Phospholipase D-modified Low Density Lipoprotein Is Taken Up by Macrophages at Increased Rate

A Possible Role for Phosphatidic Acid

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Abstract

Macrophage uptake of modified forms of LDL leads to cellular cholesterol accumulation. Upon incubation of LDL with phospholipase D (PLase D), a time- and enzyme dose-dependent production of phosphatidic acid (PA), paralleled by a rapid reduction in LDL phosphatidyl choline content (up to 65% within 15 min of incubation) was noted. No lipid peroxidation could be found in PLase D-modified LDL. Upon in vitro incubation of PLase D-LDL with copper ions, however, this modified LDL was substantially oxidized. The addition of 100 μg PA/ml to native LDL for the period of its in vitro oxidation resulted in a 63% elevation in the lipoprotein peroxides content. Incubation of PLase D-LDL with J-774A.1 macrophage-like cell line resulted in an increase in its cellular binding and degradation (up to 91 and 110%, respectively) in comparison with native LDL (via the LDL receptor). When PA was added to LDL before its incubation with the macrophages, a PA dose-dependent elevation in the cellular uptake of LDL (by up to twofold) was noted in comparison with LDL that was incubated without PA, suggesting that PA production in PLase D-LDL may be involved in the increased cellular uptake of PLase D-LDL. PLase D activity towards LDL was demonstrated in J-774A.1 macrophages. Human plasma was also shown to possess PLase D activity. Thus, PLase D modification of LDL may take place under certain pathological conditions and PLase D-LDL interaction with arterial wall macrophages can potentially lead to foam cell formation. (*J. Clin. Invest.* 1993. 91:1942-1952.) Key words: low density lipoprotein • phospholipase D • macrophage • phosphatidic acid

Introduction

Macrophage cholesterol accumulation, which is an early event in atherogenesis, could not be achieved after cell incubation with native LDL but was demonstrated after macrophage incubation with modified forms of LDL (1-4). Unlike native LDL, which is taken up by the cells via the cholesterol-regulated LDL receptor, acetyl LDL and oxidized LDL are taken up by the scavenger receptor(s), leading to cellular cholesterol accumulation (1-4). Macrophage cholesterol accumulation could also be obtained with other forms of LDL (not taken up by the scavenger receptor) that were modified in their lipids moieties by various means, including the action of several lipases on the lipoprotein (5-13).

Phospholipases exist in almost every type of cell, possess hydrophobic nature, and regulate the acyl composition of the cell membrane phospholipids (14).

The products of phospholipase action on cells (14) include second messengers (such as inositol phosphate and diacylglycerol), products of the arachidonic acid cascade (such as prostaglandins and leukotriens), and other active metabolites (such as lysophospholipids and phosphatidic acid). Phospholipase action on the surface of LDL phospholipids can "uncover" the core lipids of the lipoprotein and thus affect its interaction with cells. Phospholipase (PLase)[1] A2, which specifically attacks fatty acids at the C-2 position of the diacyl glycerol phospholipids, was shown to modify LDL to a form that was taken up by macrophages at enhanced rate (9) but adhered in a nonspecific way to fibroblasts and was not degraded to any significant extent by these cells (10). PLase C, which selectively removes the polar phosphorous moiety of the LDL phospholipids, causes the loss of its hydrophilic portion and forces the modified LDL to aggregate and to form particulate PLase C-LDL (11). PLase C-LDL is taken up via the macrophage LDL receptor by phagocytosis and was shown to form foam cells (11, 12). Sphingomyelinase-treated LDL was also shown to be taken up by macrophages at an enhanced rate (13).

PLase D, unlike PLase C, hydrolyzes only the alcohol moiety of the phospholipid (such as the choline residue) and not the phosphorous moiety and thus results in the formation of phosphatidic acid (PA). PLase D plays a role in cell activation process and its presence in plasma has been reported recently (15, 16).

The present study was undertaken to find out the effect of PLase D on the physicochemical properties of LDL and on in its interaction with cells. The results of this study show that PLase D-LDL is taken up by cells at an enhanced rate and this can be attributed to the physicochemical changes that occur in the modified lipoprotein and to the formation of PA.

Methods

Materials. PLase D (EC 3.1.4.4) of cabbage origin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). This PLase D preparation was used because, unlike PLase D from streptomyces chromofuscus, it does not contain proteases. Ficoll-Paque and Sephadex

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1. *Abbreviations used in this paper:* ACAT, acyl CoA cholesterol acyl transferase; Ac-LDL, acetyl-LDL; CE, cholesterol ester; HMDM, human monocyte-derived macrophages; HSF, human skin fibroblasts; LPDP, lipoprotein-deficient plasma; PA, phosphatidic acid; PC, phosphatidyl choline; PI, phosphatidyl inositol; PLase, phospholipase; TNBS, trinitrobenzenesulfonic acid.

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G-100 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). BioGel A 15m column was purchased from Bio-Rad Laboratories (Richmond, CA). DME, RPMI 1640 culture medium, FCS, penicillin, streptomycin, and L-glutamine were obtained from Gibco Laboratories (Grand Island, NY). Carrier-free Na I was obtained from DuPont-New England Nuclear (Boston, MA). A mAb, B1B6, towards the apolipoprotein B-100 epitopes that are located at the LDL receptor-binding domain (mapped to amino acid residues 3,214–3,506) was a generous gift from Drs. G. Schonfeld and E. Krul (Washington University, St. Louis, MO).

Cells. J-774A.1 murine macrophage-like cell line was purchased from American Type Culture Collection (Rockville, MD). J-774A.1 cells were plated at 2.5×10^5 cells per 16-mm dish in DME supplemented with 10% FCS. The cells were fed every 3 d and were used for experiments within 7 d of plating (5).

Human monocytes were isolated by density gradient centrifugation from blood derived from fasting normolipidemic subjects (17). 20 ml of blood (anticoagulated with 10 U/ml of heparin) was layered over 15 ml of Ficoll-Paque and centrifuged at 500 *g* for 30 min at 23°C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice in RPMI 1640 culture medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were plated at 3×10^5 monocytes per 16-mm dish (Primaria; Falcon Labware, Oxnard, CA) in the same medium. Human monocyte-derived macrophages (HMDM) were used within 7–10 d of plating.

Human skin fibroblasts (HSF) were cultured from punch biopsies of the skin of the anterior thigh from normal volunteers (18). Subcultures were used between passages 4 and 12. The cells were plated at 5×10^5 cells per 16-mm dishes in DME supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. After 5 d in culture, the medium was changed to DME supplemented with 10% human lipoprotein-deficient serum ($d > 1.25$ g/ml, prepared by ultrace centrifugation) for 48 h to upregulate cellular LDL receptors.

Lipoproteins. LDL was prepared from human plasma derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (19). The LDL was washed at a density of 1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) under nitrogen in the dark, at 4°C. LDL was then sterilized by filtration (0.22 nm) and used within 2 wk.

LDL was iodinated by the method of McFarlane as modified for lipoproteins (20). LDL was also radiolabeled with [^{14}C]cholesteryl ester ([^{14}C]CE) as previously described (21). LDL was acetylated by repeated additions of acetic anhydride (22) to 4 mg/ml LDL diluted 1:1 (vol/vol) with saturated ammonium acetate at 4°C. Acetic anhydride was added at 40-fold molar excess with regard to total amino acid lysine residues in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital buffer. Lipoproteins were analyzed for their composition by determination of their protein (23), unesterified and esterified cholesterol (24), triglycerides (25), and phospholipids (26) content.

Lipoproteins were analyzed for possible changes in their charge by lipoprotein electrophoresis on agarose (27). Vitamin E content of the lipoproteins was analyzed with the bathophenantroline-ferric chloride assay (28). The concentration of total carotenoids was determined in the lipoprotein fractions by HPLC of the lipid extracts (29). LDL fatty acids distribution was analyzed by gas-liquid chromatography as described elsewhere (30). Lipoprotein oxidation was performed by incubation of LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10 μM) for 24 h at 37°C. Lipoprotein oxidation was assayed by the lipid peroxidation test as well as by analysis of the conjugated dienes content of the lipoproteins (5, 31).

PLase D-modified LDL (PLase D-LDL) was prepared by incubation of LDL (1 mg of protein/ml) with 5 U/ml of PLase D (in 50 mM acetate buffer, pH 6.5) in the presence of 50 mM $CaCl_2$ for 1 h at 37°C. The enzyme reaction was terminated by the addition of 1 mM EDTA and refrigeration.

For phospholipid subclasses analysis, the LDL lipids were extracted with chloroform/methanol (2:1, vol/vol) and the lipid extract was analyzed for its content of PA, phosphatidyl choline (PC), and phosphatidyl inositol (PI) by TLC on silica gel plates. The plates were developed with a solution of chloroform/methanol/ammonium hydroxide (60:35:8, vol/vol/vol). Iodine vapors were used to visualize the lipid spots on the TLC plates using the appropriate standards. The appropriate spots of PA, PC, and PI were scraped and analyzed for their phosphorous content (26). Transphosphatidylation in plasma was studied by plasma incubation with 5 mM $CaCl_2$ and 20% methanol for 2 h at 37°C. The production of phosphatidyl methanol was visualized using the appropriate standards. Phospholipid mass was calculated by multiplying the phosphorous content by 25 for PC and PI and by 22 for PA.

For analysis of the physicochemical characteristics of PLase D-LDL and for lipoprotein–cell interaction studies, the modified lipoprotein was separated from excess phospholipases and from lipolyzed products by a second ultracentrifugation at a density of 1.210 g/ml or by passage over a Sephadex G-100 minicolumn (10×2 cm), which was then eluted with 50 mM saline-EDTA (1 mM) buffer (pH 7.4).

Metabolism of lipoprotein by cells. LDL degradation was measured after incubation of ^{125}I-labeled LDL (180–300 cpm/ng of protein) with cells for 5 h at 37°C under conditions as described for each specific experiment. The hydrolysis of LDL protein was assayed in the incubation medium by measurement of trichloroacetic acid–soluble, noniodide radioactivity (32). Cell-free LDL degradation was minimal and was subtracted from total degradation. The cell layer was washed three times with PBS and extracted by a 1-h incubation at room temperature with 0.5 ml of 0.1 N NaOH for measurement of protein by the method of Lowry et al. (23) and for determination of cell-associated I-labeled lipoproteins. The mAb B1B6, which binds to the LDL receptor-binding domains on LDL apo B-100, was used to assess binding of the lipoprotein to the LDL receptor (33).

High affinity binding of lipoproteins to cells was studied by incubation of ^{125}I-labeled lipoproteins for 4 h at 4°C. After extensive washing (4×) with PBS, cells were extracted by incubation with 0.1 N NaOH for 1 h at room temperature and the bound radiolabeled LDL was counted (34).

LDL cholesterol uptake by cells was estimated by measurement of the stimulation of ^{3}[H]oleate incorporation into cholesteryl ester (35). The cells were incubated for 18 h with the lipoproteins, followed by medium removal and a further incubation of the cells with the radiolabeled oleate (0.2 mM, 10 μCi/ml ^{3}[H]oleate in the presence of 0.07 mM fatty acid–free albumin) for 2 h at 37°C. The cells were then washed twice with PBS at 4°C and incubated for 30 min with 1 ml of hexane/isopropyl alcohol (3:2, vol/vol) in a 16-mm dish at room temperature to extract cellular lipids. After two more washes with these solvents, the pooled lipid extract was dried under nitrogen and resolubilized in chloroform. The labeled CE was isolated by TLC on silica gel plates using hexane/diethyl ether/acetic acid solution (130:40:1.5, vol/vol/vol). Cellular content of unesterified cholesterol and CE was determined after lipid extraction (24).

Heparin Sepharose affinity chromatography. Subfractionation of LDL and PLase D–LDL by heparin Sepharose was carried out as previously described (33). Sepharose complexed with heparin (Affi-gel Heparin; Bio-Rad Laboratories) was packed into a small column (10×2 cm). The column was saturated with BSA and equilibrated to 0.05 M NaCl, 2 mM phosphate buffer (pH 7.4). 1 mg lipoprotein protein was applied to the column and elution was begun at a flow rate of 30 ml/h. After the unbound fraction was eluted (and the absorbance at 280 nm had decreased to baseline), the retained fraction (bound) was eluted with 0.8 M NaCl. Recovery of the lipoprotein protein ranged between 84 and 89%. The capacity of the column for LDL binding was more than double the loads used.

Solid-phase competitive binding radioimmunoassay. Radioimmunoassay of LDL and PLase D–LDL was performed in microtiter plates (33). The plates were coated with 150 μl of purified mAb B1B6 (10 μg/ml) overnight and then wells were blocked with 3% BSA–PBS.

Serial dilutions of the lipoproteins in 1% BSA–PBS were added followed by the addition of a constant amount of I-labeled LDL (500 ng). After incubation for 4 h at room temperature, the wells were washed three times with PBS and binding (B) was determined. The maximal binding (Bo) was determined in wells where competing lipoprotein was not added and the results were expressed as the B/Bo ratio.

Other assays. SDS-PAGE was performed with 3–10% gradient gel using mercaptoethanol as the reducing agent (36). Electrophoresis was performed at constant current (5 mA) for 16 h. The gels were stained with 0.1% Coomassie brilliant blue R and destained with 10% acetic acid. Nondenaturing polyacrylamide gradient gel electrophoresis of the lipoprotein preparations was performed on 3–10% gels to compare their relative sizes and possible aggregation of PLase D–LDL (37). Lipoprotein aggregation was also measured in samples of ^{125}I-labeled LDL and PLase D–^{125}I-labeled LDL in the original samples as well as in the supernatant fractions that were obtained after centrifugation (10,000 *g* for 10 min) of these samples. Lipoprotein aggregation was calculated as the percent of total LDL radioactivity (38). Another analysis for the possible presence of aggregates in PLase D–LDL was performed by gel filtration chromatography using a Bio Gel A15m column (39). ^{125}I-labeled lipoprotein samples (10 mg of protein in 3 ml) were loaded on top of the column (85 × 1.6 cm) and 2-ml fractions were collected using 0.9% NaCl, 0.01% Na_2 EDTA, 0.01% NaN_3, pH 7.4, as the eluant, at a flow rate of 10 ml/h. The radioactivity was then measured in each fraction. Free lysin amino groups in LDL were estimated with trinitrobenzenesulfonic acid (TNBS). LDL (50 μg protein) was mixed with 1 ml of 4% $NaHCO_3$, pH 8.4, and 50 μl of 0.1% TNBS and heated for 1 h at 37°C, after which the absorbance at 340 nm was measured (40).

LDL-free fatty acid content was analyzed colorimetrically, using a NEFA C kit (Wako Pure Chemicals, Osaka, Japan), on the basis of fatty acid acylation with CoA. The acyl-CoA is then oxidized to yield hydrogen peroxide, which is measured by an enzymatic colorimetric assay.

Statistical analysis. Statistical analysis was performed using the nonpaired Student's *t* test. Results are given as mean±SD.

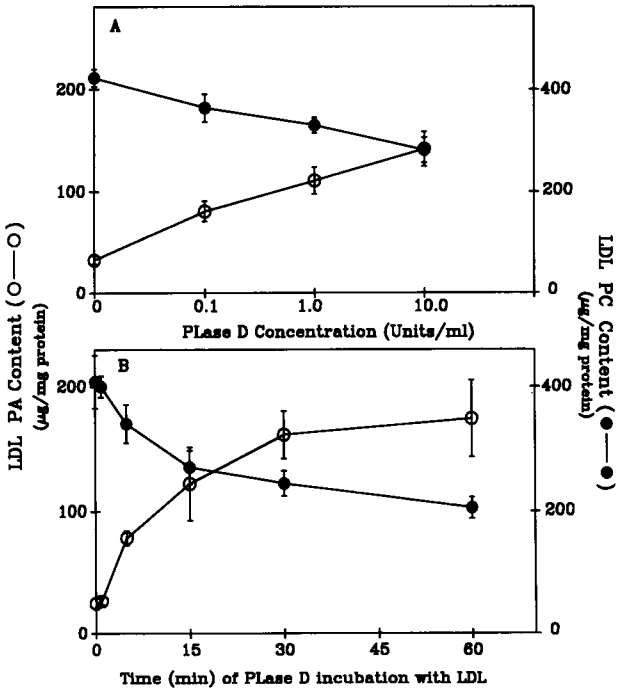

Figure 1. Dose response (*A*) and time course (*B*) of LDL modification with phospholipase D. (*A*) LDL at 1 mg protein/ml was incubated with increasing concentrations of PLase D for 1 h at 37°C followed by lipoprotein separation on Sephadex G-100 minicolumn and analysis of the LDL content of phosphatidyl choline (●) and phosphatidic acid (○). (*B*) LDL (1 mg protein/ml) was incubated with PLase D (5 U/ml) and samples were collected at various time intervals for analysis of LDL PC and PA content as described in Methods. Results are given as the mean±SD (n = 3).

Results

Physicochemical characteristics of PLase D–LDL. Incubation of 1 mg protein/ml LDL with increasing concentrations of PLase D for 1 h at 37°C resulted in a dose-dependent reduction in the lipoprotein PC content (by up to 35%) paralleled by a substantial elevation in LDL PA content (Fig. 1 *A*). Upon using 0.25–1.00 mg protein/ml LDL, the effect of PLase D on the production of PA was not significantly changed (data not shown). A time-dependent effect of PLase D (5 U/ml) on the content of LDL PC and PA was noted and within 15 min of incubation 35% of the LDL PC was hydrolized (Fig. 1 *B*). Choline (which is released from the LDL PC after lipoprotein incubation with PLase D) did not affect the production of PA when added to the incubation system (LDL + PLase D) at concentrations up to 100 μg/ml (data not shown). Since the activity of PLase D is dependent on the presence of calcium ions, the effect of increasing concentrations of $CaCl_2$ on LDL content of PC and PA was analyzed. Maximal effect was achieved with 50 mM $CaCl_2$ (the ratio of PA to PC increased from 0.14±0.03 in the absence of calcium ions to 0.66±0.07 in the presence of calcium ions [n = 3]) with no further effect at higher calcium concentrations (up to 150 mM $CaCl_2$ was studied).

The composition of PLase D–LDL was analyzed (Table I). On using 5 U/ml PLase D, LDL phosphatidyl choline content was significantly reduced (by 36%) with no significant changes in the other LDL lipid constituents (Table I). The relative

Table I. Composition Analysis of PLase D–LDL

	LDL	PLase D–LDL
Lipids and protein (% by weight)		
Phospholipids	18.2±2.1	11.6±1.3*
Cholesteryl ester	40.1±3.2	41.2±2.9
Unesterified cholesterol	9.9±1.1	10.6±0.9
Triglycerides	5.1±0.4	4.8±0.3
Proteins	26.2±2.3	31.4±2.2*
Fatty acids (mol %)		
Palmitic acid (16:0)	27±3	24±2
Palmitoleic acid (16:1)	7±2	9±2
Stearic acid (18:0)	9±2	7±2
Oleic acid (18:1)	18±3	16±3
Linoleic acid (18:2)	35±3	40±4‡
Arachidonic acid (20:4)	4±2	4±2
Antioxidants (μg/mg protein)		
Vitamin E	1.52±0.20	1.11±0.15‡
Carotenoids	0.22±0.05	0.14±0.04‡

PLase D–LDL was obtained by incubation of 1 mg protein/ml LDL with 5 U/ml PLase D for 1 h at 37°C followed by lipoprotein reseparation by ultracentrifugation. PLase D–LDL and control LDL were analyzed for their lipids, protein, fatty acids, and antioxidant content. Results are given as mean±SD. * $P < 0.01$, ‡ $P < 0.05$ (vs. LDL, n = 3).

content of PLase D–LDL polyunsaturated fatty acid linoleate was increased by 14% (Table I) and LDL content of the antioxidants vitamin E and carotenoids were reduced in PLase D–LDL by 20 and 25%, respectively, in comparison with native LDL (Table I). These latter changes however were not statistically very significant ($P < 0.05$). TNBS reactivity (which measures the content of free epsilon amino lysine groups on the LDL apo B-100) of PLase D–LDL was similar to that of native LDL (data not shown). On heparin Sepharose minicolumn, 72±5% of the PLase D–LDL protein was recovered in the unbound fraction in comparison with only 6±4% of native LDL that was obtained in this fraction under similar chromatography conditions ($n = 3$).

Analysis of PLase D–LDL on SDS-PAGE revealed no fragmentation of the modified lipoprotein and on nondenatured gradient gel electrophoresis, the size of PLase D–LDL was found to be somewhat smaller than that of native LDL (the PLase D–LDL particle migrated 13.5±0.3 mm from the top of the gel in comparison with a migration of 12.2±0.3 mm for native LDL), and no particles with molecular weight higher than that of native LDL could be found, suggesting that lipoprotein aggregation did not take place in PLase D–LDL. Furthermore, on using ^{125}I-labeled LDL and PLase D–^{125}I-labeled LDL, analysis of the radioactivity after high speed centrifugation (10,000 *g* for 10 min) in the supernatant compared with that of the whole sample revealed 1.5±0.3 and 1.7±0.5% aggregation for LDL and PLase D–LDL, respectively ($n = 3$). As the centrifugation procedure may not remove small aggregates (up to ~ 500 nm in diameter) and since small aggregates of modified LDL could still account for a substantial increase in LDL degradation, we have also performed column chromatography analysis on a Bio Gel A 15m column (4% agarose, 200–400 mesh beads) of 1 mg protein/ml ^{125}I-labeled LDL or PLase D–^{125}I-labeled LDL (using 5 U/ml of PLase D). Both preparations were eluted as a single peak with no residual radioactivity present at higher or at lower particle size (^{125}I-labeled LDL was eluted at eluant peak volume of 95±5 ml and PLase D–^{125}I-labeled LDL, at 99±3 ml [$n = 3$]). No radioactivity could be found in fractions collected before the lipoprotein peak, when concentrations up to 15 U/ml PLase D were used. When higher concentrations of PLase D were used, however, some aggregates appeared. Only when using 25 U/ml PLase D was some residual radioactivity associated with fractions collected at 83±5 ml of the eluant volume (before the main lipoprotein peak), which accounted for 2.8±0.5% of the total lipoprotein radioactivity ($n = 3$). As in our experiments, the concentration of PLase D was only 5 U/ml, thus, no aggregates were present in the modified LDL used in the present study. The electrophoretic mobility of PLase D–LDL on cellulose acetate was increased by 28±5% in comparison with native LDL (from 1.3±0.2 [LDL] to 1.7±0.1 cm [PLase D–LDL] from the origin, respectively, $n = 3$). No lipolysis of PLase D–LDL esterified fatty acids could be found, as the content of LDL-associated free fatty acids in native LDL and in PLase D–LDL were similar (87±16 vs. 101±23 mol of free fatty acids/mol apo B-100, respectively). PLase D–LDL was not oxidized (data not shown) as analyzed by the peroxide and conjugated dienes assays (30). When PLase D–LDL was oxidized in the presence of copper ions, however, the content of lipoprotein-associated peroxides increased in a dose-dependent fashion (of the enzymatic activity) by up to 153% elevation (when 10 U/ml of PLase D was used) in comparison with native LDL (Table II). Similarly, the conjugated dienes content of LDL increased from 185±14 nmol/mg LDL protein in the absence of PLase D to 285±21 and 311±17 nmol/mg LDL protein when 5 and 10 U/ml of the enzyme were used, respectively ($P < 0.01$, $n = 3$). In vitro oxidation of native LDL by its incubation with 10 μM $CuSO_4$ for 24 h at 37°C in the presence of increasing concentrations of PA resulted in an increment in LDL-associated peroxides from 116±20 (in the absence of PA) to 166±17 and 189±21 nmol/mg LDL protein in the presence of 50 and 100 μg PA/ml, respectively ($P < 0.01$, $n = 3$). Similarly, LDL-associated conjugated dienes increased from 177±23 to 250±23 and 288±17 nmol/mg LDL protein, respectively ($P < 0.01$, $n = 3$).

Table II. The Susceptibility of PLase D–LDL to In Vitro Oxidation

PLase D concentration	Lipoprotein oxidation
U/ml	*nmol peroxides/mg protein*
0	135±14
0.1	153±16
0.5	167±11*
1.0	199±21*
5.0	226±31*
10.0	249±23*

The samples of PLase D–LDL were prepared by incubation of increasing concentrations of PLase D with LDL (1 mg protein/ml) for 1 h at 37°C. The lipoproteins were dialyzed against PBS and each sample was then oxidized by incubation with 10 μM $CuSO_4$ at 37°C for 24 h. At the end of the incubation, lipoprotein peroxides were analyzed. Results are given as mean±SD ($n = 3$). * $P < 0.01$ (vs. 0 concentration).

The effect of PLase D on oxidized LDL was also studied. Upon incubation of 5 U/ml PLase D with 1 mg protein/ml of either native LDL or oxidized LDL (produced by LDL incubation with 10 μM $CuSO_4$ for 24 h at 37°C), the reduction in the lipoprotein PC content as well as the elevation in its PA content were similar for both lipoproteins (data not shown), suggesting that PLase D activity towards LDL was not affected by lipoprotein oxidation.

Macrophage uptake of PLase D–LDL. Upon using increasing concentrations of PLase D to modify the LDL, macrophage degradation of the lipoprotein was increased in a dose-dependent fashion by up to 108% when a concentration of 10 U/ml of the enzyme was used (Fig. 2 *A*). Similarly, upon increasing the time of LDL modification with PLase D, the degradation of the modified lipoprotein by macrophages increased with time of incubation by up to 117% in comparison with native LDL (Fig. 2 *B*). We next studied the binding (at 4°C) of PLase D–^{125}I-labeled LDL to J-774A.1 macrophages in comparison with ^{125}I-labeled LDL. At all lipoprotein concentrations, PLase D–LDL demonstrated increased binding to the cells (by 50–91%) in comparison with the nonmodified LDL (Fig. 3 *A*). Analysis of macrophage degradation of PLase D–LDL over a concentration range between 25 and 100 μg protein/ml revealed a 81–110% increased macrophage degradation of PLase D–LDL in comparison with native LDL (Fig. 3 *B*). Cellular degradation of PLase D–LDL was completely inhibited by 50 μM of chloroquin, the inhibitor of lysosomal enzymes (data

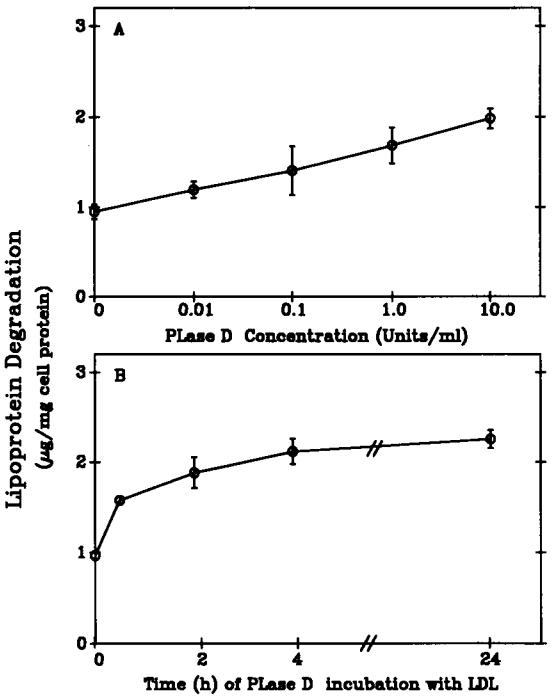

Figure 2. Dose response (*A*) and time course (*B*) of the effect of PLase D modification of LDL on cellular degradation of PLase D–LDL by J-774A.1 macrophages. Experimental protocol was as described in Fig. 1. The lipoprotein samples obtained after LDL modification with PLase D were added (25 μg protein/ml) to the cells and incubated for 5 h at 37°C before determination of cellular degradation of the lipoprotein. Results represent mean±SD (n = 3).

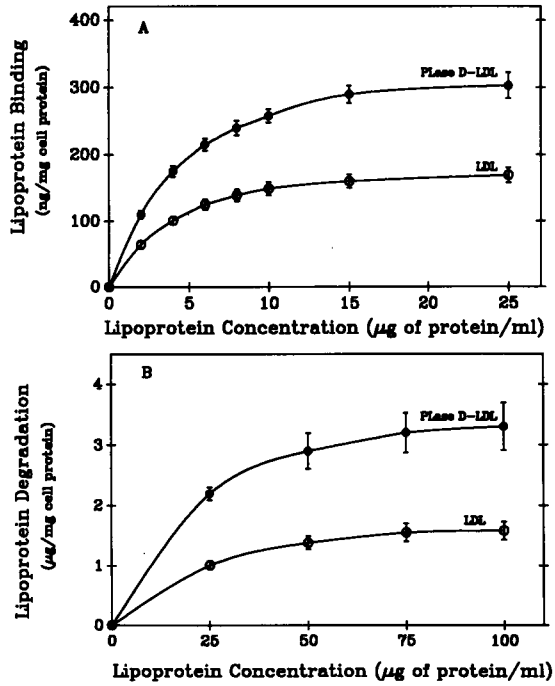

Figure 3. Cellular binding (*A*) and degradation (*B*) of ^{125}I-labeled LDL and PLase D-^{125}I-labeled LDL by J-774A.1 macrophages. Cells were incubated with increasing concentrations of ^{125}I-labeled LDL (○) or PLase D-^{125}I-labeled LDL (●) for 4 h at 4°C (*A*) or for 5 h at 37°C (*B*) before the analysis of lipoprotein binding and degradation, respectively. Results are given as the mean±SD (n = 3).

not shown), suggesting lysosomal involvement in PLase D–LDL cellular degradation.

Cellular cholesterol esterification rate was increased after macrophage incubation with 25 μg protein/ml PLase D–LDL by 170% over control cells (which were incubated without lipoproteins). Similar concentration of native LDL increased macrophage cholesterol esterification rate by only 75%, (values of 0.16±0.03, 0.28±0.04, and 0.43±0.04 nmol esterified cholesterol/mg cell protein per 2 h, n = 3, were obtained for control cells, cells incubated with LDL, or cells incubated with PLase D–LDL, respectively). Similarly, macrophage cholesterol mass increased from 34±4 to 46±5 and 63±5 μg/mg cell protein (n = 6) in cells that were incubated for 24 h without lipoproteins or with 100 μg of protein/ml of native LDL or PLase D–LDL, respectively. The elevation in cellular cholesterol after incubation with PLase D–LDL was mainly (78%) in the CE fraction (data not shown).

Cellular degradation of PLase D–LDL (25 μg protein/ml) was also elevated in comparison with native LDL by two other cells: HMDM and HSF, which do not possess the scavenger receptor, by 59 and 71%, respectively (Table III).

The cellular receptor responsible for the uptake of PLase D–LDL. To ascertain whether PLase D–LDL was taken up via cellular receptors (the LDL or the scavenger receptor), the degradation of PLase D-^{125}I-labeled LDL was studied in the presence of 50-fold excess unlabeled LDL, acetyl-LDL (Ac-LDL), fucoidin, and mAb B1B6 (Table IV). The unlabeled LDL reduced PLase D-^{125}I-labeled LDL cellular degradation by 89% whereas unlabeled Ac-LDL and fucoidin, which bind to the scavenger receptor, had only minimal effect on PLase D–LDL cellular degradation (Table IV). Preincubation of PLase D-^{125}I-labeled LDL with mAb B1B6 (directed against epitopes of the LDL receptor-binding domains on the LDL apo B-100 molecule) inhibited cellular degradation of the labeled lipoprotein by 80% (Table IV). The involvement of the macrophage LDL receptor in the cellular uptake of PLase D–LDL was further assessed in J-774A.1 macrophages that were preincubated for 24 h at 37°C with 10% lipoprotein-deficient serum (to upregulate the LDL receptor), as well as in cells that were loaded with cholesterol (cellular cholesterol increased from 33±8 to 74±11 μg/mg cell protein) by preincubation of the cells with 100 μg/ml of Ac-LDL (to downregulate the LDL receptor). In control cells (with no pretreatment), PLase D-^{125}I-labeled LDL (25 μg protein/ml) degradation rate was 1,335±131 ng/mg cell protein per 5 h whereas, in the upregulated cells and in

Table III. Cellular Degradation of PLase D–LDL and LDL by Various Cells

Cell type	Lipoprotein degradation	
	LDL	PLase D–LDL
	ng protein/mg cell protein	
J-774	901±81	1,698±104* (+88%)
HMDM	688±61	1,095±80* (+59%)
HSF	1,099±95	1,881±117* (+71%)

Cells were incubated with 25 μg protein/ml ^{125}I-labeled lipoproteins for 5 h at 37°C before analysis of lipoprotein cellular degradation. Results are mean±SD (n = 3). * $P < 0.01$ (vs. LDL).

Table IV. Involvement of the LDL Receptor in the Cellular Uptake of PLase D-LDL

Additions	Lipoprotein degradation	
	^{125}I-labeled LDL	PLase D-^{125}I-labeled LDL
	ng protein/mg cell protein	
None (control)	612±61	1,099±63
LDL	99±13*	119±51*
Ac-LDL	589±60	905±66
Fucoidin	631±73	1113±77
mAb B1B6	139±31*	219±47*

J-774A.1 macrophages were incubated with 10 μg protein/ml ^{125}I-labeled LDL or of PLase D-^{125}I-labeled LDL, in the absence (control) or presence of 50-fold excess of unlabeled LDL or Ac-LDL, or 50 μg/ml fucoidin, or 20 μg/ml mAb B1B6. Cellular degradation was measured as described in Methods. Results are given as mean±SD (n = 3). * $p < 0.01$ (vs. control).

the downregulated macrophages, degradation rates of PLase D-^{125}I-labeled LDL were 1,699±171 and 779±61 ng/mg cell protein per 5 h ($P < 0.01$, $n = 3$), respectively. In a similar experiment, macrophage degradation of native LDL increased in the upregulated cells by 37% and was reduced in the downregulated cells by 59% (data not shown). Similarly, the immunoreactivity of PLase D-LDL in comparison with native LDL against mAb B1B6 (directed against the LDL receptor-binding domains of the LDL apo B-100) was also increased. The ED_{50} (lipoprotein concentration required to reduce the ^{125}I-labeled LDL radioactivity in the immunecoplex by 50%) for native LDL and for PLase D-LDL were 9.1±1.6 and 6.0±0.9 μg protein/ml, respectively ($P < 0.01$, $n = 3$).

Cellular processing of PLase D-LDL. Cellular processing of the lipoproteins was studied over 5 h of macrophage incubation with the lipoproteins. No oxidation of the lipoproteins could be found over this period of incubation (25–39 nmol peroxides/mg protein). Cellular degradation (Fig. 4 *A*) and cell association (Fig. 4 *B*) of PLase D-LDL was elevated by ~ 1.8-fold in comparison with native LDL over all time points (Fig. 4). The cellular processing of PLase D-LDL was studied in a pulse-chase experiment. J-774A.1 macrophages were incubated with ^{125}I-labeled LDL or with PLase D-^{125}I-labeled LDL (25 μg protein/ml) for 4 h (pulse period) followed by cell wash and a further incubation of the cells in fresh medium for 2 h (chase period). Fig. 5 demonstrated that the rate of the reduction in cell-associated PLase D-LDL along the first hour of the chase period was 3.5-fold higher than that of native LDL (0.60±0.07 vs. 0.17±0.03 μg LDL protein/mg cell protein per 1 h). We further assessed the processing of PLase D-LDL by the macrophages using lipoproteins that were labeled in the cholesterol of their CE moiety ([^{14}C]CE). The kinetics of the hydrolysis of the lipoprotein CE in cells where acyl CoA cholesterol acyl transferase (ACAT) was inhibited (with 50 μM of progesteron) was studied. Under these conditions, lysosomal hydrolysis of the lipoprotein CE occurred but reesterification of the released unesterified cholesterol by ACAT was blocked. This experiment allows for quantitation of the cellular processing of the lipoprotein CE. Table V demonstrates that the reduction in the cellular [^{14}C]CE was significantly greater in

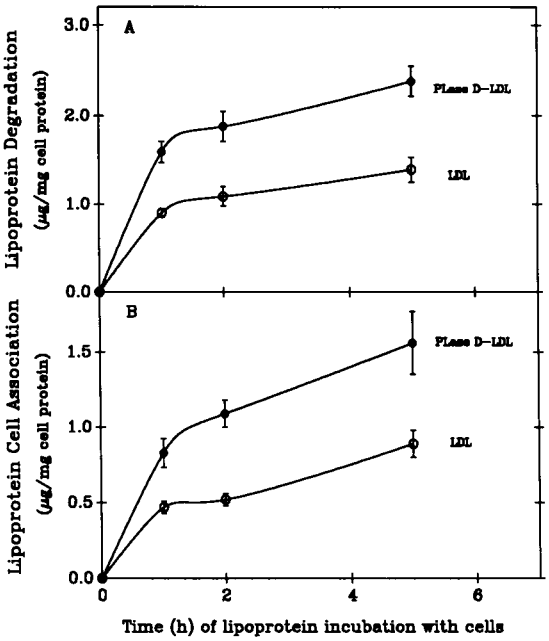

Figure 4. The effect of the time of incubation of ^{125}I-labeled LDL or PLase D-^{125}I-labeled LDL with cells on lipoprotein cellular degradation (*A*) and on lipoprotein cell association (*B*). J-774A.1 macrophages were incubated with 50 μg protein/ml ^{125}I-labeled LDL (○) or PLase D-^{125}I-labeled LDL (●) for up to 5 h at 37°C and at the indicated time points the degradation (*A*) and cell association (*B*) of the lipoproteins were determined. Results represent mean±SD (n = 3).

J-774A.1 macrophages that were preincubated with PLase D-[^{14}C]-CE LDL in comparison with [^{14}C]CE-LDL. After 1 h of chase incubation, cellular [^{14}C]CE reduced by 67% in cells that were preincubated with PLase D-LDL in comparison with only 12% reduction in cells that were preincubated with LDL (Table V). The ^{14}C-labeled unesterified cholesterol was increased in parallel to the reduction in the [^{14}C]CE and, after 1 h of the chase incubation, 78 and 26% increments in cellular radioactivity of ^{14}C-labeled unesterified cholesterol were ob-

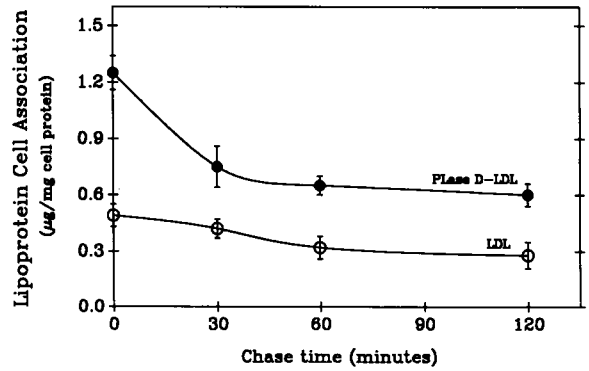

Figure 5. Time course of the lipoprotein processing by J-774A.1 macrophages. Cells were incubated with 25 μg protein/ml ^{125}I-labeled LDL (○) or PLase D-^{125}I-labeled LDL (●) for 4 h at 37°C (pulse period). The cells were washed then with PBS and further incubated in DME at 37°C for the indicated time points (chase period). Cellular association of the labeled lipoproteins was then analyzed. Results represent mean±SD (n = 3).

Table V. Cellular Processing of the CE Moiety in LDL and PLase D-LDL

Lipoprotein used in the preincubation	Time of chase incubation (min)				
	0	15	30	60	120
	cpm/mg cell protein				
LDL	4,151±121	4,005±101	3,811±138*	3,641±117*	3,355±111*
PLase D-LDL	6,851±153	4,831±144*	3,367±101*	2,283±95*	2,073±99*

J-774A.1 macrophages were preincubated with 25 μg protein/ml LDL or PLase D-LDL that were labeled in their core with [^{14}C]CE. Cells were incubated with the lipoproteins in the presence of 50 μM of progesteron to inhibit ACAT activity (pulse). After 2 h of incubation, cells were washed with PBS and further incubated in DME containing 50 μM progesteron at 37°C (chase). At the indicated time points, cells were collected for analysis of cellular content of the labeled CE. Results are given as mean±SD (n = 3). * $P < 0.01$ (vs. 0 time).

tained in cells that were prelabeled by incubation with PLase D–LDL or with LDL, respectively (data not shown).

The effect of PA on macrophage uptake of LDL. Since PLase D action on LDL produced PA on the surface of the lipoprotein, we studied the effect of adding increasing concentrations of PA to ^{125}I-labeled LDL (10 μg protein/ml) before its incubation with J-774A.1 macrophages on LDL cellular association and degradation (Fig. 6). PA was found to increase cellular uptake of LDL in a dose-dependent fashion by up to 114% when a concentration of 100 μg PA/ml was used (Fig. 6). Similarly, the cellular degradation rates of the LDL were increased from 0.93±0.07 μg LDL protein/mg cell protein to 1.35±0.11 and 1.99±0.17 μg LDL protein/mg cell protein when using 25 and 100 μg/ml of PA, respectively (n = 3).

PLase D activity in macrophages and in plasma. A possible physiological relevance for PLase D–LDL is suggested from the fact that macrophages demonstrated PLase D activity towards LDL (Fig. 7). Upon incubation of J-774A.1 macrophages with LDL (1 mg protein/ml) in the presence of 5 mM $CaCl_2$ at 37°C for 24 h, a 179% elevation in LDL PA content and a 27% reduction in LDL PC content were noted in comparison with LDL that was incubated without cells (Fig. 7).

Upon incubation of fresh plasma at 37°C for up to 1 h in the presence of 5 mM $CaCl_2$, a time-dependent formation of PA was noted and it was paralleled by up to a 20% reduction in plasma PC content (Table VI). Similarly, a $CaCl_2$ dose study revealed that after 1 h of plasma incubation at 37°C, a dose-dependent effect of calcium ions on the elevation in plasma PA concentration was noted (Table VII). This was accompanied by a substantial reduction in plasma PC concentration (Table VII). Furthermore, plasma incubation for only 10 min in the presence of 10 mM $CaCl_2$ resulted in the reduction of plasma PI from 355±27 to 253±17 μg/ml (26%, n = 3) whereas plasma PC concentration was reduced at the same time from 1,217±27 to only 1,111±47 μg/ml (9%, n = 3). Upon incubation of plasma for 1 h at 37°C in the presence of 5 mM $CaCl_2$ and 5 mM EDTA, the formation of plasma PA was blocked. Similar results were obtained with the thiol blocking agent, p-hydroxymercuriphenylsulfonic acid (2 mM) or with 10 μM of 1,10 phenanthroline, the transition metal ions chelator (data not shown).

Incubation of native LDL in the presence of 5 mM $CaCl_2$ for up to 1 h at 37°C, however, did not affect its PC and PA content (data not shown), suggesting that PLase D activity is not associated with LDL. Upon incubation of 1 mg LDL protein/ml with 10% (vol/vol) lipoprotein-deficient plasma (LPDP) in the presence of 5 mM $CaCl_2$ for 1 h at 37°C, however, the LDL PC content was reduced from 310±21 to 274±17 μg/ml whereas PA concentration increased from 63±7 to 105±9 μg/ml (n = 3, $P < 0.01$), suggesting again the

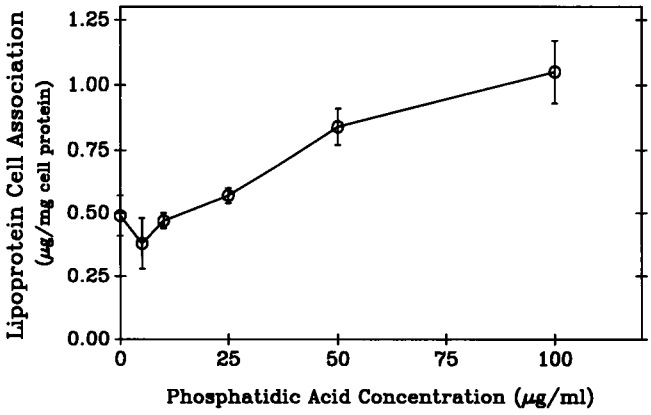

Figure 6. The effect of PA on cell association of LDL to J-774A.1 macrophages. Cells were incubated for 5 h at 37°C with 10 μg protein/ml ^{125}I-labeled LDL that was premixed with increasing concentrations of PA. Cellular association of the labeled lipoprotein was then measured. Results are given as mean±SD (n = 3).

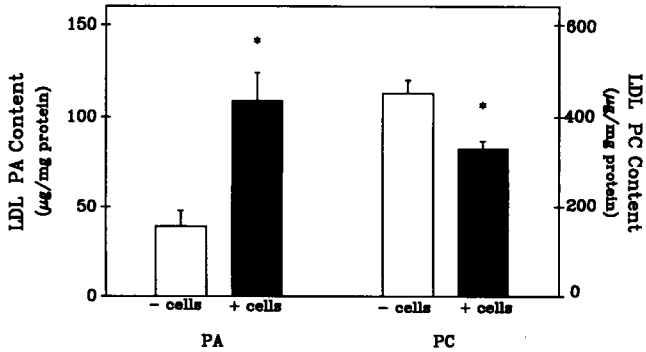

Figure 7. PLase D activity of J-774A.1 macrophages towards LDL. LDL (1 mg protein/ml) was incubated without (−*cells*) or with J-774A.1 macrophages (+*cells*) in the presence of 5 mM $CaCl_2$ for 24 h at 37°C. At the end of the incubation, the content of LDL phosphatidic acid (*PA*) and phosphatidyl choline (*PC*) was determined. Results are given as mean±SD. * $P < 0.01$ (+*cells* vs. −*cells*, n = 3).

Table VI. Time Study of PLase D Activity in Plasma

Time of incubation	PA	PC
min	*μg/ml plasma*	
0	103±11	1,172±93
5	121±9*	1,130±81
15	153±21*	1,059±39*
30	211±17*	986±33*
60	239±33*	944±20*

Fresh plasma obtained from healthy subjects was incubated for up to 1 h at 37°C in the presence of 5 mM $CaCl_2$. Samples were removed at the indicated time points and analyzed for their content of PC and PA as described in Methods. Results represent the mean±SD (n = 6). * $P < 0.01$ (vs. 0 time).

presence of PLase D activity in LPDP. To confirm our finding of PLase D activity in plasma, transphosphatidylation (which is a reaction unique to PLase D) was assessed in plasma.

Upon incubation of plasma in the presence of 5 mM $CaCl_2$ and 20% methanol for 2 h at 37°C, the formation of phosphatidyl methanol was demonstrated by TLC analysis (using a standard of phosphatidyl methanol that was prepared by a 2-h incubation of phosphatidyl ethanolamine with purified PLase D in the presence of 20% methanol at 37°C). This transphosphatidylation reaction indeed supports our finding of PLase D activity in plasma.

Further characterization of the plasma PLase D enzymatic activity was carried out by analysis of its pH sensitivity and heat stability. Plasma incubation with 10 mM $CaCl_2$ for 1 h at 37°C at pH 7.4 resulted in the elevation of PA concentration from 117±18 to 244±11 μg/ml (n = 3). Upon raising the pH to 8.0 or reducing the pH to 7.0 (by plasma dialysis against 25 mM phosphate buffer, 150 mM NaCl with the appropriate pH values), the plasma concentrations of PA were 200±17 and 255±13 μg/ml, respectively (n = 3), and thus pH 7.0 was optimal for plasma PLase D activation. Plasma PLase D activity was heat labile and at pH 7.4 under the above conditions PA production was reduced from 250±19 to 193±15 μg/ml and to 130±19 μg/ml in plasma samples that were preheated for 5 min to 55 or 100°C, respectively.

Table VII. Calcium Ions Concentration Study of PLase Activity in Plasma

$CaCl_2$ concentration	PA	PI	PC
mM	*μg/ml*	*μg/ml*	*μg/ml*
0	181±9	330±13	1,205±43
5	254±7*	267±10*	981±40*
10	289±11*	223±12*	923±31*
50	309±9*	207±11*	900±23*

Plasma was incubated for 1 h at 37°C with increasing concentrations of $CaCl_2$. At the end of the incubation, plasma PC, PI, and PA were determined. Results are the means±SD of three experiments. * $P < 0.01$ (vs. 0 concentration).

Discussion

Enhanced cellular uptake of PLase D–LDL in comparison with native LDL was demonstrated in the present study. This effect could be the result of alterations in the lipoprotein composition or in its physicochemical properties or of phospholipolysis products remaining with the modified LDL particle. Relevant changes in PLase D–LDL include the formation of PA on the surface of the lipoprotein, the loss of heparin-binding capability, and the increased immunoreactivity of PLase D–LDL towards mAb B1B6 (which is directed against the LDL receptor binding domains on apo B-100). Cellular uptake of LDL was shown to be affected by the lipoprotein lipid composition (2, 5–13), which in turn, affects the epitope expression on the LDL apo B-100. Changes in epitope expression were shown to affect both heparin-binding capacity and the immunoreactivity of the lipoprotein against the LDL receptor-binding domains (5, 8, 10, 33). Both the inability of PLase D–LDL to bind to heparin and its increased immunoreactivity against mAb B1B6 can contribute to the increased cellular uptake of PLase D–LDL in comparison with native LDL. Thus, the significance of these changes lies in its possible contribution to macrophage cholesterol accumulation and foam cell formation. Since LDL aggregation was shown to increase its cellular uptake (11, 12), a careful analysis of aggregates in PLase D–LDL was performed, but no aggregation of the lipoprotein could be found. PLase D–LDL, as did oxidized LDL, demonstrated increased electrophoretic mobility on cellulose acetate, did not bind to heparin, and contained reduced content of antioxidants. However, unlike oxidized LDL (2, 40), PLase D–LDL did not show reduced TNBS reactivity, its apo B-100 was not fragmented, and it was not oxidized as demonstrated by analysis of the content of its peroxides and conjugated dienes.

Although PLase D–LDL was not oxidized, it demonstrated increased susceptibility to undergo lipid peroxidation after incubation with copper ions. Thus, PLase D–LDL may resemble the minimally oxidized LDL (41). The susceptibility of LDL to undergo lipid peroxidation is dependent on intrinsic as well as extrinsic factors (42, 43). The LDL antioxidants vitamin E and β-carotene were shown to protect the lipoprotein from lipid peroxidation (42) whereas low lipoprotein content of these antioxidants (as in PLase D–LDL) can contribute to its increased propensity for oxidation (42, 43). In addition, the LDL polyunsaturated fatty acids linoleic acid and arachidonic acid were shown to enhance LDL oxidizability (42). In PLase D–LDL, both the increased content of linoleic acid and the location of this polyunsaturated fatty acid in the core CE moiety of LDL probably have contributed to its increased oxidizability (43). The reduced antioxidant content in PLase D–LDL in comparison with native LDL (which could have resulted from conformational changes in PLase D–LDL) and the increased percentage of linoleic acid (paralleled by some reduction in palmitic, stearic, and oleic acids that could have resulted from some nonspecific fatty acid lipolysis) could possibly have contributed to the increased susceptibility of PLase D–LDL to undergo lipid peroxidation (42, 43).

The somewhat smaller size of PLase D–LDL in comparison to native LDL may be also responsible for its increased propensity for oxidation, as it was recently shown that the smaller and denser particles of LDL are more prone to oxidation than the larger LDL particles (44).

The formation of PA on the surface of the modified lipoprotein could have also contributed to this effect. In the present study PA was shown to possess the ability to increase the propensity of LDL to oxidation and, thus, PA may be responsible for the enhanced susceptibility of PLase D–LDL to oxidation. In neutrophils a correlation was shown between the generation of PA and the production of superoxides (45). PA, which is rapidly produced by a variety of stimulated cells, elicited NADPH-dependent superoxide anion production in neutrophils by a direct interaction with NADPH oxidase (45). Although PLase D–LDL did not show lipid peroxidation even after its incubation with macrophages, it is possible that the increased susceptibility of PLase D–LDL to undergo lipid peroxidation in the presence of copper ions can contribute to the oxidation of PLase D–LDL by macrophages under oxidative stress such as that existing in the atherosclerotic lesion. Thus, arterial wall macrophages under such conditions can possibly produce oxidized PLase D–LDL. Oxidized PLase D–LDL can further contribute to macrophage cholesterol accumulation by its cellular uptake via the scavenger receptor (2) in addition to the increased uptake of the nonoxidized PLase D–LDL via the LDL receptor.

Lipid modifications of LDL were previously reported (5–13). It was shown that a reduction in the lipoprotein core triglycerides (7) or CE (46) content by LDL incubation with lipoprotein lipase or cholesterol esterase resulted in an enhanced or reduced cellular uptake of the modified LDLs, respectively. Modifications of the LDL surface lipids (unesterified cholesterol and phospholipids) were previously studied. Upon oxidation of the LDL unesterified cholesterol with cholesterol oxidase, cholestenone was formed on the surface of the lipoprotein and the cholesterol oxidase–LDL was taken up by macrophages at an increased rate (5).

Besides unesterified cholesterol, phospholipids also contribute to the surface coat of the lipoprotein. Phospholipase treatment of LDL was shown to result in the formation of a modified form of LDL with altered physicochemical properties depending on the type of phospholipase used (9–13). Both PLase C and PLase D act on the phosphoryl group of phospholipids, but PLase D, unlike PLase C, does not remove the phosphorous moiety of the phospholipid. PLase D removes only the alcohol portion of the phospholipid, resulting in the formation of PA on the surface of the lipoprotein (14).

PA was shown in the present study to increase cellular uptake of LDL in a dose-dependent fashion and this may have resulted from the action of PA as a "second messenger" as was demonstrated in other cells (47, 48). Indeed, pretreatment of the cells with PA resulted in a significant enhancement of LDL uptake by macrophages and this phenomenon may be related to PA-mediated increased synthesis of LDL receptors. PLase D–LDL uptake by macrophage was mediated via the LDL receptor and not via the scavenger receptor. This was suggested from the competition of excess unlabeled LDL (but not Ac-LDL) with PLase D-^{125}I-labeled LDL for cellular degradation as well as from the ability of mAb B1B6 (directed against the LDL receptor binding domain on apo B-100) and the inability of fucoidin (a scavenger receptor ligand) to affect PLase D-^{125}I-labeled LDL cellular degradation. Furthermore, macrophage uptake of PLase D–LDL, like native LDL, was subjected to cellular cholesterol regulation (1) of the LDL receptor (up- and down-regulation). The ability of HSF (which do not contain scavenger receptors) to degrade PLase D–LDL at enhanced rate in comparison with native LDL also rules out the involvement of the scavenger receptor in the uptake of PLase D–LDL. Increased macrophage binding and degradation of PLase D–LDL in comparison with native LDL was shown over a range of lipoprotein concentrations. However, the saturation of the cellular binding and degradation of PLase D–LDL at higher lipoprotein concentrations compared with those obtained with native LDL may suggest that, in addition to the LDL receptor–mediated uptake of PLase D–LDL, a nonreceptor cellular uptake of this modified lipoprotein may be also involved. The dose- and time-dependent effects of PLase D on LDL PA and PC content as well as on the cellular uptake of PLase D–LDL were not exactly paralleled. During 30 min of LDL incubation with PLase D, LDL PC content was almost maximally reduced (with a parallel increase in LDL PA content). This modified LDL, however, after 30 min of incubation, increased macrophage degradation in comparison with native LDL to only half of its maximal effect (obtained after 24 h of incubation). This phenomenon may suggest that the stimulation of cellular degradation of PLase D–LDL relative to native LDL is dependent not only on the lipoprotein PA and PC content but also on some other changes in PLase D–LDL. These changes, as pointed out, could be related to the decrease in heparin binding, the increased immunoreactivity against mAb B1B6, the elevated levels of linoleic acid, and the reduced content of the lipoprotein antioxidants. Differential cellular processing of lipoproteins may have contributed to the ability of PLase D–LDL to stimulate intracellular cholesterol esterification more than native LDL as was previously shown for B-VLDL (49). This is suggested since the kinetic of macrophage uptake and degradation of PLase D–LDL was more rapid than that of native LDL. Furthermore, in the pulse–chase experiment, the rapid reduction in the cell association of PLase D–LDL in comparison with native LDL also suggests a different intracellular processing for the native and the modified lipoproteins. As both native LDL and PLase D–LDL are internalized via the LDL receptor, and since there were no aggregates in PLase D–LDL, the accelerated cellular catabolism of the modified LDL is probably the result of increased susceptibility to lysosomal enzymes. A physiological relevance of PLase D–LDL is suggested since PLase D activity was demonstrated in the present study both in macrophages and plasma. Under the experimental conditions of macrophage incubation with relatively high calcium ions concentration, no cell death occurred and thus the PLase D was probably secreted from the cells. This calcium ion–mediated PLase D release from macrophages may not occur normally in vivo. However, under conditions such as increased local concentration of calcium ions (activation by second messengers) it may be operated. No PLase D activity could be found in association with native LDL but plasma was shown to possess significant PLase D activity, as was recently shown for another type of PLase D (15, 16). Considerable similarities are shared by the plasma glycosylphosphatidylinositol and PLase D activities of phagocytes (15, 16, 47, 48). PLase D activity possesses broad substrate specificity and is able to hydrolyze choline-, ethanolamine-, and inositol-containing phosphoglycerides. PLase D from human neutrophils and monocytes, such as plasma PLase D but not endothelial cells PLase D, was shown to be calcium dependent (15, 16, 48). In the present study, plasma PLase D activity was found to be calcium dependent and act on both PI and PC. It is of interest that plasma PLase D activity demonstrated much higher

affinity towards plasma PI in comparison with plasma PC. After 1 h of plasma incubation in the presence of 10 mM $CaCl_2$, the PI was reduced by 32% in comparison with only 23% reduction in plasma PC concentration. This difference was much greater when comparing the reduction in plasma PI and PC after only 10 min of incubation (26 and 9%, respectively). Another plasma PLase D activity was recently shown to be specific for phosphatidylinositol glycan but not towards PC or PI (50). This plasma PLase D activity, however, was more heat stable and was stimulated at lower pH than the plasma PLase D activity shown in the present study. Because the transphosphatidylation reaction is unique to PLase D, the formation of phosphatidylalcohol is a useful indicator of PLase D activity. In the present study we have successfully demonstrated the formation of phosphatidyl methanol in plasma after 1 h of plasma incubation in the presence of 5 mM $CaCl_2$ and methanol. Finally, the PLase D activity of LPDP on LDL suggests again the presence of PLase D activity in plasma but not in association with plasma lipoproteins. The finding of PLase D activity in plasma is of major importance since this activity may play an important role in vivo in phospholipid metabolism.

The interactions of modified lipoproteins with cells involve different pathways, including lipoprotein uptake via the LDL receptor or the scavenger receptor(s) and also a nonreceptor internalization of the LDL cholesterol. The accumulation of cholesterol in arterial wall macrophages and the formation of foam cells during atherogenesis involve more than one mechanism (1–12, 51). LDL modifications that affect foam cell formation are associated with changes in the LDL lipids moieties as well as with the oxidation of the lipoprotein fatty acids and cholesterol constituents (1–13). PLase D modification of LDL can contribute to macrophage cholesterol accumulation by both of these mechanisms since it produces modified LDL (which is taken up by the LDL receptor at an enhanced rate) and since this modified LDL is easily oxidized to a form that is taken up at enhanced rate via the macrophage scavenger receptor, leading to foam cell formation.

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