2–5). Most recently it has been shown that vitamin E inhibits lesion progression in the apoE-knockout model and that this correlates nicely with a decrease in the extent of isoprostane formation by nonenzymatic oxidation of arachidonic acid (6). Strong support for the hypothesis is provided by the recent report that targeted disruption of the gene for 12/15-lipoxygenase profoundly inhibits atherosclerosis in the apoE-deficient mouse (7). For all of these reasons there is intense interest in the mechanisms involved in LDL oxidation and the specific changes in structure that account for OxLDL–scavenger receptor interaction.

This question has broader implications because it appears that the receptors involved in the recognition of OxLDL are, at least some of them, involved also in the recognition of damaged or dying cells (8). Sambrano and Steinberg (9) showed that oxidatively damaged erythrocytes bound to mouse peritoneal macrophages without the need for opsonization and that this binding was competitively inhibited by OxLDL. They showed that the binding and phagocytosis of apoptotic thymocytes was also inhibited by OxLDL. Thus, insights gained into the nature of the ligand(s) on OxLDL responsible for its recognition by scavenger receptors might also give insights into the nature of the ligands on the membranes of apoptotic cells responsible for their recognition by macrophages.

During oxidation of LDL there is a progressive decrease in the number of reactive lysine epsilon amino groups (10). It has been generally assumed that these amino groups are blocked by reaction with short-chain aldehydes derived from the peroxidation of polyunsaturated fatty acids (e.g., malondialdehyde or 4-hydroxynonenal) (11, 12). This conjugation of aldehydic fragments with lysine amino groups was believed to be responsible for converting native LDL into a form rec-

Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized LDL; apoB, apolipoprotein B from native LDL; OxapoB, apoB isolated from OxLDL; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TNBS, trinitrobenzenesulfonic acid; PL, phospholipid; POVPC, 1-palmitoyl-2-(5-oxovaleroyl) phosphatidylcholine; RM-OxapoB, apoB from LDL that had been reductively methylated prior to oxidation.

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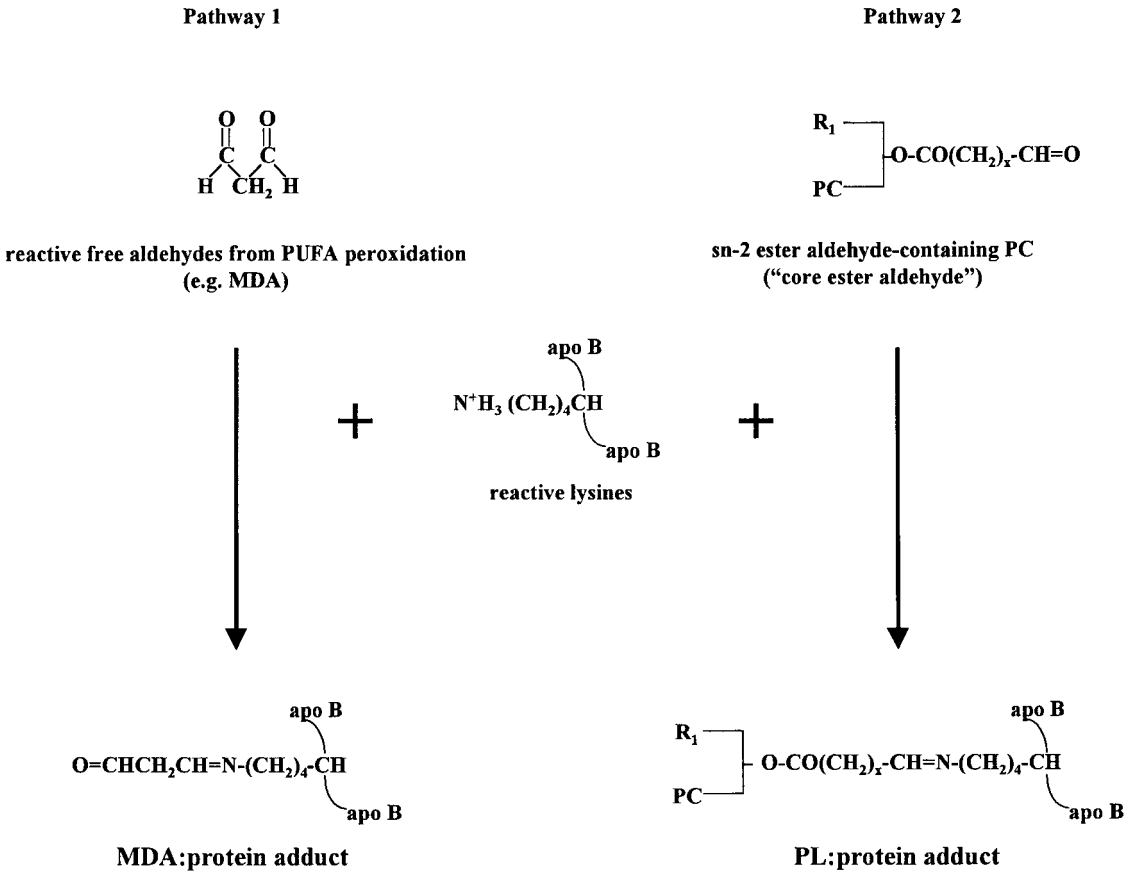

Fig. 1. Covalent adduct formation between core aldehydes and apoB. Pathway 1: The aliphatic aldehydes derived from the methyl terminus of the PUFA chain are also known to form conjugates with the lysine residues of apoB. Shown for representation is a proposed reaction of apoB with malondialdehyde (MDA). Pathway 2: Phospholipid core aldehydes, generated as a result of peroxidation of the polyunsaturated fatty acids (PUFA) present in phospholipids, bind to lysine residues of apoB.

ognized by macrophage scavenger receptors (**Fig. 1**). This was a logical conclusion drawn in part by analogy with the several modifications that were shown to generate modified LDL recognized by the scavenger receptors, i.e., acetylation (13), treatment with malondialdehyde (14), and so on. There was little or no consideration of the possibility that some of the masking of lysine amino groups might be due to conjugation with core ester aldehydes (i.e., phospholipids in which the *sn*-2 polyunsaturated fatty acid has been oxidatively degraded leaving a shortened fatty acid fragment, still esterified to the glycerol backbone, but terminating with an aldehydic function, or polyunsaturated cholesteryl esters in which the fatty acid has been similarly degraded leaving a new exposed aldehydic function) (Fig. 1). The possibility that such core ester aldehydes might play a significant role was raised by studies in this laboratory demonstrating that both the apolipoprotein moieties and the lipid moieties of OxLDL could bind to macrophage scavenger receptors and compete reciprocally with each other for that binding. It was further strengthened by the demonstration that a monoclonal antibody against oxidized phospholipids could react with both the isolated apolipoprotein and the reconstituted lipids from OxLDL (15). Moreover, this antibody strongly inhibited macrophage binding of OxLDL, as well as that of the reconstituted lipid and protein moieties of OxLDL (15, 16).

The present studies show that after exhaustive extraction of lipids the apoB of native LDL contains 0 to 4 moles of phosphorus per mole of apoB whereas that from oxidized LDL contained on average 75 moles of phosphorus per mole of apoB. The number of phospholipid molecules covalently bound was varied either by varying the time of oxidation or by treating the LDL by reductive methylation before oxidizing it to decrease the number of available free amino groups. It is shown that the ability of the apoB from oxidized LDL to compete with intact oxidized LDL or with the apoB prepared from OxLDL varied directly with the number of phospholipid molecules bound per mole of apolipoprotein. The recognition of the oxidized LDL apoB by monoclonal antibody EO6 also varied according to the number of phospholipid molecules covalently attached. Antibody EO6 has previously been shown to react with antigens present in atherosclerotic lesions (17), suggesting that oxidized phospholipid-apoB adducts are also produced in vivo.

EXPERIMENTAL PROCEDURES

Materials

CF50 membrane concentration cones were purchased from Amicon. $CuSO_4$ was obtained from Lab Chem (Pittsburgh, PA). N-octylglucoside was from Boehringer Mannheim. RPMI medium 1640 was from BioWhittaker and was supplemented with gentamycin from Omega Scientific (Tarzana, CA). Fetal bovine serum was from Gemini Biological Products (Calabasas, CA). Carrier-free $Na^{125}I$ was purchased from ICN. Lumiphos (530) was from Lumigen, Inc. (Southfield, MI). NeutrAvidin-Alkaline Phosphatase Conjugated was from Pierce (Rockford, IL) and the Alkaline Phosphatase Conjugate Substrate Kit and Kaleidoscope Prestained Standards were from BioRad (Hercules, CA). All other reagents were analytical grade.

Lipoproteins

LDL was isolated from normolipidemic donors by sequential preparative ultracentrifugation (18). For oxidation of LDL, the LDL was diluted to 100 μg protein/ml with EDTA-free phosphate-buffered saline (PBS) and incubated with 10 μm $CuSO_4$ for 18 h at 37°C. The extent of oxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS) (19) and the degree of fluorescence (excitation 350 nm, emission 433 nm) as described (12). The oxidized LDL (OxLDL) was concentrated in CF50A membrane cones (Amicon) to a protein concentration of approximately 1 mg/ml (20). EDTA and BHT were added at 0.1 mm and 20 μm, respectively, to prevent further oxidation. To stabilize Schiff bases by reduction, $NaCNBH_3$ was added to some samples of OxLDL to a final concentration of 20 mm, followed by incubation and dialysis against Tris-buffered saline (TBS) (0.01 m Tris-HCl, 0.001 m EDTA, 0.15 m NaCl, pH 7.4) for 3 h at 37°C.

Isolation of apolipoprotein B (apoB)

Lipids were extracted from native or OxLDL as previously described (21) with additional extractions to ensure that lipid components were completely removed. Briefly, HCl was added to the LDL preparations to a final concentration of 10 mm, and the lipids were extracted using chloroform–methanol 1:1. The precipitated insoluble apoB was subjected to two sequential Bligh and Dyer extractions (22), followed by washes of ethyl ether, water, and acetone. The isolated protein was solubilized in 10 mm NaOH containing octylglucoside (octylglucoside:protein ratio, 30:1), and the excess detergent was removed by extensive dialysis against TBS. ApoB from native LDL precipitates upon removal of the detergent by dialysis. Therefore, when measurements of native apoB were desired (and the isolated protein was not to be used in a cellular assay) the protein pellet was solubilized in 3% SDS. Protein concentrations were measured (20) and the protein-associated phosphorus was determined by the method of Marinetti (23). Briefly, the samples, as well as standard samples of KH_2PO_4, were dried under nitrogen in glass tubes. Perchloric acid (72%) was added and the samples were digested at 180°C for 2.5 h. After cooling, color development was initiated by incubation at 100°C for 10 min with a 1-amino-2-naphtho-4-sulfonic acid-based reagent. The presence of phosphorus in the samples was assessed by measurement of the absorbance at 800 nm and comparison to a standard curve.

Protein modification

Reductive methylation of native LDL was carried out as previously described by Lund-Katz et al. (24). Briefly, on ice, 0.15 m $NaCNBH_3$ was added to a final concentration of 20 mm to native LDL, present in saline (0.15 m NaCl, 1 mm EDTA, 0.02% NaN_3). Formaldehyde was added to result in final molar ratios of formaldehyde per lysine residues ranging from 0/1 to 10/1. This mixture, which contained about 2.5 mg apoB, equivalent to 1,622 nmols of lysine residues (357 lysines per mole apoB (24)), was incubated at 4°C for 18 h. The reaction was stopped by dialysis against saline and then against PBS. The number of reactive epsilon-amino groups before and after methylation was determined by reactivity with trinitrobenzenesulfonic acid (TNBS) using a revision of the method of Habeeb (25). In brief, 50 μg of protein was diluted to 250 μl with dH_2O in glass tubes. Five hundred μl of 4% bicarbonate buffer, pH 8.5, and 50 μl of 0.1% TNBS were added with vortexing and the samples were incubated at 37°C for 60 min. Two hundred μl 1 N HCl and 100 μl 10% SDS were then added with vortexing and the samples were incubated at room temperature for an additional 15 min. Absorbances were determined at 340 nm and were compared to that of standard samples containing known amounts of valine. The reductively methylated samples were oxidized and extracted as described above.

Phospholipid (PL) associated with the apoB from oxidized LDL (OxapoB) was saponified by incubation of the protein with 1 m KOH in 95% methanol for 1 h at 37°C. After the incubation, 6 N HCl was added dropwise until an acidic pH was reached and $CHCl_3$ was added to extract lipids. Over 99% of the phosphorus originally associated with the OxapoB was now recovered in the aqueous phase. The aqueous phase was also analyzed for choline content using an enzymatic colorimetric method, Phospholipids B (Wako Chemicals USA). The saponified protein was solubilized as described above and dialyzed.

During oxidation of LDL, apoB is degraded to yield a spectrum of lower molecular weight polypeptide fragments. The chromatographic patterns of OxapoB recovered after saponification were shown to be comparable to those of non-saponified OxapoB (SDS-PAGE analysis and visualization with Coomassie blue stain). The chloroform phase from the saponification/extraction was collected for fatty acid analysis. The fatty acids were transmethylated (26) and analyzed in a Varian gas chromatograph model 3700, equipped with a column of 10% Silar 5 CP on 100/120 Gas Chrom Q2. Fatty acid profiles were determined from the total fatty acid recovered as well as from an internal standard containing known amounts of specific fatty acids.

Western blot

OxapoB (10 μg) samples were electrophoresed on SDS 4–12% tris-glycine polyacrylamide gels (Novex) for 2.5 h at 120 V in an Xcell II Mini-Cell apparatus (Novex); Kaleidoscope Prestained Standard (BioRad) was also applied for molecular weight estimation. Proteins were transferred to nitrocellulose membrane and visualization of protein with 0.1% Ponceau S (Sigma) confirmed complete and equivalent transfer for all samples tested. The membrane was incubated with Super Block (Pierce) for 1 h and washed thoroughly with TBS (0.05% Tween-20). Biotinylated antibody EO6 (100 μg) was diluted to 25 ml with TBS-containing 3% bovine serum albumin (0.05% Tween-20) and incubated with the membrane for 18 h at room temperature. The membrane was washed ten times with TBS-containing 0.05% Tween-20 and incubated with alkaline phosphatase-labeled avidin (Pierce) for 45 min. The bound antibodies were detected by alkaline phosphatase visualization by the Alkaline Phosphatase Conjugate Substrate Kit (BioRad).

Cellular binding assay

Native LDL and prepared OxapoB were radiolabeled with ^{125}I by the method of Salacinski et al. (27). Both were exhaustively dialyzed against PBS to remove free ^{125}I and the ^{125}I-labeled LDL was oxidized as described above. The specific activities of the

ligands were generally between 250 and 300 cpm/ng protein, as determined from TCA-precipitable protein.

Swiss-Webster female mice were injected intraperitoneally with 2 ml of thioglycollate medium and the peritoneal macrophages were harvested 3 days later. The macrophages were plated in 24-well plates at a density of 1.2 million cells/well in RPMI-1640 medium supplemented with gentamycin and containing 5% fetal bovine serum. The cells were washed 4 h after plating and were incubated overnight at 37°C in the RPMI-based media. For the binding assay, the cells were washed on ice and incubated at 4°C for 3 h with the appropriate ligand in the presence or absence of competitors. After the incubation, the cells were washed three times with PBS, twice with 1% BSA-PBS, and once with PBS. Monolayers were partially solubilized with 0.2 N NaOH and sonicated with a hand-held sonicator; aliquots were removed for determination of the ^{125}I and protein content.

Chemiluminescent immunoassay for antibody binding (15)

Antigens were plated overnight in 96-well round-bottomed High Binding Microfluor (Dynex) microtitration plates at concentrations ranging from 1.25 to 40 μg/ml. The wells were washed three times with PBS, blocked with 1% BSA-PBS for 10 min, and washed again three times with PBS. The monoclonal antibody EO6 (15), which recognizes OxLDL and oxidized phospholipids, including POVPC, was applied at 5 μg/ml in 1% BSA-PBS and was incubated for 1 h at room temperature. After three washes, alkaline-phosphatase-labeled goat anti-mouse IgM (Sigma) in 1% BSA-PBS was applied and again a 1-h incubation at room temperature was carried out. The bound EO6 was determined using LumiPhos (530) substrate by chemiluminescent technique (28).

For competition immunoassays, a fixed concentration of EO6 (2 μg/ml) was incubated in the absence or presence of competitors ranging in concentration from 0 to 50 μg/ml overnight at 4°C in Eppendorf tubes. The following day, the tubes were centrifuged at 13000 rpm for 15 min at 4°C to pellet immune complexes and aliquots of the supernates, containing free antibody, were incubated with plated OxapoB in microtitration plates, which had been washed and blocked as described above.

RESULTS

Covalent binding of phosphorus to apoB as a result of oxidation

ApoB was isolated from OxLDL (OxapoB) by exhaustive extraction of lipids (see Methods) and then analyzed for the presence of covalently bound phosphorus, as determined by the amount of inorganic phosphorus present after perchlorate digestion. Despite the exhaustive extraction of the lipids from OxapoB, 78 ± 15 moles of phosphorus were found to remain associated with each mole of oxidized protein (**Fig. 2**). In contrast, apoB recovered from native LDL under the same conditions showed 4 ± 3 moles of phosphorus per mole of protein.

The amount of tightly bound phosphorus increased with the extent of LDL oxidation (**Fig. 3**). LDL was oxidized at 37°C in the presence of 10 μm $CuSO_4$ for time periods ranging from 0 to 18 h, at the conclusion of which BHT and EDTA were added and the samples were maintained at 4°C. TBARS analysis and the development of fluorescence were used to follow the degree of oxidation and

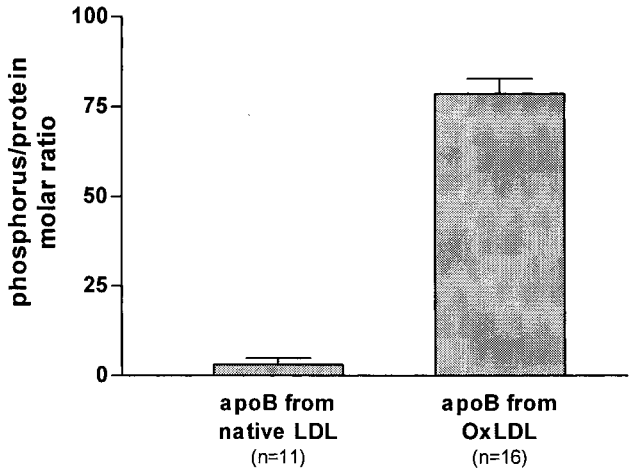

Fig. 2. Phosphorus/protein ratio of delipidated apoB recovered from native LDL or from ox LDL. Shown is the average ± SE of 11 or 16 determinations for apoB isolated from native or oxidized LDL, respectively. For determination of the ratio, an average apoB molecular mass of 550 kD was assumed.

both increased as the time of oxidation increased (Fig. 3). Under these conditions, both TBARS and fluorescence values reached a maximum between 6 and 18 h, as expected from previous studies (10). In parallel to the extent of LDL oxidation, there was a progressive increase in the number of phosphorus molecules associated with the isolated apoB.

During oxidation of LDL there is a progressive decrease in amino groups reactive with TNBS. It has been generally assumed that this reflected conjugation of fatty acid degradation products (e.g., malondialdehyde or 4-hydroxynonenol) with lysine epsilon amino groups (Fig. 1, pathway 1). We wanted to know to what extent, if at all, the bound PL contributed to blocking of lysine epsilon-amino groups. To this end, we first blocked lysine epsilon-amino groups by reductive methylation of native LDL, which should reduce the number of PL molecules that can become bound to apoB during a subsequent oxidation. Reductive methylation of native LDL reduced the number of TNBS reactive amino groups by 72%. This reductively methylated LDL was then oxidized in parallel with unmodified native LDL. The TBARS values were not significantly different, 40 and 44 nmol MDA/mg protein, respectively. However, the amount of protein-bound phosphorus in the case of the reductively methylated OxLDL was sharply reduced (**Fig. 4**). The apoB from LDL that had been reductively methylated prior to oxidation (RM-Oxapo B) contained only 12 ± 2 moles of phosphorus per mole of protein while the apoB from untreated OxLDL contained 58 ± 6 moles of phosphorus per mole of protein. Thus, at least 80 % of the protein-bound phosphorus appears to be attached at lysine residues.

To further characterize the putative bound PL, we prepared OxLDL, reduced it (prior to lipid extraction) with $NaCNBH_3$ to stabilize Schiff bases, extracted lipids as described, and then treated the apolipoprotein with dilute alkali. If the linkage occurs between a fragmented *sn*-2-

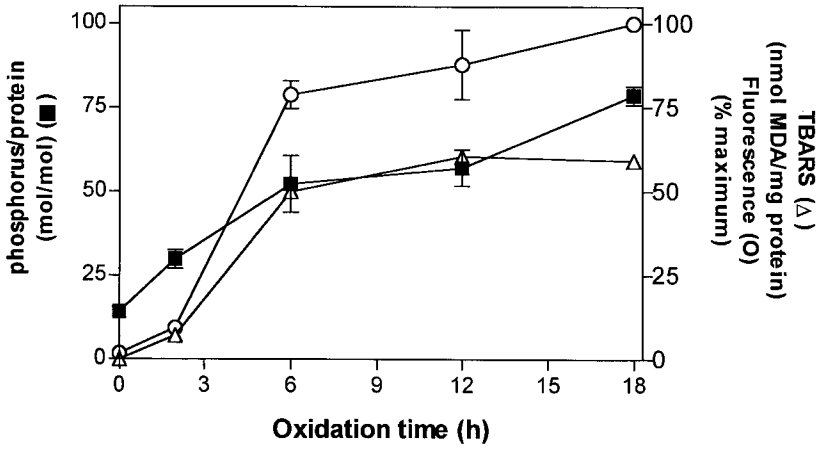

Fig. 3. Phosphorus tightly bound to apoB increases as the extent of LDL oxidation increases. Shown are specific characteristics of LDL or apoB after oxidation for periods ranging from 0 to 18 h. TBARS values (△) were determined on the intact LDL particles (n = 2). The phosphorus/protein ratio (□) was determined on apoB isolated from the LDL that had been oxidized for varying lengths of time (n = 3). Fluorescence (○) was also determined on the isolated apoB samples using an excitation wavelength of 350 nm and an emission wavelength of 433 nm. Fluorescence results are expressed as percent of control with the 18 h time-point set at 100% (n = 1).

fatty acid aldehyde of the oxidized PL and a lysine residue of apoB, as we propose, saponification should result in the release of the fatty acid from the *sn*-1 position, while the *sn*-2 fatty acid fragment should remain attached to the protein (because the Schiff base linkage was stabilized by reduction). In fact, we found a release of primarily 14:0, 16:0, and 18:0 fatty acids and the sum corresponded closely to the amount of phosphorus released. The molar ratio of total fatty acids recovered from the OxapoB to total protein-associated phosphorus was 0.84:1. Other fatty acids (16:1 and 18:1) were detected in negligible amounts (<4% of total fatty acids recovered). As a result of saponification, choline and phosphorus were released in equimolar amounts (choline:phosphorus, 0.98:1). After the saponification, no detectable phosphorus remained associated with the apoB. Moreover, this apoB no longer reacted with monoclonal antibody EO6, either by direct immunoassay nor on Western blotting (**Fig. 5**). Note that the alkali treatment used to release bound phospholipid did not affect the distribution or staining intensities of the protein bands. These data establish that oxidized PL is covalently linked to oxidized apoB and suggest that it is required for EO6 recognition of the apoB from oxidized LDL.

Oxidized phospholipid adducts as ligands for macrophage recognition

We next investigated the extent to which the protein-lipid adducts in OxLDL play a role in the recognition of OxLDL apoB by macrophage scavenger receptors. ApoB from oxidized LDL and apoB from LDL that had been reductively methylated prior to oxidation (RM-OxapoB) were compared as competitors for macrophage binding of either ^{125}I-labeled OxLDL or ^{125}I-labeled OxapoB (**Fig. 6**). OxapoB at 50 μg/ml inhibited the binding of 4 μg/ml of

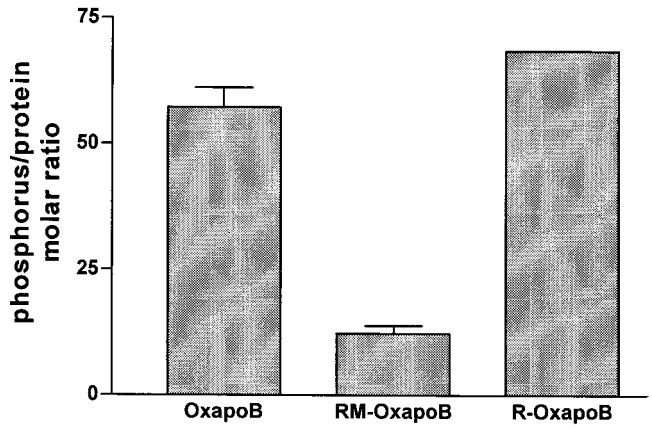

Fig. 4. Effect of prior reductive methylation (RM) of LDL on the amount of phosphorus binding to apoB during subsequent oxidation. Shown is the phosphorus/protein ratio determined for apoB isolated from OxLDL (OxapoB), apoB isolated from LDL which had been reductively methylated prior to oxidation (RM-OxapoB), and apoB that had been carried through the reductive methylation procedure but without any formaldehyde added (R-OxapoB). The phosphorus/protein ratio was determined in triplicate and is expressed as the mean ± SD.

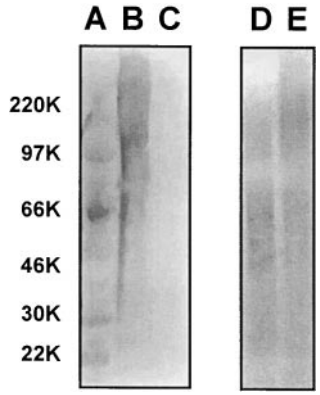

Fig. 5. Western blot analysis of EO6 reactivity with OxapoB post saponification to remove covalently attached PL. Samples were electrophoresed under non-reducing conditions and analyzed for EO6 reactivity by Western blot as described in the Methods. Lane A: standards; lane B: OxapoB; lane C: OxapoB after the saponification treatment which removed all associated PL. Ten μg of protein was applied to lanes B and C; equivalent transfer was confirmed by staining of the nitrocellulose membrane with Ponceau S prior to the Western analysis. Lanes D and E demonstrate Coomassie blue staining of a gel containing OxapoB (lane D) and OxapoB after the saponification treatment (lane E) after electrophoresis under the same conditions as described for the Western blot.

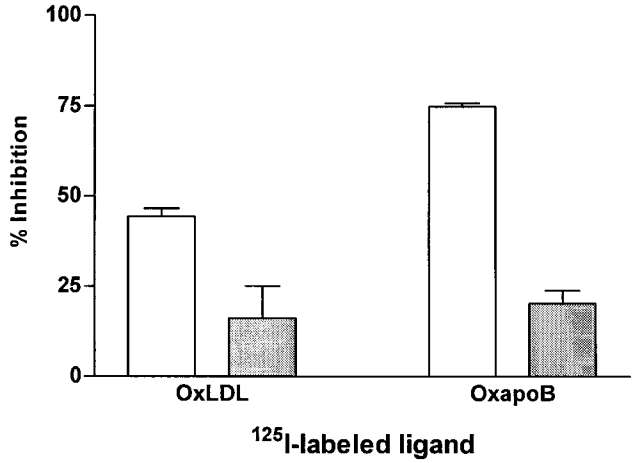

Fig. 6. ApoB from LDL reductively methylated prior to oxidation is less effective than OxapoB as an inhibitor of macrophage binding of either ^{125}I-labeled OxLDL or of ^{125}I-labeled OxapoB. ^{125}I-labeled OxLDL (4 μg/ml) or ^{125}I-labeled OxapoB (4 μg/ml) were incubated with elicited mouse peritoneal macrophages in the presence or absence of 50 μg/ml of OxapoB (open bars) or apoB isolated from LDL that had been reductively methylated prior to oxidation (RM-OxapoB) (shaded bars) (3 h at 4°C). Values are the mean ± SD of triplicate measurements and are expressed as percent inhibition. Control total binding values were 0.32 ± 0.03 μg ^{125}I-labeled OxLDL bound/mg cell protein and 0.38 ± 0.01 μg ^{125}I-labeled OxapoB bound/mg cell protein.

OxLDL by 50%, whereas RM-OxapoB at the same concentration inhibited binding by only about 20% (Fig. 6, left). The RM-OxapoB, which in this experiment had only 40 moles of PL associated per mole of protein compared to 80 moles of PL for the untreated OxapoB sample, was significantly less effective in inhibiting the OxLDL binding ($P = 0.04$, *t*-test). This difference was even more apparent when the two proteins were compared as competitors for the binding of ^{125}I-labeled OxapoB (Fig. 6, right). Unlabeled OxapoB was able to inhibit approximately 75% of the binding of labeled OxapoB. In contrast, unlabeled RM-OxapoB inhibited less than 25% of the labeled OxapoB binding. Again, the reduced ability of RM-OxapoB to compete was very significant, with a P value of 0.002 as determined by *t*-test. Clearly, the protein with the greater PL:protein content was a better ligand for macrophage scavenger receptors.

To further characterize the importance of the protein-lipid adducts, we blocked epsilon-lysine amino groups by reductive methylation to different degrees in order to progressively reduce the number of sites to which PL molecules could bind during subsequent oxidation. TBARS values as a result of this oxidation were all equivalent, despite the reductive methylation (ranging from 33 to 38 nmol MDA/mg). The lipids of these samples were exhaustively extracted and the apoB was isolated. The phosphorus:protein molar ratios ranged from 12:1 to 56:1, the highest being in the control sample that had no formaldehyde added during the methylation procedure. In this experiment, apoB from OxLDL prepared in the standard manner (i.e., no reductive methylation step) had 51 moles of PL associated with the protein. As shown in **Table 1**, the effectiveness of these OxapoB preparations as competitors for binding to the monoclonal antibody EO6 and for binding of ^{125}I-labeled OxapoB to macrophages correlated directly with the number of moles of phosphorus associated.

Previous studies have shown that during oxidation of LDL, the apoB undergoes nonenzymatic fragmentation, yielding a broad spectrum of polypeptides (29). That was confirmed in the present studies. A 4–12% SDS-PAGE electrophoretic separation followed by Coomassie blue staining revealed that all of the oxidized samples consisted of a mixture of peptides ranging from 12 kD to 400 kD (data not shown). We have previously shown that monoclonal antibody EO6 reacts with a large number of these fragments in a Western blot (15). We therefore quantified the reactivity of the unfractionated peptide mixture with EO6 (using a chemiluminescence immunoassay) to see how it related to the number of moles of PL attached per mole of apoB. As **Fig. 7** demonstrates, the mock reductively methylated OxapoB (▼) and the 50% modified sample (□) reacted with EO6 in exactly the same way as refer-

TABLE 1. Effect of OxapoB with differing degrees of PL incorporated on interactions with mAb EO6 and mouse peritoneal macrophages

	Binding of EO6 to OxapoB		Binding of ^{125}I-OxapoB to macrophage	
Competitor (PL:protein mole ratio)	RLU (± < 0.5%)	% Competition	Absolute Binding (μg/mg cell protein)	% Competition
no competitor	133613	—	0.25 ± 0.02	—
56:1	15610	88 ± 3	0.07 ± 0.01	72 ± 2
31:1	35944	73 ± 1	0.18 ± 0.02	28 ± 6
13:1	56457	57 ± 4	0.21 ± 0.02	16 ± 4
12:1	54584	59 ± 1	0.24 ± 0.02	4 ± 3

EO6 competition by the modified apoB was determined by a competition immunoassay as described in Methods. Fifty μg/ml of competitor was preincubated with 2 μg/ml of EO6 and the resulting free antibody was incubated with plated OxapoB. Percent competition was determined through comparison to RLU detected in the absence of competitor. All values are the mean of triplicate measurements ± SD; RLU, relative light units.

Binding of ^{125}I-labeled OxapoB to elicited mouse peritoneal macrophages was measured as described in Methods. Four μg/ml radiolabeled ligand was incubated with the macrophages for 3 h at 4°C in the presence of 50 μg/ml of the indicated competitor. Percent competition was determined through comparison to absolute binding in the absence of competitor. All values are the mean of triplicate measurements ± SD.

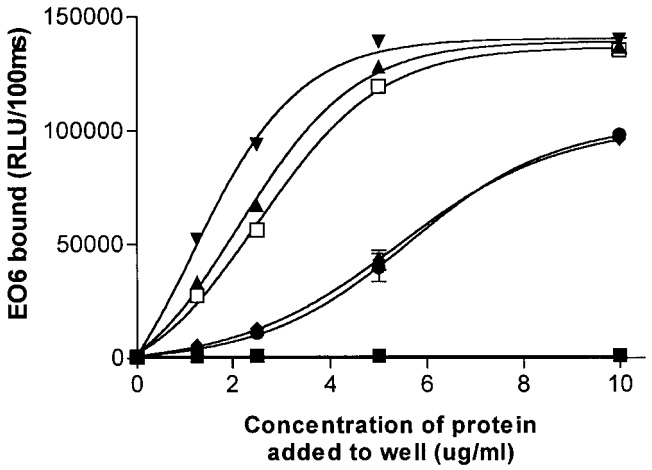

Fig. 7. Reactivity of EO6 with modified forms of OxapoB. EO6 binding to various plated proteins was carried out by a chemiluminescent immunoassay. OxapoB was plated for comparison and as a positive control (▲). ApoB isolated from LDL that had been reductively methylated to various extents prior to oxidation were also plated; these proteins had phospholipid/protein molar ratios of 56:1 (▼), 30:1 (□), 13:1 (●), and 12:1 (◆). Native LDL (■) was used as a negative control. Data points represent the mean of triplicate measurements ± SD (error bars smaller than symbol when not observed).

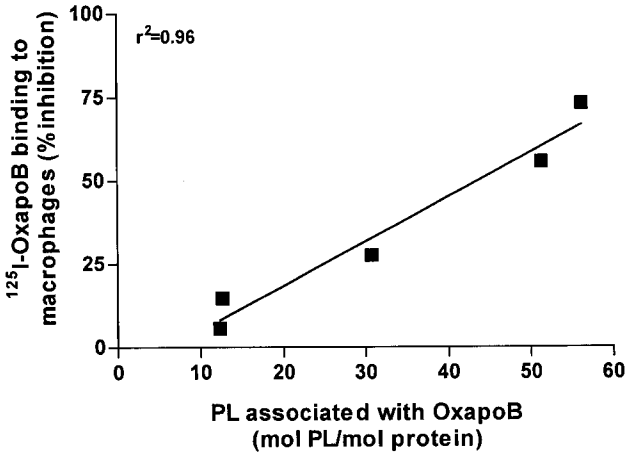

Fig. 8. Correlation of the extent of PL association with OxapoB and its ability to compete for ^{125}I-labeled OxapoB binding to elicited mouse peritoneal macrophages. ^{125}I-labeled OxapoB (4 μg/ml) was incubated in the presence or absence of 10–50 μg/ml of OxapoB samples described in Fig. 5 (3 h at 4°C). Shown is the correlation between the amount of phospholipid associated with OxapoB and the ability of the protein at 50 μg/ml to inhibit the binding of ^{125}I-labeled OxapoB. The correlation is significant with a r^2 value of 0.96. The total binding value in the absence of competitor was 0.25 ± 0.02 μg ^{125}I-labeled OxapoB/mg cell protein.

ence OxapoB (▲), reaching 50% saturation binding at about 1 μg/ml of plated protein. In contrast, reduction of the associated PL by 79%, as in the samples containing only 12 or 13 moles of PL per mole of protein (●, ◆), led to a dramatic reduction in EO6 binding, reaching 50% saturation binding at about 5 μg/ml of protein added to the plate. Native LDL (■) served as a negative control. To further confirm these results, we measured the ability of the variously modified OxapoB samples to compete for the ability of EO6 to bind to OxapoB. OxapoB with a PL:protein ratio of 56:1 was able to compete for 88 ± 3% of the EO6 binding to plated OxapoB while the proteins containing lesser amounts of PL exhibited a progressive decrease in competition (Table 1). This is consistent with the data of Fig. 6 and suggests that the PL:protein adduct is the epitope recognized by EO6. Furthermore, these results are consistent with those observed for the saponified samples described above, which displayed total loss of EO6 reactivity upon removal of the attached PL.

These same RM-OxapoB samples were used as competitors for ^{125}I-labeled OxapoB binding to elicited mouse peritoneal macrophages. The samples exhibited different degrees of competition for the ligand (added at 4 μg/ml) over a wide concentration range (10–100 μg/ml) (data not shown). Similar to the results examining EO6 competition, the ability of the OxapoB to compete for ^{125}I-labeled OxapoB binding was most efficient when the 56:1 (PL:protein) sample was used (Table 1). In fact, the effectiveness of the proteins as competitor was found to correlate very significantly with the molar ratio of PL:protein (**Fig. 8**; $r^2 = 0.96$). This result offers strong evidence that the association of oxidized PL with the lysine residues of apoB is a determining characteristic in the ability of OxapoB to participate as a ligand for macrophage scavenger receptors. However, this by no means rules out an important role for other lipid-lysine adducts, which would also be decreased by prior reductive methylation.

DISCUSSION

It is critically important to ask whether the phosphorus remaining associated with apoB in OxLDL was in fact covalently linked to it. Lipid-protein bonding can be quite strong short of covalent linkage. The evidence for covalent linkage can be summarized as follows. First, the exhaustive lipid extraction procedure used left almost no phosphorus attached to the apoB from native LDL even though LDL contains approximately 700 moles of lipid phosphorus per mole of apoB (30). Second, the amount of protein-bound phosphorus increased with the time of LDL oxidation in a fashion parallel to the increase in the number of lysine epsilon amino groups blocked. Third, the amount of apoB-associated phosphorus was reduced by 80% when the LDL had been subjected to reductive methylation to block lysine amino groups prior to oxidation. This treatment does not alter the charge on the protein and should not affect noncovalent binding of phospholipids. Fourth, saponification of the apoB from OxLDL (reduced with $NaCNBH_3$ prior to extraction to stabilize the Schiff base linkages) released fatty acids, choline, and phosphate in almost exactly the expected stoichiometric quantities. Fifth, the reactivity of the apoB from OxLDL with monoclonal antibody EO6 was lost after the saponifi-

cation treatment. Previous studies have shown that this antibody recognizes oxidized phospholipids (15) and that the phosphocholine moiety headgroup is required for reactivity (S. Hörkkö, P. Friedman, E. Dennis, D. Steinberg, and J. L. Witztum, unpublished results).

On average there were about 75 moles of phosphorus attached per mole of apoB after overnight copper-catalyzed oxidation. The overall decrease in the number of TNBS-reactive amino groups averaged 250 moles per mole of apoB. Thus, fully 30% of the conjugated lysine amino groups appeared to be attributable to phospholipid core aldehydes, i.e., phospholipids in which there is an aldehydic fatty acid fragment still esterified to the glycerol back bone (Fig. 1, pathway 2). The remainder are presumably attributable to conjugation with cholesteryl ester core aldehydes and with nonester fragments from polyunsaturated fatty acids, including malondialdehyde, 4-hydroxynonenal, and the many other aldehydic fragments that have been demonstrated (Fig. 1, pathway 1) (30, 31). The extent of lipid aldehyde conjugation with apoB was surprisingly high. The concentration of these aldehydes, if expressed relative to the total volume of the incubation mixture, would be rather low and might not be expected to yield this degree of conjugation. However, as has been pointed out by Esterbauer et al. (32), the lipid aldehydes remain in the lipid phase of the LDL and in that phase their concentrations can be quite high. 4-Hydroxynonenal, for example, has been estimated to reach concentrations of up to 150 mm in the lipid phase of the LDL particles (33).

The possibility that lipid ester core aldehydes might bind to the protein moiety during LDL oxidation has received little attention. Esterbauer et al. (30) mentioned it as a theoretical possibility in his 1992 review but never approached the problem experimentally. Steinbrecher et al. (10) have quantified the extent to which lysine residues are blocked during LDL oxidation and correlated this with biological properties of OxLDL. In a later study, Steinbrecher (12) incorporated phospholipids labeled in the *sn*-2 fatty acid into LDL prior to oxidation and showed that some of the fatty acid radioactivity became tightly associated with the apoB. The nature of the adducts was not determined. They could have represented either or both adducts of short-chain aldehydic fragments derived from polyunsaturated fatty acids released from the *sn*-2 position or they could have represented core lipid ester aldehydes. Ravandi et al. (34) have synthesized and characterized phosphatidylcholine core lipid ester aldehydes and shown that they react readily, as expected, with free amino acids and with protein. Kamido et al. (35) isolated and characterized PC core aldehydes in lipids extracted from OxLDL. Conjugates with the apoB were not demonstrated. Karakatsani et al. (36) extracted the lipids from OxLDL by four successive extractions with ether/ethanol. The delipidated apoB was resolubilized in SDS and the amount of phosphorus remaining attached was determined. The value for apoB from native LDL was 25.3 ± 9.6 nmol/mg apoB and for OxLDL it was 68.0 ± 20; this increase was statistically significant. The nature of the adduct was not further elucidated chemically but the authors suggested that the formation of a Schiff base with a core aldehyde was a likely possibility. Finally, Tertov et al. (37) have previously demonstrated that apoB prepared by extraction of lipids from OxLDL retains bound phosphorus, but they recovered only 4.78 mol/mol apoB compared to our finding of 50–70 mol/mol apoB. They used a different lipid extraction procedure so we repeated the experiment using their extraction procedure and found approximately 50 moles of phosphorus/mol apoB. The reasons for their failure to find more conjugated phosphorus are not known.

The importance of this extensive "decoration" of apoB with oxidized phospholipids stems from their potential importance as ligands for recognition of OxLDL by macrophage scavenger receptors (16, 38). Undoubtedly other lipid-protein adducts and other oxidized lipids will be shown to play a role in the recognition of OxLDL and of apoptotic cells by macrophage receptors. However, the fact that monoclonal antibody EO6 can inhibit macrophage binding and uptake of intact OxLDL by as much as 91%, and can also inhibit the uptake of both the isolated lipids and protein of OxLDL, suggests that oxidized phospholipids play a major role. The conditions for oxidation in vivo may be very different from those used in the current work, however, the relevance of these findings in vivo is attested to by the fact that EO6 reacts with epitopes in circulating human LDL and in human atherosclerotic lesions (17). Itabe et al. (39) immunized mice with material from a human atheroma and cloned an antibody (DHL3) with anti-phospholipid activity very similar to that of EO6. This antibody also reacted with circulating oxidized human LDL and with human atherosclerotic lesions. These findings suggest that in vivo LDL oxidation also yields phospholipid-apoB conjugates but further studies of OxLDL extracted from lesions will be needed to confirm this. The recent demonstration that the protein and lipid moieties of OxLDL exhibit reciprocal inhibition of binding to the macrophage (16), and the data presented in the current work, strongly supported the hypothesis that there is at least one class of receptors present on the macrophage surface which is able to recognize both the protein and lipid moieties of OxLDL due to a commonly recognized epitope of oxidized phospholipid. Studies in this laboratory using CD36-transfected cells show that this is the case specifically for the CD36 receptor (40) and studies are in progress testing additional OxLDL receptors.

Because OxLDL, EO6, and a model oxidized phospholipid-protein adduct (POVPC-BSA) can all inhibit the phagocytosis of apoptotic cells (8), the same oxidized PL-protein adduct likely serves as a ligand for macrophage recognition of apoptotic cells as well. It is clear that there are several different receptors on the macrophage that recognize OxLDL and/or apoptotic cells so it will be important to ask whether the specific ligands they recognize are the same or different. The protein moiety of OxLDL may be recognized by multiple classes of receptors, some recognizing the oxidized PL-apoB adducts characteristic of OxapoB, while others may recognize other modifications of apoB.

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