Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase

Urs P. Steinbrecher and P. Haydn Pritchard*

Departments of Medicine and Pathology,* University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

Abstract Degradation of phosphatidylcholine to lysophosphatidylcholine occurs during oxidative modification of low density lipoproteins (LDL). In this study, we have shown that this phospholipid hydrolysis is brought about by an LDL-associated phospholipase A_2 that can hydrolyze oxidized but not intact LDL phosphatidylcholine. The chemical nature of the oxidized phospholipids that can act as substrates for this enzyme was not fully characterized, but we hypothesized that the specificity of the enzyme for oxidized LDL phosphatidylcholine might be explained by fragmentation of polyunsaturated *sn*-2 fatty acyl groups in LDL phosphatidylcholine during oxidation. To facilitate characterization of this enzyme, we therefore selected a fluorescent phosphatidylcholine substrate that had a short-chain, polar residue in the *sn*-2 position: 1-palmitoyl 2-(6-[7-nitrobenzoxadiazolyl]amino) caproyl phosphatidylcholine, (C_6NBD PC). This substrate was efficiently hydrolyzed by LDL, but the dodecanoyl analogue of C_6NBD PC, which differed only in that a 12-carbon rather than a 6-carbon acyl derivative was present in the *sn*-2 position, was not hydrolyzed. The phospholipase activity was heat-stable, calcium-independent, and was inhibited by the serine esterase inhibitors phenylmethylsulfonylfluoride and diisopropylfluorophosphate, but was resistant to *p*-bromophenacylbromide and dithiobisnitrobenzoic acid. The phospholipid hydrolysis could not be attributed to the action of lecithin:cholesterol acyltransferase or lipoprotein lipase. Nearly all of the activity in EDTA-anticoagulated normal plasma was physically associated with apoB-containing lipoproteins, but this apoprotein was not essential as enzyme activity was present in plasma from abetalipoproteinemic patients. These properties are very similar to those recently reported for human plasma platelet-activating factor (PAF) acetylhydrolase. In the present study, we found that acylhydrolase activity against C_6NBD PC, PAF, and oxidized phosphatidylcholine copurified through gel filtration and ion-exchange chromatography. Substrate competition was demonstrated between C_6NBD PC, PAF, and oxidized 2-arachidonyl phosphatidylcholine, suggesting that a single enzyme was active against all three substrates. The enzyme had an apparent molecular weight of 40,000–45,000 by high pressure gel exclusion chromatography. Inhibition of this activity with diisopropylfluorophosphate prior to oxidative modification of LDL prevented phospholipid hydrolysis but did not affect the production of thiobarbituric acid reactive compounds or the change in electrophoretic mobility. In addition, this inhibition of phospholipase did not prevent the rapid degradation of oxidized LDL by macrophages. The importance of this enzyme in the metabolism of PAF in plasma is widely recognized. However, the present results suggest that it could also play a role in the metabolism of oxidized phospholipids in lipoproteins.—**Steinbrecher, U. P., and P. H. Pritchard.** Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase. *J. Lipid Res.* 1989. **30:** 305–315.

Supplementary key words LDL oxidation • phospholipase A_2 • PAF acetylhydrolase • fluorescent substrate • peroxidized lipids

Oxidative modification of low-density lipoproteins (LDL) by exposure to redox-active metal ions, or by incubation with cultured arterial endothelial cells, is accompanied by degradation of phosphatidylcholine (1, 2). The products of this phospholipid degradation are 2-lysophosphatidylcholine and polar fragments derived from the *sn*-2 acyl group (1, 3). Because phospholipid hydrolysis is observed when LDL is oxidized by exposure to 5 μM $CuSO_4$ in the absence of cells or other plasma components, we previously suggested that a phospholipase A_2 activity may be associated with LDL (1, 4). Furthermore, Parthasarathy and colleagues (4) obtained evidence that this phospholipid hydrolysis may be important for recognition of oxidized LDL by the acetyl LDL receptor of macrophages. Because no phospholipid hydrolysis is observed if LDL is incubated under conditions that do not permit oxidation, we hypothesized that oxidation either modifies LDL phospholipids in a way that renders them susceptible to hydrolysis by an enzyme that is inactive against the intact phospholipids, or that oxidation activates a previously

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; DTNB, dithiobisnitrobenzoic acid; HPLC, high-pressure liquid chromatography; C_6NBD PC, 1-palmitoyl 2-(6-[7-nitrobenzoxadiazoyl]amino) caproyl phosphatidylcholine; *p*BPB, *p*-bromophenacylbromide; DFP, diisopropylfluorophosphate; MDA, malondialdehyde.

latent enzyme, allowing it to hydrolyze intact (or oxidized) phospholipids. Precedents for such an increase in phospholipase activity in response to oxidation have been reported in microsomal as well as in lysosomal preparations (5, 6).

Polyunsaturated fatty acyl residues are the components of phosphatidylcholine that are most susceptible to oxidation. The chemistry of polyunsaturated fatty acid oxidation is rather complex, and a large number of products have been identified even in simple systems such as peroxidation of methyl linolenate (7, 8). With the complex mixture of phospholipids present in LDL, the number of potential products becomes very large. Depending on the conditions of oxidation, one might expect to find fatty acyl hydroperoxides, epoxides, alcohols, and fragmentation products including aldehydes, alkanes, and cyclic compounds (9, 10). Our initial approach to the characterization of this enzyme involved the use of autoxidized 2-arachidonoyl phosphatidylcholine as substrate. Although this substrate allowed us to detect phospholipase activity in native LDL, its heterogeneity made it unsuitable for further characterization of the enzyme. We speculated that the reason oxidized phosphatidylcholine acted as a substrate for this enzyme was related to oxidative modification of the polyunsaturated *sn*-2 fatty acid, leaving a short-chain and/or polar oxidation product. Hence, we selected a fluorescent compound, 1-palmitoyl 2-[-(7-nitrobenzoxadiazolyl)amino] caproyl phosphatidylcholine or C_6NBD PC for use as a model substrate because of its resemblance to these properties of phosphatidylcholine oxidation products. This substrate was efficiently hydrolyzed by LDL, and facilitated characterization of the LDL-associated phospholipase. The apparent molecular weight and inhibitor sensitivity of the C_6NBD PC acylhydrolase were found to be essentially identical to those recently reported for plasma platelet-activating factor (PAF) acetylhydrolase (11–14), and substrate competition was demonstrated between C_6NBD PC and PAF. Enzyme activity toward these two substrates was shown to copurify with that for oxidized phosphatidylcholine, suggesting that the same enzyme (i.e., PAF acetylhydrolase) was acting on all three substrates. Although the major physiologic role of this enzyme is believed to be the metabolism of PAF (11–14), the present finding that it can also act on oxidized phospholipids suggests a possible role in the metabolism of peroxidized lipids in lipoproteins.

MATERIALS AND METHODS

Chemicals

Na[^{125}I] was purchased from New England Nuclear, Lachine, P.Q., and [^{3}H]acetic anhydride was from Amersham Corp. 1-Palmitoyl 2-[12-(13-nitrobenzoxadiazolyl)-amino]caproyl phosphatidylcholine (C_6NBD PC), 1-palmitoyl 2-[12-(13-nitrobenzoxadiazolyl)amino] dodecanoyl phosphatidylcholine (C_{12}NBD PC), 6(7-nitrobenzoxadiazolyl) aminocaproic acid (C_6NBD fatty acid), and 2-arachidonoyl phosphatidylcholine were obtained from Avanti Polar Lipids, Birmingham, AL. Autoxidized 2-arachidonoyl phosphatidylcholine was prepared as follows: 10 mg phosphatidylcholine was dried in a 13 × 100 mm screw-top borosilicate test tube, to which 500 μl benzene was added and then vortexed for 1 min to yield reverse-phase micelles (15). The tube was flushed with O_2, sealed, and heated to 100°C for 60 min, resulting in a brown solution. The benzene was removed under reduced pressure, and then the oxidized phospholipid was dispersed in PBS and briefly sonicated to yield an optically clear solution. 1-O-Alkyl 2-[acetyl-^{3}H]-*sn*-glycero-3-phosphocholine was synthesized by reacting L-2-lyso PAF (Sigma Chemical Co., St. Louis, MO) with [^{3}H]acetic anhydride as previously described (16). The product was purified by thin-layer chromatography on silica gel G plates developed in chloroform–methanol–acetic acid–water 50:25:8:4 (by vol). The radioactive band comigrating with authentic PAF was scraped from the plate and eluted. Over 90% of the radioactivity in the product was released by snake venom phospholipase A_2. Phenylmethylsulfonylfluoride (PMSF), dithiobisnitrobenzoic acid (DTNB), and *p*-bromophenacylbromide (*p*BPB) and diisopropylfluorophosphate (DFP) were from Sigma Chemical Co. Iodoacetamide was supplied by Aldrich Chemical Co., Milwaukee, WI. Phospholipase A_2 from *Crotalus adamanteus* was obtained from Serdary Research Laboratories, London, Ontario. Sepharose CL4B, CNBr-activated Sepharose 4B, and DEAE-Sepharose CL6B were purchased from Pharmacia (Dorval, P.Q.). Bio-Gel A-5m was from Bio-Rad (Mississauga, Ontario). Cell culture media and fetal bovine serum were supplied by Gibco (Mississauga, Ontario). All other chemicals and solvents were of reagent grade or better, and were obtained from Fisher Scientific (Vancouver, B.C.), Sigma Chemical Co., or British Drug Houses (Vancouver, B.C.).

Lipoproteins

Lipoproteins were isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma obtained from fasting normal human volunteers (17). Isolation and partial purification of phospholipase activity from LDL was accomplished using donor plasma provided by Dr. Noel Buskard, Director of the Red Cross Transfusion Service, Vancouver, B.C. Plasma samples from patients with abetalipoproteinemia were obtained through the courtesy of Dr. Roger Illingworth, Portland, OR. Radioiodination of LDL was carried out using the iodine monochloride method (18), and resulted in specific activities between 80 and 200 cpm/ng. Lipid labeling was less than 7%, and

over 99% of radioactivity was precipitable with 10% trichloroacetic acid.

Assays

C_6NBD PC acylhydrolase activity was measured by incubating 10 nmol of substrate at 37°C with plasma or lipoprotein fractions in 1 ml Dulbecco's phosphate-buffered saline (PBS). In some experiments, the buffer also contained 1 mM Ca^{2+} but, as noted below, this did not alter the rate of substrate hydrolysis. Reactions were terminated by vortexing for 1 min with 1.2 ml methanol and 1.2 ml chloroform. The mixtures were centrifuged at 2000 *g* for 15 min to separate the phases and the fluorescence of the aqueous layer was measured with a Turner 430 fluorimeter at excitation 470 nm and emission 533 nm. The mass of fluorescent substrate hydrolyzed was calculated using a standard curve generated by serial dilutions of C_6NBD fatty acid in methanol. PAF acetylhydrolase activity was measured in plasma or lipoprotein eluate fractions by a method similar to that described by Blank et al. (11) using 1-O-alkyl-2-[^{3}H-acetyl]-*sn*-glycero-3 phosphocholine as a substrate. The assay mixture contained 50 μM [^{3}H-acetyl] PAF (2–5 Ci/mol), and plasma or lipoprotein fractions in a final volume of 0.4 ml Dulbecco's PBS. The mixtures were incubated for 10 min at 37°C and the lipids were extracted by the method of Folch, Lees, and Sloane Stanley (19). The amount of ^{3}H recovered in the aqueous phase was determined by liquid scintillation counting. The assays were linear for up to 15 min using 10 μl of human plasma as the enzyme source. Cholesterol was measured enzymatically with kits supplied by Boehringer Mannheim Corp. (Dorval, P.Q.). Protein was measured by the method of Lowry et al. (20) using bovine albumin as the standard, with 0.05% deoxycholate added to the assay mixtures to minimize turbidity. Lipoprotein electrophoresis was carried out using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine serum albumin at a final concentration of 20 mg/ml was added to dilute lipoprotein samples to ensure reproducible migration distances. Lipid peroxides were estimated as the fluorescent reaction product with thiobarbituric acid (1). Freshly diluted tetramethoxypropane was used as the standard, and results are expressed as nmol malondialdehyde (MDA) per mg protein. For phospholipid analysis, duplicate aliquots of LDL were extracted with $CHCl_3$–CH_3OH (21). Phospholipids were separated by thin-layer chromatography on silica gel G plates developed in $CHCl_3$–CH_3OH–H_2O 65:35:7 and the bands were visualized with iodine vapor. Lysophosphatidylcholine and phosphatidylcholine zones were scraped into test tubes, digested with $HClO_4$, and assayed for phosphorus content (22).

Cultured cells

Resident peritoneal macrophages were obtained from female Swiss mice by peritoneal lavage with ice-cold Dulbecco's PBS as previously described (1). The cells were suspended in alpha MEM with 10% fetal bovine serum and were plated in 12-well plastic culture plates (10^6 cells/dish). Nonadherent cells were removed after 1 hr by medium exchange and the adherent macrophages were cultured overnight prior to use in experiments. LDL degradation by cells was determined after incubation of various concentrations of ^{125}I-labeled LDL in serum-free medium with macrophages for 5 hr at 37°C. ^{125}I-Labeled LDL degradation products were assayed in culture medium as the content of trichloroacetic acid-soluble noniodide radioactivity (1).

Chromatographic methods

Human plasma (2 ml) was fractionated by gel exclusion chromatography over a 1.5 × 40 cm column of Bio-Gel A-5m eluted with 150 mM NaCl, 10 mM Tris, 0.3 mM EDTA, and 0.3% NaN_3, pH 7.4, at a flow rate of 8 ml/hr. Fractions of 2 ml volume were collected and assayed for cholesterol and phospholipase activity. The column was calibrated with ultracentrifugally isolated human VLDL, LDL, and HDL. Immunoaffinity chromatography of plasma was done using a monoclonal antibody to human LDL (MB-24) that was generously provided by Dr. L. K. Curtiss, Scripps Clinic Research Foundation, La Jolla, CA. Murine ascites fluid containing the antibody was purified by chromatography over LDL immobilized on Sepharose 4B. Ten mg of purified monoclonal antibody was then coupled to 3 ml CNBr-activated Sepharose 4B. Five hundred μl of human plasma was passed over the column, and the nonbound material was collected. The column was washed with phosphate-buffered saline and the bound fraction was eluted with 3 M NaSCN and collected. The starting plasma as well as the nonbound and the retained fractions were then assayed for phospholipase activity. The fluorescent material released from C_6NBD PC during incubation with LDL was analyzed by high-pressure liquid chromatography on a Waters 0.4 × 10 cm μBondapak C18 column and eluted at 1 ml/min with 20% CH_3CN in 20 mM phosphate, pH 7.0. Absorbance of the effluent was monitored at 535 nm.

Partial purification of phospholipase from LDL

Thirty to forty mg protein of ultracentrifugally isolated human LDL was treated with 15 mM deoxycholate. The mixture was applied to a 1.6 × 40 cm column of Sephacryl S-300 equilibrated with 10 mM deoxycholate, 50 mM NaCl, and 50 mM Na_2CO_3, pH 10.0. The column was eluted with the same buffer at 1 ml/min, and effluent

fractions were assayed for phospholipase activity. The peak fractions of enzyme activity were pooled, and concentrated on Amicon CF25 ultrafiltration membrane cones (Amicon Corp., Danvers, MA). Detergent exchange was accomplished by washing twice with 8 mM cholamidopropyldimethylammoniopropane sulfonate (CHAPS), 25 mM Tris, pH 7.4. The sample was then applied to a 1 × 40 cm column of DEAE Sepharose CL6B and eluted with a linear gradient of 0–200 mM NaCl in 8 mM CHAPS, 50 mM Tris, pH 7.4. The phospholipase activity in eluted fractions was measured, and the peak fractions were pooled and concentrated on CF 25 membrane cones. Estimation of molecular weight was done using a 0.6 × 30 cm Waters TSK SW300 size exclusion column eluted at 0.5 ml/min with 8 mM CHAPS, 50 mM Tris, pH 7.4. This column was calibrated with phosphorylase b, bovine albumin, catalase, ovalbumin, superoxide dismutase, carbonic anhydrase, trypsin inhibitor, lactalbumin, and cytochrome c. Phospholipase activity and absorbance at 280 nm of effluent fractions were determined.

Ethical approval for these studies was granted by human studies and animal use committees of the University of British Columbia.

RESULTS AND DISCUSSION

The hydrolysis of phospholipid that occurs during the oxidation of LDL could be due to nonenzymatic fragmentation of phospholipid, activation of latent phospholipase activity by oxidation, or the generation of oxidatively altered phospholipids that subsequently serve as substrates for a phospholipase that was inactive against intact phospholipids in LDL. To determine which of these possibilities was correct, we compared the rate of hydrolysis of the oxidized phosphatidylcholine by native LDL with that of the intact non-oxidized phospholipid. The results shown in **Fig. 1** indicate that native LDL was capable of hydrolyzing only oxidized phosphatidylcholine, in accord with previously reported observations (4). However, the oxidized phospholipid was clearly heterogeneous when analyzed by reverse-phase HPLC (not shown) and it was therefore not possible to calculate true substrate concentration with this material. As noted above, we suspected that the phospholipase present in LDL was specific for short-chain acyl residues at the *sn*-2 position. Therefore, a commercially available fluorescent phospholipid, C_6NBD PC, was chosen as a model substrate. This compound has a short-chain acyl group at the *sn*-2 position and the nitrobenzoxadiazole fluorophore linked to this short-chain fatty acid permitted substrate hydrolysis to be conveniently monitored as fluorescence remaining in the aqueous phase after chloroform–methanol extraction. **Fig. 2** shows that the phospholipase associated with LDL was capable of hydrolyzing C_6NBD PC, but that no hydrolysis occurred when the substrate differed only in that a 12-carbon (rather than a 6-carbon) fatty acid derivative was esterified at the 2-position. Control experiments indicated that the rate of hydrolysis of C_{12}NBD PC by snake venom A_2 was similar to that of C_6NBD PC (**Table 1**). Therefore, it appears that the LDL-associated phospholipase is specific for short-chain acyl residues. The water-soluble fluorescent product was shown to be C_6NBD fatty acid by coelution with authentic C_6NDB fatty acid on reverse-phase HPLC.

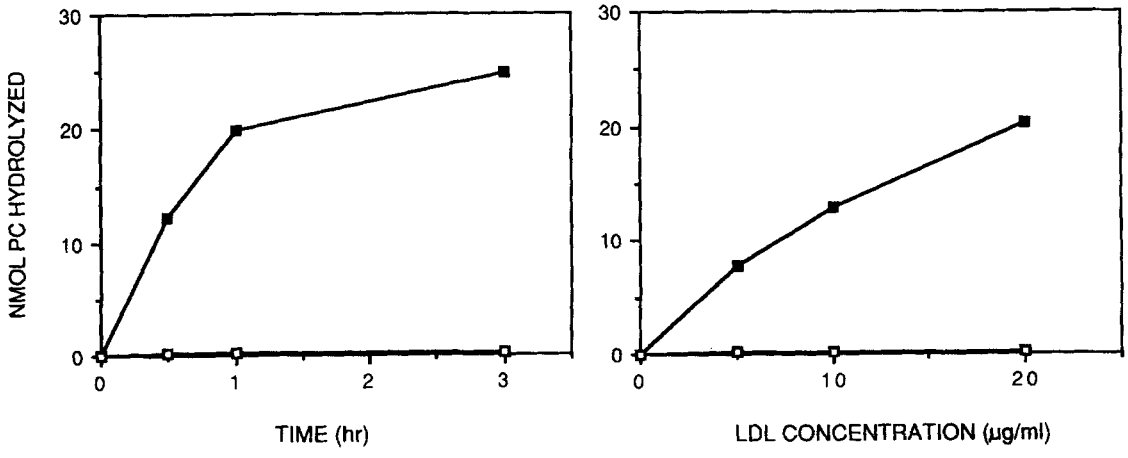

Fig. 1. Hydrolysis of oxidized phosphatidylcholine by LDL. For each point 250 nmol oxidized 2-arachidonoyl phosphatidylcholine (■), or intact 2-arachidonoyl phosphatidylcholine (□) was dispersed in 0.5 ml PBS and briefly sonicated to yield optically clear micellar solutions. The amount of lysophosphatidylcholine formed during incubation with LDL was quantified by thin-layer chromatography and phosphorus analysis. Panel A shows the time course of hydrolysis with 20 μg LDL as enzyme source. Panel B shows the effect of increasing LDL concentration at constant substrate concentration, with 1 hr incubation time.

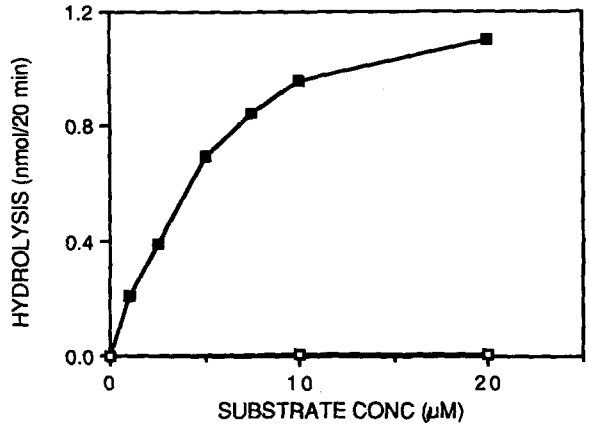

Fig. 2. Hydrolysis of C_6NBD PC but not C_{12}NBD PC by LDL. The indicated amount of C_6NBD PC (■) or C_{12}NBD PC (□) was incubated for 30 min at 37°C with 20 μg LDL in 1 ml PBS, and then extracted with $CHCl_3$-CH_3OH. The amount of fluorescent fatty acid in the aqueous phase was determined as described in Methods. Results are means of duplicate incubations.

TABLE 1. Recovery of fluorescence in the aqueous phase after hydrolysis of C_6NBD PC or C_{12}NBD PC

Additions to Incubation	nmol Fluorescent Fatty Acid in Aqueous Phase
0.2 nmol C_6NBD fatty acid	0.17
0.5 nmol C_6NBD fatty acid	0.45
1.0 nmol C_6NBD fatty acid	0.86
10 nmol C_6NBD PC	0.05
10 nmol C_{12}NBD PC	0.06
10 nmol C_6NBD PC + 4 U venom PLA_2	1.85
10 nmol C_{12}NBD PC + 4 U venom PLA_2	1.22

To validate the separation of substrate and product by $CHCl_3$-MeOH extraction, the indicated amounts of C_6NBD fatty acid were added to 1 ml PBS, and then extracted with $CHCl_3$-MeOH as described in Methods. The amount of fatty acid that partitioned into the aqueous phase was then determined by fluorescence measurement. To demonstrate that both C_6NBD PC and C_{12}NBD PC could serve as phospholipase substrates, each was incubated for 20 min at 37°C with snake venom phospholipase A_2 and then extracted for fluorescence measurement as above.

Thus, the release of the fluorophore into the aqueous fraction was due to hydrolysis of the *sn*-2 ester bond rather than cleavage between the fatty acid and the nitrobenzoxadiazole group. To show that the aqueous phase fluorescence accurately reflected the amount of C_6NBD fatty acid formed, varying amounts of authentic C_6NBD fatty acid were carried through the extraction procedure used in the assay. Results shown in Table 1 indicate that about 90% of C_6NBD fatty acid partitioned into the aqueous phase. Assay conditions were optimized on the basis of results shown in Fig. 2 and **Fig. 3**; subsequent experiments were done at pH 7.4 with a 20-min incubation at 37°C, 10 nmol substrate per ml, and, except where otherwise stated, 20 μg/ml LDL protein. Enzyme activity was unaltered by 10 mM EDTA, or by addition of 1 mM Ca^{2+} in the absence of EDTA. The effect of various inhibitors on C_6NBD PC hydrolysis is illustrated in **Fig. 4.** The serine esterase inhibitors PMSF and DFP were both inhibitory but complete inactivation of the enzyme required relatively high concentrations. The histidine reagent *p*BPB did not inhibit activity. Variable inhibition was noted with high concentrations of DTNB, but this was minimal when high-phosphate buffer was used to prevent precipitation of DTNB. Reduced, alkylated LDL retained full enzyme activity. In parallel control experiments, plasma lecithin:cholesterol acyltransferase activity was almost completely inhibited by 100 μM DTNB, 100 μM DFP, or 100 μM PMSF, and snake venom phospholipase A_2 was inhibited more than 90% by 200 μM *p*BPB. These inhibitor

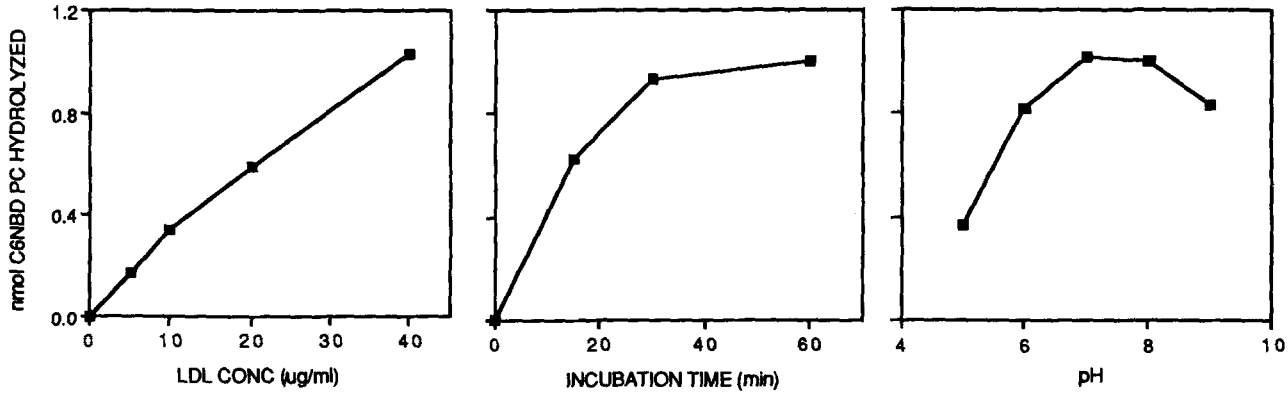

Fig. 3. Characteristics of the assay for C_6NBD PC hydrolysis by LDL. Left panel: 10 μmol C_6NBD PC was incubated for 30 min at 37°C with the indicated amount of LDL in 1 ml PBS, pH 7.4, and then the amount of substrate hydrolyzed was measured as described in Methods. Middle panel: 10 nmol C_6NBD PC and 20 μg LDL in 2 ml PBS, pH 7.4 were incubated at 37°C for the indicated time, and the amount of substrate hydrolysis was measured. Right panel: 10 nmol of C_6NBD PC and 20 μg LDL were incubated for 30 min at 37°C in 1 ml PBS adjusted to the indicated pH, and then the amount of substrate hydrolysis was determined. Results are means of duplicates.

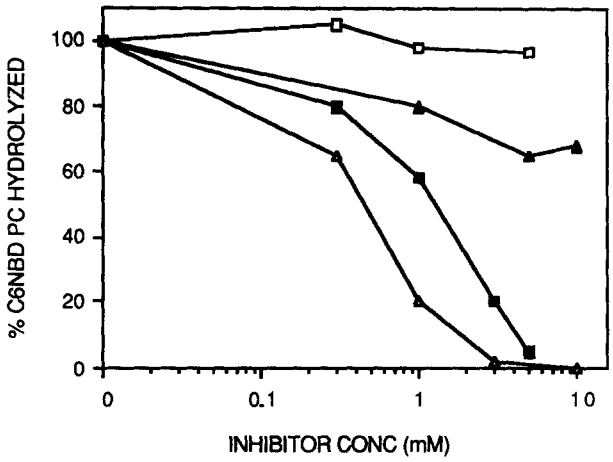

Fig. 4. Inhibitor profiles of LDL-associated C_6NBD PC acylhydrolase. Twenty μg LDL in 1 ml PBS, pH 7.4, was preincubated for 30 min at 20°C with the indicated concentration of pBPB (□), DTNB (▲), PMSF (■), or DFP (△), and then 10 nmol of C_6NBD PC was added. After 20 min incubation with substrate, the amount of hydrolysis was measured as described in Methods. Results are means of duplicate incubations.

profiles, as well as the finding that the LDL phospholipase activity was stable to heating for 1 hr at 60°C, suggested that this phospholipase activity could not be attributed to lecithin:cholesterol acyltransferase. Because lipoprotein lipase has some degree of activity against phospholipids (23), it was also considered as a potential source of activity against C_6NBC PC. However, there was no difference in C_6NBD PC hydrolysis with plasma obtained from a normal subject before and 30 min after intravenous injection of 70 I.U./kg heparin.

The above studies indicated that the LDL-associated phospholipase A_2 was calcium-independent, heat-stable, had a neutral pH optimum, acted only on short-chain acyl residues, and was inhibited with DFP or PMSF but resistant to pBPB and DTNB. Essentially identical properties have been reported for the plasma PAF acetylhydrolase (12–15). **Fig. 5** shows that the inhibitor profiles for LDL PAF acetylhydrolase activity were nearly identical to the profiles shown for C_6NBD PC acylhydrolase (Fig. 4). More direct evidence that both substrates were interacting with the same enzymatic site was obtained in substrate competition experiments (**Fig. 6**). These showed that PAF inhibited C_6NBD PC hydrolysis by LDL and conversely, C_6NBD PC inhibited PAF hydrolysis. Oxidized 2-arachidonoyl phosphatidylcholine inhibited the hydrolysis of both C_6NBD PC and PAF. However, relatively high concentrations were required, suggesting that either the K_m for oxidized phospholipid was high in comparison to that of the two labeled substrates, or more likely, that the oxidized phospholipid was heterogeneous, and only a fraction of the oxidation products actually fulfilled the structural requirements for interacting with the enzymatic site. Dimyristoyl phosphatidylcholine produced very little inhibition, suggesting that a substrate dilution effect was unlikely to account for the inhibition seen with oxidized phosphatidylcholine. **Fig. 7** shows that acylhydrolase activity for both PAF and C_6NBD PC was located predominantly with the LDL fraction of plasma by gel exclusion chromatography. Similarly, the specific activity ratio toward the two substrates was constant using various lipoprotein fractions isolated from EDTA-anticoagulated plasma by ultracentrifugation as the enzyme source (**Fig. 8**). In a few experiments the amount of enzyme activity in the d > 1.21 g/ml fraction was substantially higher (up to 30%) than shown in this figure; this dissociation of enzyme from LDL was most evident in citrate-anticoagulated plasma. To verify that the enzyme was physically associated with apoB-containing particles, human EDTA-plasma was chromatographed over immobilized monoclonal antibody to apoB. Over 85% of acylhydrolase activity was removed by this procedure, and most of the activity was recovered in LDL eluted from the column with 3 M NaSCN. However, apoB is not essential for enzyme activity, as the activity in plasma from four abetalipoproteinemic individuals was indistinguishable from that of four normolipidemic controls (9.1 ± 1.8 vs 8.9 ± 0.9 pmol/μl per min, mean ± SD). When acylhydrolase was further purified from LDL by detergent solubilization and chromatography over Sephacryl S-300 and DEAE Sepharose, there was apparent co-purification of activity against the two substrates (**Fig. 9**). The CHAPS-solubilized enzyme had an apparent molecular weight of 40,000–45,000 by high-pressure gel exclusion chromatography on TSK SW300, in agreement with values of 42,700–45,000 reported by Stafforini, Prescott, and McIntyre (14). This copurification of activities provides

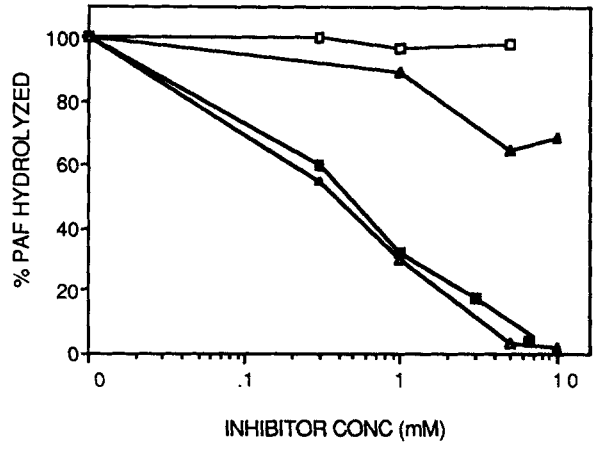

Fig. 5. Inhibitor profiles of LDL-associated PAF acetylhydrolase. Twenty μg LDL in 0.4 ml PBS, pH 7.4, was preincubated for 30 min with the indicated concentration of pBPB (□), DTNB (▲), PMSF (■), or DFP (△), and then 20 nmol [^{3}H-acetyl]PAF was added. After 20 min incubation with substrate, the amount of hydrolysis was measured as described in Methods. Results are means of duplicate incubations.

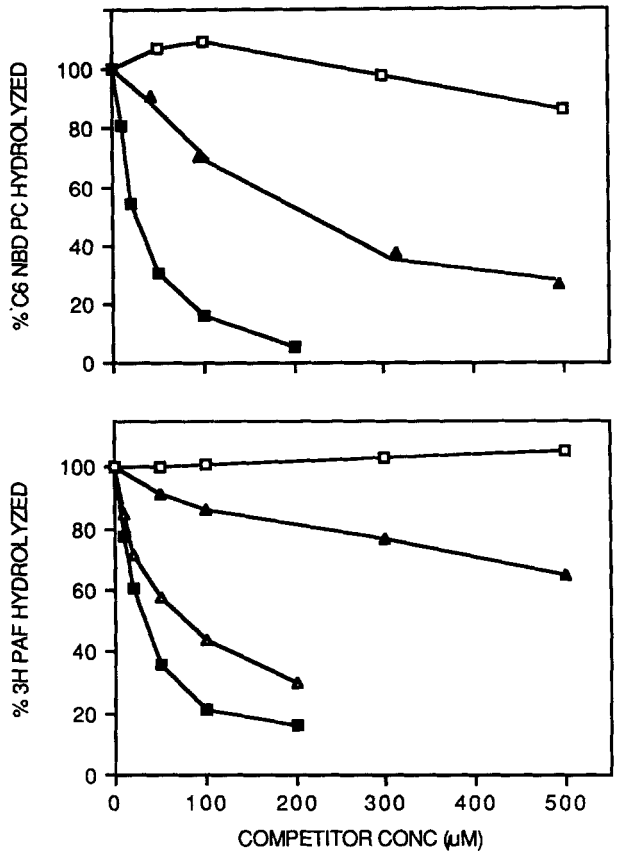

Fig. 6. Substrate competition for acylhydrolase. Top panel: 10 nmol of C_6NBD PC was added to assay tubes containing 20 μg LDL and varying amounts of PAF (■), dimyristoylphosphatidylcholine (□), or oxidized 2-arachidonoyl phosphatidylcholine (▲), and after 20 min the amount of C_6NBD PC hydrolyzed was determined. The value in absence of competitor was 0.063 nmol/min. Lower panel: 20 nmol of [^{3}H]PAF was added to assay tubes containing 20 μg LDL and varying amounts of C_6NBD PC (△), unlabeled PAF (■), oxidized 2-arachidonoylphosphatidylcholine (▲), or dimyristoylphosphatidylcholine (□), and after 20 min the amount of [3HPAF] hydrolyzed was determined. The value in the absence of competitor was 0.32 nmol/min.

further evidence that the hydrolysis of C_6NBD PC and PAF is attributable to the same enzyme. The ratio of specific acylhydrolase activities toward the two substrates appeared to change somewhat during purification. However, this does not necessarily reflect a true change in enzyme kinetics because the substrate concentration in proximity to the enzyme is unknown. Both substrates were added in excess of their critical bilayer concentrations (2×10^{-7} M for C_6NBD PC, 3×10^{-6} M for PAF), but the concentration of each substrate in the vicinity of the enzyme can vary as the nature of the lipid interface and/or interaction with micellar substrate is altered during successive solubilization and purification steps. A detailed discussion of the problems associated with kinetic analysis of interfacial enzymes and micellar substrates has been presented by Dennis (24) and Jarvis, Cain, and Dennis (25). In the present study, it is possible that the lipid interface in whole LDL provided an optimal environment for enzyme–substrate interaction, and that the decrease in total activity seen with purification may be a consequence of effects on the lipid domain and substrate presentation rather than on the enzyme itself.

To verify that the PAF and C_6NBD PC acylhydrolase activity was the same as that responsible for hydrolyzing oxidized phosphatidylcholine in LDL, we used several different sources of oxidized phospholipid as substrates to test for enzyme activity. The first was autoxidized 2-arachidonoyl phosphatidylcholine (generated as described in Materials and Methods), and the second was obtained by pretreating LDL with DFP to inhibit intrinsic acylhydrolase, and then oxidizing the LDL phospholipids by exposure to 5 μM $CuSO_4$ for 16 hr. Equivalent amounts of native LDL or partially purified PAF acylhydrolase were then added, and after 60 min the mixture was extracted with $CHCl_3$–CH_3OH. The phospholipids were separated by thin-layer chromatography and quantified by phosphorus assay. Because of the possibility that the oxidized LDL phospholipids that can act as substrates for the phospholipase were labile, we also used a third approach which involved addition of 20 μg of LDL or 5 μg of partially purified acetylhydrolase to 200 μg DFP-pretreated LDL, and then oxidizing the mixture by incubation with 5 μM Cu^{2+} for 16 hr at 37°C. Generation of lysophosphatidylcholine was then measured as above. **Table 2** shows that the acylhydrolase activity against oxidized

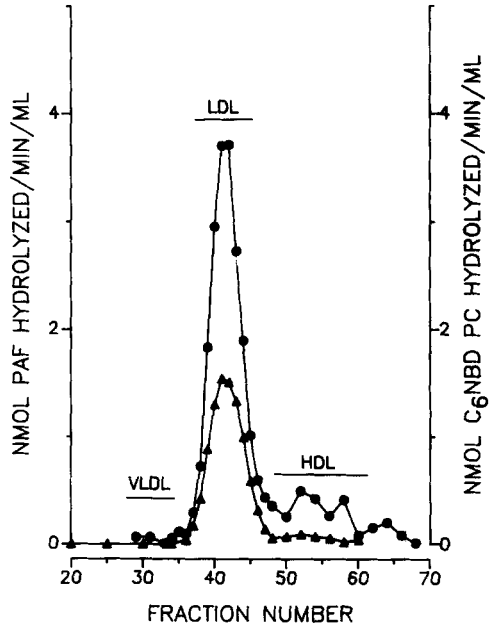

Fig. 7. Coelution of PAF acetylhydrolase and C_6NBD PC acylhydrolase activity with LDL on gel filtration chromatography. Two ml of human plasma was fractionated using a 1.5 × 40 cm column of Bio-Gel A-5m, and individual fractions were assayed for C_{12}NBD PC acylhydrolase activity and PAF acetylhydrolase activity as described in Methods. Elution volumes of individual lipoprotein classes are indicated by horizontal bars. C_6NBD PC acylhydrolase (▲); PAF acetylhydrolase (●).

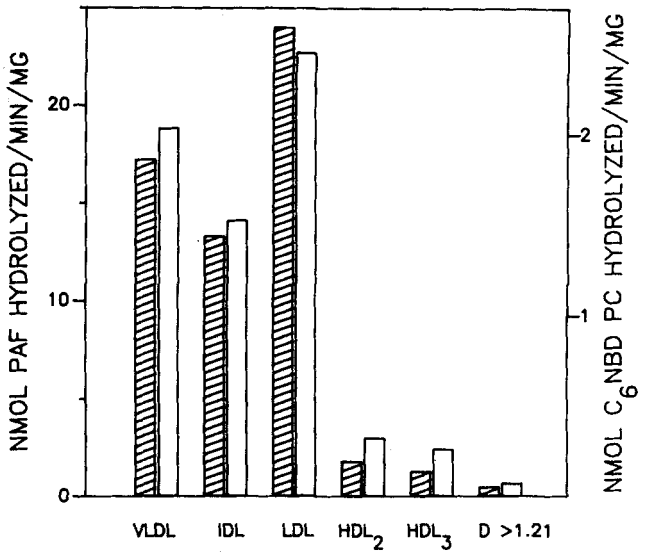

Fig. 8. Activity of C_6NBD PC acetylhydrolase and PAF acylhydrolase in various plasma fractions. Lipoprotein classes were isolated from plasma by sequential ultracentrifugation. Twenty to 50 μg protein of each lipoprotein, of the d < 1.21 g/ml infranatant was then assayed for C_6NBD PC acylhydrolase (hatched bars) and PAF acetylhydrolase (open bars) activities.

phospholipids copurified with C_6NBD PC and PAF acylhydrolase. As noted above, the hydrolysis rates with the oxidized phospholipid substrates were obtained under different conditions of incubation and with different substrate concentrations. In addition, the acylhydrolase is labile under the oxidation conditions used in the third approach discussed above. Therefore, the absolute rates shown are not directly comparable and the only conclusion that we draw from these results is that the activity toward all of these substrates increases roughly in parallel through successive purification steps.

In a previous study, Parthasarathy and colleagues (4) found that both the phospholipid hydrolysis and the susceptibility to degradation by macrophages associated with oxidative modification of LDL could be inhibited by *p*BPB or 1-bromooctan-2-one, an aliphatic phospholipase inhibitor. However, the above results show that LDL phospholipase activity is resistant to *p*BPB. To determine whether the apparent inhibitory effect of *p*BPB noted in the earlier study might nevertheless result from a specific inhibition of phospholipase rather than to some other effect of *p*BPB, ^{125}I-labeled LDL was preincubated for 4 hr with 10 mM DFP to inactivate the acylhydrolase, dialyzed, and then oxidized by exposure to 5 μM Cu^{2+}. Residual acylhydrolase activity against C_6NBD PC was less than 1% of control. As shown in **Table 3**, oxidation of the DFP-pretreated or control LDL resulted in similar increases in thiobarbituric acid reactivity and electrophoretic mobility. However, phospholipid hydrolysis was markedly inhibited by DFP pretreatment. Of note, degradation of the oxidized DFP-pretreated LDL by macrophages was as rapid as the untreated oxidized LDL (**Fig. 10**), indicating that phospholipid hydrolysis may not be essential for recognition of oxidized LDL by macrophages. There are a number of possible explanations for these discrepant results between the two studies. First, the apparent inhibition of *p*BPB in the earlier study may have been due to an antioxidant effect of *p*BPB (resulting in decreased phospholipid oxidation and therefore generation of less substrate for the acylhydrolase) rather than to inhibition of the enzyme itself. Second, it is possible that the failure of *p*BPB to inhibit acylhydrolase in the present study was because of a difference in incubation conditions. We verified that

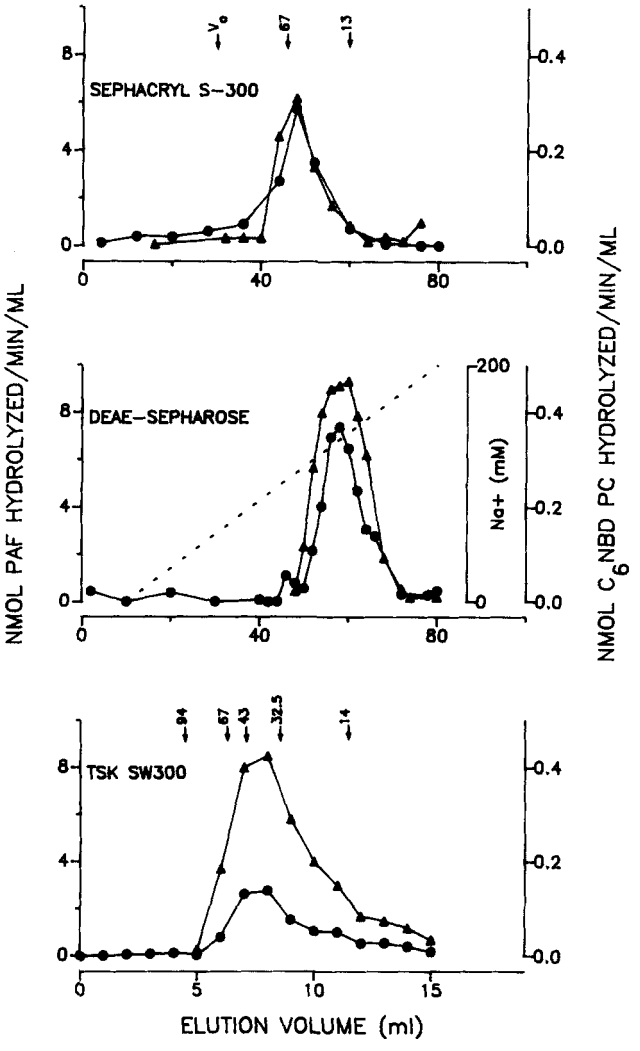

Fig. 9. Copurification of PAF acetylhydrolase and C_6NBD PC acylhydrolase. LDL (30 mg) was treated with 15 mM sodium deoxycholate and then chromatographed over Sephacryl S-300 as described in Methods. Peak fractions of enzyme activity were concentrated on ultrafiltration cones and detergent was exchanged for CHAPS. A second separation over DEAE Sepharose CL6B was then carried out. The peak fractions were again concentrated and analyzed by HPLC size-exclusion chromatography. Aliquots of fractions from each chromatographic step were assayed for C_6NBD PC acylhydrolase (▲) and PAF acetylhydrolase (●). Note the different scales for the two substrates.

TABLE 2. Copurification of acylhydrolase activity for oxidized phospholipids with C_6NBD PC and PAF acylhydrolase

	Substrate				
	Oxidized APC	Oxidized DFP-LDL	DFP-LDL + $CuSO_4$	PAF	C_6NBD PC
	phosphatidylcholine hydrolyzed (nmol/mg/min)				
Plasma	.12	.017	–	.39	.044
LDL	6.5	1.0	1.0	12.3	2.6 (39%)
S-300 peak	36.3	3.1	1.36	24.3	16 (37%)
DEAE peak	43.6	5.9	1.75	57.8	24.2 (11%)

Assays with oxidized 2-arachidonoyl phosphatidylcholine, C_6NBD PC, and PAF were performed as described in Methods and in the legend to Fig. 1. DFP-LDL was obtained by preincubating LDL for 4 hr with 10 mM DFP, and had no detectable C_6 NBD PD acylhydrolase activity. This material was oxidized by exposure to 10 μM $CuSO_4$ for 16 hr at 37°C, and was subsequently incubated with the indicated enzyme source at a concentration of 200 μg/ml ("oxidized DFP-LDL"). The results shown in the third column below were with DFP-LDL (200 μg/ml) added to the enzyme source *prior* to oxidation by exposure to 5 μM $CuSO_4$ for 16 hr at 37°C. Plasma inhibited oxidation of LDL, and therefore no hydrolysis of phosphatidylcholine was observed with this assay. All assays were done with 5–10 μl plasma, 20 μg LDL, and 3–5 μg partially purified enzyme. Values shown are means of duplicates that varied less than ± 10%. The percentage of total enzyme activity recovered is indicated in parentheses.

snake venom phospholipase A_2 was completely inhibited by *p*BPB under conditions that did not affect LDL acylhydrolase. However, it has been reported that the ability of *p*BPB to inhibit peroxidation-stimulated microsomal phospholipase A activity is dependent on the presence of glutathione (26). The present experiments were done in phosphate-buffered saline without thiols whereas the earlier studies were done in F-10 culture medium. A third possibility is that two phospholipases, both active against oxidized phospholipids, are associated with LDL and that one of these is sensitive to *p*BPB but cannot act on C_6NBD PC or PAF. Finally, it is possible that, although phospholipid hydrolysis was inhibited by about 75% in DFP-pretreated LDL, this may not have been sufficient to eliminate the modification of apoB that leads to rapid degradation by macrophages.

In conclusion, the present studies have provided further characterization of an unusual phospholipase A_2 that exists in association with plasma LDL. This enzyme shows remarkable specificity for short-chain acyl groups, and can apparently hydrolyze certain oxidized phosphatidylcholines as well as PAF and synthetic analogues such as C_6NBD PC. The importance of this enzyme in regulating the effects of PAF is widely recognized (27–31). However, the present results suggest that this enzyme might also play a role in the metabolism of oxidized phospholipids in lipoproteins. The potential pathogenetic importance of this action on oxidized phospholipids is emphasized by the recent report of Quinn, Parthasarathy, and Steinberg (32) that lysophosphatidylcholine can act as a chemotactic factor for human monocytes. In view of recent evidence that lipoprotein oxidation may play an important role in atherosclerosis (33, 34), these results suggest one potential mechanism by which this might occur: oxidation of LDL in the artery wall would lead to lysophosphatidylcholine formation, with recruitment of monocytes into the intima.

TABLE 3. Inhibition of phospholipid hydrolysis during LDL oxidation by DFP

	Electrophoretic Mobility	TBA Reactivity nmol MDA/mg	LysoPC:SM Molar Ratio	C_6NBD PC Acylhydrolase
				pmol/μg/min
Control LDL				
– DFP	0.29	2.6	0.082	2.4
+ DFP	0.31	1.2	0.059	0.05
Oxidized LDL				
– DFP	0.90	42.6	0.67	N.D.
+ DFP	0.81	42.2	0.194	N.D.

Untreated LDL and LDL pretreated with 10 mM DFP were analyzed for electrophoretic mobility, thiobarbituric acid (TBA) reactivity, phospholipid composition, and C_6NBD PC acylhydrolase activity as described in Methods; similar assays were repeated after oxidation of these LDLs by exposure to 5 μM Cu^{2+} for 20 hr. Electrophoretic mobility is expressed relative to that of bovine albumin. Phospholipid hydrolysis is expressed as the lysophosphatidylcholine:sphingomyelin (lysoPC SM) molar ratio because the content of sphingomyelin was not altered by LDL oxidation. Values shown are means of duplicate determinations that varied less than ± 10%; N.D., not determined.

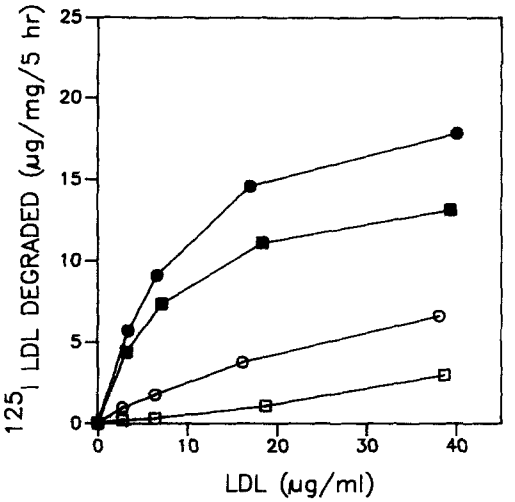

Fig. 10. Degradation of oxidized DFP-treated LDL by macrophages. ^{125}I-Labeled LDL was pretreated with 10 mM DFP to inhibit acylhydrolase and was oxidized by exposure to 5 µM $CuSO_4$ for 20 hr. The indicated concentration of oxidized DFP-treated LDL (●) was then incubated with cultured mouse peritoneal macrophages. After 5 hr the medium was assayed for degradation products (trichloroacetic acid-soluble noniodide radioactivity). The positive control was non-pretreated oxidized LDL (■), and negative controls included DFP-pretreated nonoxidized LDL (○), and native LDL (□). Each point represents the mean of triplicate dishes. Physical and chemical characterization of these LDLs is shown in Table 3.

These monocytes could internalize the modified lipoproteins and thereby accumulate cholesterol (35), or could release "active oxygen" intermediates that might amplify lipoprotein peroxidation, resulting in further intimal damage and influx of inflammatory cells (36).

We are indebted to Drs. Dennis Voelker, Joseph Witztum, Dennis Vance, and Sampath Parthasarathy for valuable discussions and advice. Skilled technical assistance was provided by Marilee Lougheed and Cathy Crowley. Text processing was done by Phyllis Wiesen. Grant support for these studies was from the Medical Research Council of Canada and the B.C. Heart Foundation.

Manuscript received 17 September 1987, in revised form 16 April 1988, and in re-revised form 9 August 1988.

REFERENCES

1. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
2. Steinbrecher, U. P.; J. L. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis.* **7:** 135–143.
3. Steinbrecher, U. P. 1987. Oxidation of human low density lipoproteins results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
4. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial-cell induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
5. Sevanian, A., S. F. Muakkassah-Kelly, and S. Montestruque. 1985. The influence of phospholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxides. *Arch. Biochem. Biophys.* **223:** 441–452.
6. Weglicki, W. B., B. F. Dickens, and I. Tong Mak. 1984. Enhanced lysosomal phospholipid degradation and lysophospholipid production due to free radicals. *Biochem. Biophys. Res. Commun.* **124:** 229–235.
7. Fujimoto, K., W. E. Neff, and E. N. Frankel. 1984. The reaction of DNA with lipid oxidation products, metals, and reducing agents. *Biochim. Biophys. Acta.* **795:** 100–107.
8. Frankel, E. N. 1982. Volatile lipid oxidation products. *Prog. Lipid Res.* **22:** 1–33.
9. Wu, G-S., R. Stein, and J. F. Mead. 1982. Autoxidation of phosphatidylcholine liposomes. *Lipids.* **17:** 403–413.
10. Esterbauer, H., K. H. Cheesman, M. U. Dianzani, G. Poli, and T. F. Slater. 1982. Separation and characterization of products of lipid peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes. *Biochem. J.* **208:** 129–140.
11. Blank, M. C., T. C. Lee, V. Fitzgerald, and F. Snyder. 1981. A specific acetylhydrolase for 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). *J. Biol. Chem.* **256:** 175–178.
12. Wardlow, M. L., C. P. Cox, K. E. Meng, D. E. Greene, and R. S. Farr. 1986. Substrate specificity and partial characterization of the PAF-acylhydrolase in human serum that rapidly inactivates platelet activating factor. *J. Immunol.* **136:** 3441–3446.
13. Stafforini, D. M., T. M. McIntyre, M. E. Carter, and S. M. Prescott. 1987. Human plasma platelet-activating factor acetylhydrolase: association with lipoprotein particles and role in the degradation of platelet-activating factor. *J. Biol. Chem.* **262:** 4215–4222.
14. Stafforini, D. M., S. M. Prescott, and T. M. McIntyre. 1987. Human plasma platelet-activating factor acetylhydrolase: purification and properties. *J. Biol. Chem.* **262:** 4223–4230.
15. Yamamoto, Y., E. Niki, Y. Kamiya, and H. Shimasaki. 1984. Oxidation of lipids. 7. Oxidation of phosphatidylcholines in homogeneous solution and in water dispersion. *Biochim. Biophys. Acta.* **795:** 332–340.
16. Pritchard, P. H., A. Chonn, and C. C. H. Yeung. 1985. The degradation of platelet-activating factor in the plasma of a patient with familial high density lipoprotein deficiency (Tangier disease). *Blood.* **66:** 1476–1478.
17. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **43:** 1345–1353.
18. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. *Biochim. Biophys. Acta.* **260:** 212–221.
19. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–509.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
21. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
22. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
23. Fielding, P. E., V. G. Shore, and C. J. Fielding. 1977. Lipoprotein lipase. Isolation and characterization of a second enzyme species from postheparin plasma. *Biochemistry.* **16:** 1896–1900.
24. Dennis, E. A. 1983. Phospholipases. *In* The Enzymes. Vol. XVI. Boyer, P. D., editor. Academic Press, New York. 307–353.
25. Jarvis, A. A., C. Cain, and E. A. Dennis. 1984. Purification and characterization of a lysophospholipase from human amnionic membranes. *J. Biol. Chem.* **259:** 15188–15195.
26. Beckman, J. K., S. M. Borowitz, and I. M. Burr. 1987. The role of phospholipase A activity in rat liver microsomal lipid peroxidation. *J. Biol. Chem.* **262:** 1479–1481.
27. Hanahan, D. J. 1986. Platelet activating factor: a biologically active phosphoglyceride. *Annu. Rev. Biochem.* **55:** 483–509.
28. Pinckard, R. N., R. S. Farr, and D. J. Hanahan. 1979. Physicochemical and functional identity of platelet activating factor released in vivo during IgE anaphylaxis with PAF released in vitro from IgE-sensitized basophils. *J. Immunol.* **123:** 1847–1857.
29. Farr, R. S., C. P. Cox, M. L. Wardlow, and R. Jorgensen. 1980. Preliminary studies of an acid-labile factor in human sera that inactivates platelet-activating factor. *Clin. Immunol. Immunopathol.* **15:** 318–330.
30. Farr, R. S., M. L. Wardlow, C. P. Cox, K. E. Meng, and D. E. Greene. 1983. Human serum acid-labile factor is an acylhydrolase that inactivates platelet activating factor. *Fed. Proc.* **42:** 3120–3122.
31. Blank, M. L., M. N. Hall, E. A. Cress, and F. Snyder. 1983. Inactivation of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine by a plasma acetylhydrolase: higher values in hypertensive rats. *Biochem. Biophys. Res. Commun.* **113:** 666–671.
32. Quinn, M. T., S. Parthasarathy, and D. Steinberg. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA.* **85:** 2995–2998.
33. Kita, T., Y. Nagano, M. Yokode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida, and C. Kawai. 1987. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model of hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **84:** 5928–5931.
34. Carew, T., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
35. Steinberg, D. 1983. Lipoproteins and atherosclerosis: a look back and a look ahead. *Arteriosclerosis.* **3:** 283–301.
36. Quinn, M. T., S. Parthasarathy, L. G. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoprotein: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* **84:** 2995–2998.