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Prevention of phospholipase-C induced aggregation of low density lipoprotein by amphipathic apolipoproteins

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Phospholipase C (PL-C) digestion of human low density lipoprotein (LDL) results in hydrolytic cleavage of the phosphocholine head group of phosphatidylcholine, thereby generating diacylglycerol. Loss of amphiphillic surface lipids and/or accumulation of diacylglycerol causes LDL samples to develop turbidity. Examination of PL-C treated LDL by electron microscopy revealed a progressive aggregation of LDL as a function of phosphatidylcholine hydrolysis: fused particles, clusters, and multiple stacked aggregates were observed. Lipid analysis of untreated and aggregated LDL confirmed that the phosphatidylcholine content of the latter had decreased with a corresponding increase in diacylglycerol. It is likely that phospholipolysis created hydrophobic gaps within the surface monolayer of LDL, thereby inducing LDL fusion and aggregation. When amphipathic α-helix-containing apolipoproteins, such as human apoA-I or *Manduca sexta* apolipophorin III (apoLp-III) were present, PL-C treated LDL did not aggregate. Compositional analysis of apolipoprotein-containing PL-C LDL showed that phospholipolysis was not affected by the presence of apolipoproteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of lipoproteins re-isolated following incubation with PL-C revealed an association of apoA-I or apoLp-III with PL-C digested LDL. Electron microscopy showed no major morphological differences between native LDL and apoprotein stabilized PL-C treated LDL and the average particle diameter of apoA-I stabilized PL-C LDL was 22.5 ± 2.2 nm versus 22.8 ± 1.6 nm for control LDL. Incubation of tritium-labeled apoLp-III with LDL and PL-C demonstrated that association of apoLp-III with PL-C LDL correlated with the extent of phospholipid hydrolysis, the apolipoproteins apparently being recruited to compensate for the increased hydrophobic surface created by conversion of phosphatidylcholine into diacylglycerol. The results suggest that transient association of amphipathic apolipoproteins with damaged or unstable LDL may provide a mechanism to obviate formation of atherogenic LDL aggregates in vivo.

Lipoprotein; Phospholipase; Apolipoprotein; Amphipathic; Phospholipid; Diacylglycerol

1. INTRODUCTION

In studies of facilitated lipid transfer between insect high density lipophorin (HDLp) and human low density lipoprotein (LDL) we found that LDL is an excellent acceptor of HDLp-derived diacylglycerol (DAG) [1]. DAG transfer, facilitated by *Manduca sexta* lipid transfer particle (LTP), was not accompanied by apolipoprotein exchange or transfer and lipid enriched LDL were created. When the amount of donor HDLp was increased relative to LDL, continued DAG accumulation led to LDL particle aggregation [2]. Facilitated DAG transfer-induced aggregation of LDL was prevented, however, when assays were conducted in the presence of amphipathic α-helix containing apolipoproteins, such as *Manduca sexta* apolipophorin III (apoLp-III) or nonB human very low density lipoprotein (VLDL) apolipoproteins. In this case, DAG transfer resulted in apolipoprotein association and formation of larger VLDL-like particles. In this reaction expansion of LDL volume resulting from DAG accumulation created new lipid/water interface that induced apolipoprotein binding. A similar phenomenon, known to occur under physiological conditions, pertains to hormone-induced flight-related lipoprotein transformations in insects such as *M. sexta* or *Locusta migratoria* [3,4]. During flight, the DAG content of pre-existing hemolymph HDLp increases by 3-fold, converting the particle to low density lipophorin [5]. Concomitant with DAG accumulation, reversible association of multiple copies of apoLp-III occurs, which is thought to stabilize the structure of low density lipophorin [6,7].

Creation of DAG at the expense of phosphatidylcholine in the surface monolayer of lipoproteins by the action of phospholipase C (PL-C) resembles DAG accumulation in lipoproteins induced by LTP-mediated lipid transfer. Treatment of LDL with PL-C [8] or sphingomyelinase [9] induces formation of aggregates which represent multi-valent (i.e. they contain multiple copies of apoB) ligands for the LDL receptor. Uptake of these complexes by macrophages in vitro greatly in-

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Abbreviations: DAG, diacylglycerol; apo, apolipoprotein; apoLp-III, apolipophorin III; PL-C, phospholipase C; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDLp, high density lipophorin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

creases the amount of cholesterol taken up per internalization event and, therefore, results in formation of foam cells. These data, together with reports of extracellular phospholipase activities [10,11], suggest aggregation of LDL may be an important step in development of atherosclerosis. In the present study we have performed experiments to investigate if amphipathic apolipoproteins can stabilize LDL particles digested by PL-C, thereby conferring resistance to formation of atherogenic LDL aggregates.

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Phosphatidylcholine specific phospholipase C from *Bacillus cereus* (Grade I; <0.05% sphingomyelinase activity) was purchased from Boehringer Mannheim; [^{3}H]succinimidyl propionate was from Amersham; and Sephadex gel filtration matrices were supplied by Pharmacia. All other reagents were obtained from Sigma or Fischer. Human LDL (density 1.006–1.063 g/ml) was obtained from fasting human plasma by sequential density gradient ultracentrifugation [12]. Lipoprotein preparations were stored in an argon atmosphere at 4°C. Human apolipoprotein A-I (apoA-I) was prepared according to Ryan et al. [13] and *M. sexta* apoLp-III was purified as described by Wells et al. [14].

2.2. *Analytical methods*

Protein concentrations were determined with the bicinchoninic acid assay (Pierce Chemical Co.), using bovine serum albumin as standard. SDS-PAGE was performed in 4–18% acrylamide gradient slab gels at 30 mA for 3.5 h and stained with Coomassie brilliant bleu. ApoA-I was quantitated by construction of a standard curve from known amounts of isolated apoA-I electrophoresed on an acrylamide slab in parallel with apoA-I stabilized PL-C treated LDL samples. The stained gel was scanned on a Cannag TLC Scanner II. Lipid analysis was performed using enzymatic kits for choline containing phospholipids, unesterified and esterified cholesterol, and neutral glycerolipids (Boehringer Mannheim). Where indicated lipids were extracted with 2:1 chloroform/methanol (v/v) and separated by thin-layer chromatography prior to lipid analysis. Electron microscopy was performed in a Philips EM 420 as described previously [15]. Samples (40 μg/ml) were negatively stained with 2% sodium phosphotungstate (pH 7.0) and photographed at magnifications (calibrated) of 13,000 or 55,000. The diameters of lipoprotein particles were measured on 3 times enlarged photographic prints. ApoLp-III was labeled as follows: 30 μl [^{3}H]succinimidyl propionate (1 mCi/ml) in toluene was dried under N_2. Three mg apoLp-III, dissolved in 0.3 ml 50 mM Tris-HCl (pH 8.5), was added to the dried tube. After agitating the tube in ice for 10 min the reaction was stopped by adding 200 μl of 0.1 M glycine (pH 8.5). The reaction mixture was dialyzed against 150 mM NaCl, 50 mM Tris-HCl (pH 8.0) for 72 h before use.

2.3 *Phospholipase assays*

Routine PL-C assays were conducted in microtiter plate wells containing 100 μg LDL in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM Ca^{2+} was incubated in the presence or absence of 100 μg apoA-I or apoLp-III. PL-C was added to initiate the reaction. Absorbances at 340 nm were recorded on a SLT Labinstruments Microtiter Reader [16] to monitor the development of solution turbidity. In some cases assays were conducted in glass tubes with scaled up substrate concentrations. In order to examine apoLp-III binding as a function of PL-C hydrolysis, [^{3}H]apoLp-III was included in incubations. At specified intervals the reaction was stopped by adding 0.1 M EDTA and transferring to ice. The reaction solution was adjusted to a density 1.063 g/ml with KBr, transferred to a 13 ml Beckman Quick Seal tube and centrifuged at 40,000 rpm for 24 h in a Beckman 70.1 Ti rotor. After centrifugation the radioactivity and cholesterol content of LDL was determined.

3. RESULTS

When LDL was incubated with PL-C, the light scattering of the solution (measured as absorbance at 340 nm) increased in a sigmoidal manner as a function of time (Fig. 1). The initial lag period suggests that phospholipid depletion and/or DAG accumulation beyond a threshold is required before sample turbidity develops [16]. In an identical reaction mixture, in which the absorbance at 340 nm was allowed to reach 1.0 OD units, lipids were extracted with chloroform/methanol (3:1) and separated by thin-layer chromatography. Densitometric scanning indicated significant phosphatidylcholine hydrolysis had occurred (compared to control LDL). Electron microscopic examination of LDL treated with PL-C for various times revealed a progressive aggregation of LDL particles. Fig. 2 shows representative micrographs of LDL following incubation with PL-C as a function of time. At time zero (panel A), LDL particles are relatively homogeneous in size (22.8 ± 1.6 nm) with no sign of aggregation. Panel B corresponds to PL-C LDL with an $A_{340\,nm}$ = 0.21. These particles show the beginning of the aggregation process with small strings and clusters present. Panel C shows an intermediate stage of aggregate formation ($A_{340\,nm}$ = 0.82). Although there are many normal-sized LDL particles, an appreciable number of aggregated clusters (which appear as dark blotches) have formed. As PL-C catalyzed phospholipolysis continues, increasing aggregation of LDL particles results in further fusion and formation of stacked clusters. Panel D shows that at the

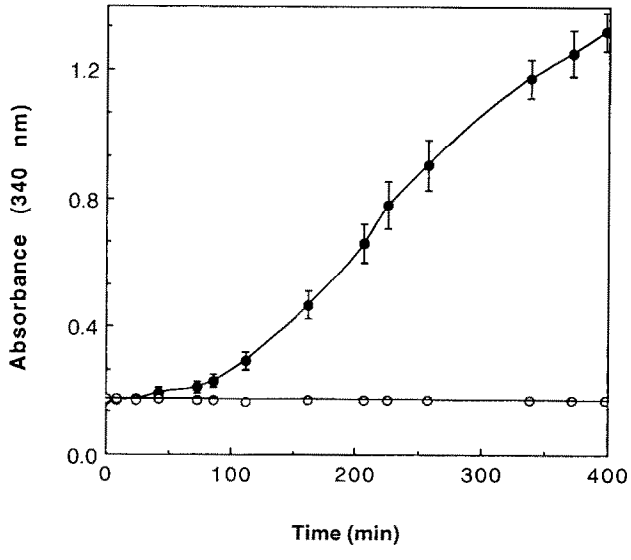

Fig. 1. The effect of PL-C on LDL sample turbidity. 200 μg of LDL was incubated at 22°C with 500 milliunits PC-specific PL-C in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM Ca^{2+}. Absorbance was measured at 340 nm. Closed circles = PL-C treated LDL; open circles = control LDL.

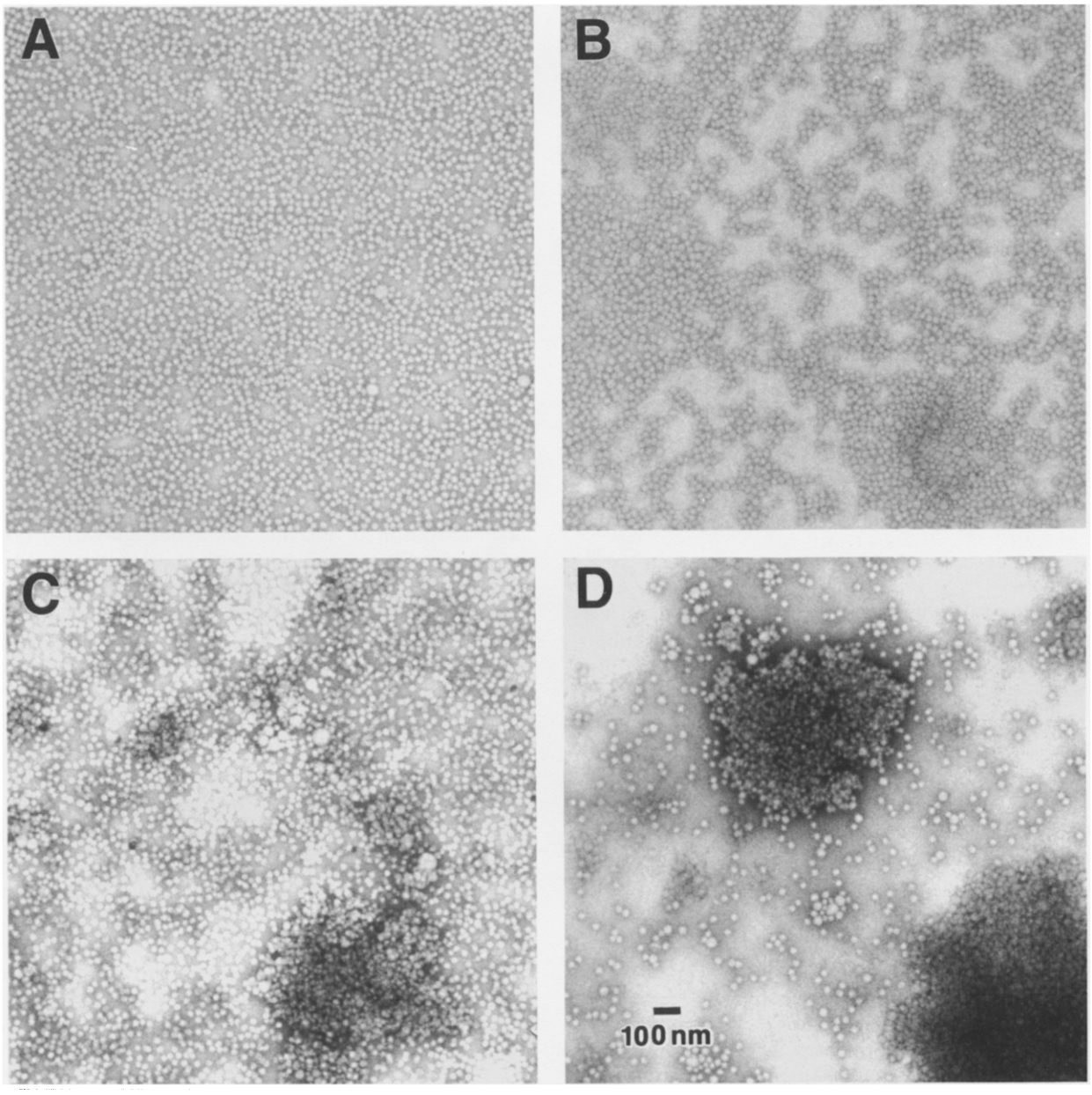

Fig. 2. Morphological characterization of PL-C treated LDL as a function of time. Incubation conditions were the same as in Fig. 1 except that the reaction was carried out at 37°C. At different intervals, an aliquot of the sample was removed and diluted 10-fold with 0.1 M EDTA to stop the reaction. Samples were then negatively stained with 2% sodium phosphotungstate and photographed in the electron microscope. (A) Before addition of PL-C ($A_{340\ nm}$ = 0.0). (B) 35 min ($A_{340\ nm}$ = 0.3). (C) 65 min ($A_{340\ nm}$ = 0.7). (D) 285 min ($A_{340\ nm}$ = 1.23).

end stage of the reaction ($A_{340\ nm}$ = 1.32) most LDL particles are piled up into large, multilayered aggregates, with relatively few free particles remaining.

When amphipathic surface apolipoproteins, such as human apoA-I or *M. sexta* apoLp-III were included in the incubation medium containing LDL and PL-C, turbidity did not develop (Fig. 3). When apolipoproteins were added to samples of aggregated LDL, however, there was no effect, suggesting the aggregation process is irreversible. Taken together, the results suggest that either PL-C catalyzed phospholipolysis was inhibited or LDL aggregation was prevented in the presence of amphipathic apolipoproteins. Quantitative lipid analysis, shown in Table I, verified the loss of phospholipid and revealed a corresponding increase in the content of DAG (measured as glycerolipid). Since cholesterol is unaffected by PL-C, the ratios of glycerolipid/cholesterol and phospholipid/cholesterol in different samples were compared. Whereas the ratio of phospholipid to total cholesterol decreased in the presence of PL-C, the glycerolipid/total cholesterol ratio increased. Comparison of the lipid composition of the control and apolipoprotein-containing samples showed the decrease in the ratio of phospholipid to total cholesterol and the in-

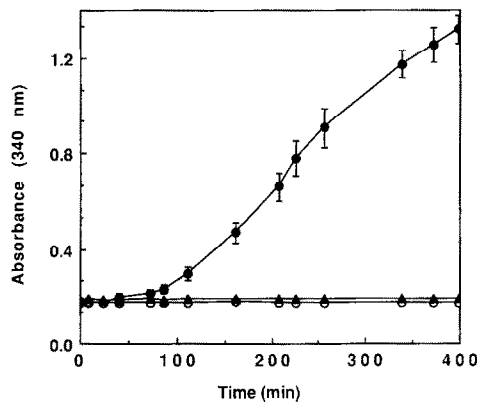

Fig. 3. The effect of apolipoproteins on PL-C induced turbidity of LDL. 200 μg of LDL was incubated at 22°C with 500 milliunits PL-C in the presence or absence of the water-soluble apolipoproteins, apoA-I or apoLp-III (200 μg each). Absorbance was monitored at 340 nm. Closed circles=PL-C treated LDL without apolipoproteins; open squares=phospholipase C treated LDL in the presence of apoA-I; closed diamonds=phospholipase C treated LDL in the presence of apoLp-III.

crease in the ratio of glycerolipid/total cholesterol was similar to changes observed in the absence of apolipoprotein, indicating phospholipolysis was not inhibited by the presence of apoA-I or apoLp-III.

Electron microscopy of LDL samples incubated with PL-C in the presence of apoA-I showed no aggregation of LDL particles whereas in the absence of apolipoproteins, PL-C treated LDL formed aggregates and fused into larger particles with diameters of 60 nm or greater (Fig. 4). Thus, amphipathic α-helical apolipoproteins have a dramatic effect in the maintenance of LDL particle integrity in the face of amphiphilic surface lipid depletion. In addition to LDL panel C contains evidence of small spherical entities (see arrows). These represent self-associated oligomers of apoA-I present in excess of that required to stabilize PL-C treated LDL. Lipid analysis of these complexes, following separation from PL- C treated LDL by Sephadex G-150 gel permeation chromatography, revealed that they are not associated with lipid. In order to determine if apolipoproteins associated with the surface of PL-C treated LDL, re-isolated LDL-containing fractions were subjected to SDS-PAGE. In the presence of human apoA-I, the gel electrophoretic pattern of PL-C LDL samples showed not only apoB but also the presence of apoA-I (Fig. 5). These data suggest that apoA-I (and apoLp-III) stabilizes PL-C treated LDL particles through a binding interaction. Quantitation of apoA-1 binding to LDL particles following incubation with PL-C was performed by densitometric scanning of SDS- PAGE gels. The data, which revealed 1.5 apoA-I bound per LDL following treatment with 2.8 units PL-C/mg LDL protein for 180 min at 22°C (A_{340} = 1.11), suggests that up to two apoA-I molecules can bind to the surface of PL-C-treated LDL.

In order to investigate the apparent correlation between the extent of PL-C induced phosphatidylcholine hydrolysis and amphipathic apolipoprotein association, [^{3}H]apoLp-III was incubated with LDL and PL-C. At specified intervals an aliquot of the reaction mixture was removed and phospholipolysis stopped by adding EDTA and transferring to ice. After separation of LDL from PL-C and unbound apoLp-III by density gradient ultracentrifugation, LDL containing fractions were analyzed for cholesterol content and radioactivity. The ratio of cpm [^{3}H]apoLp-III/cholesterol increased as a function of time (Fig. 6), consistent with increasing turbidity of a control assay, incubated in parallel, in the absence of apoLp-III. This indicated that apolipoprotein association with LDL is coupled to hydrolysis of phosphatidylcholine by PL-C. Likewise, when LDL samples were incubated with increasing amounts of PL-C in reactions that did not contain apolipoprotein, the absorbance increase at 340 nm was proportional to the concentration of enzyme (data not shown). Furthermore, in the presence of [^{3}H]apoLp-III, the amount of radioactivity associated with LDL per μg total cholesterol showed a similar correlation with the amount of PL-C added (data not shown). These experimental results demonstrate that binding of apoLp-III (and apoA-I) to the surface of LDL is dependent upon the extent of PL-C catalyzed phosphatidylcholine hydrolysis.

4. DISCUSSION

PL-C catalyzes the hydrolytic removal of the phosphocholine moiety of phosphatidylcholine, thereby generating DAG. Accumulation of DAG, with its much smaller and far less polar head group, in the surface monolayer of lipoproteins would be expected to result in particle instability. In this regard it has been shown that incorporation of small quantities of DAG into bilayer membranes promotes a lamellar to reverse hexag-

Table I

Effect of PL-C and apolipoproteins on the composition of LDL

	LDL apoA-I	LDL PL-C	LDL PL-C apoA-I	LDL PL-C apoLp-III
Total CH (mg/ml)	340 ± 30	460 ± 40	450 ± 30	410 ± 40
GL (mg/ml)	30 ± 2	83 ± 6	65 ± 5	78 ± 6
PL (mg/ml)	95 ± 8	60 ± 5	75 ± 6	50 ± 5
Protein (mg/ml)	210 ± 20	230 ± 20	250 ± 20	240 ± 20
PL/CH	0.28	0.13	0.17	0.12
GL/CH	0.09	0.18	0.15	0.19

CH=cholesterol; GL=glycerolipids; PL=phospholipids. After reaction LDL was re-isolated by gel filtration chromatography. Total cholesterol, glycerolipids, phospholipids and protein were analyzed as described in the text.

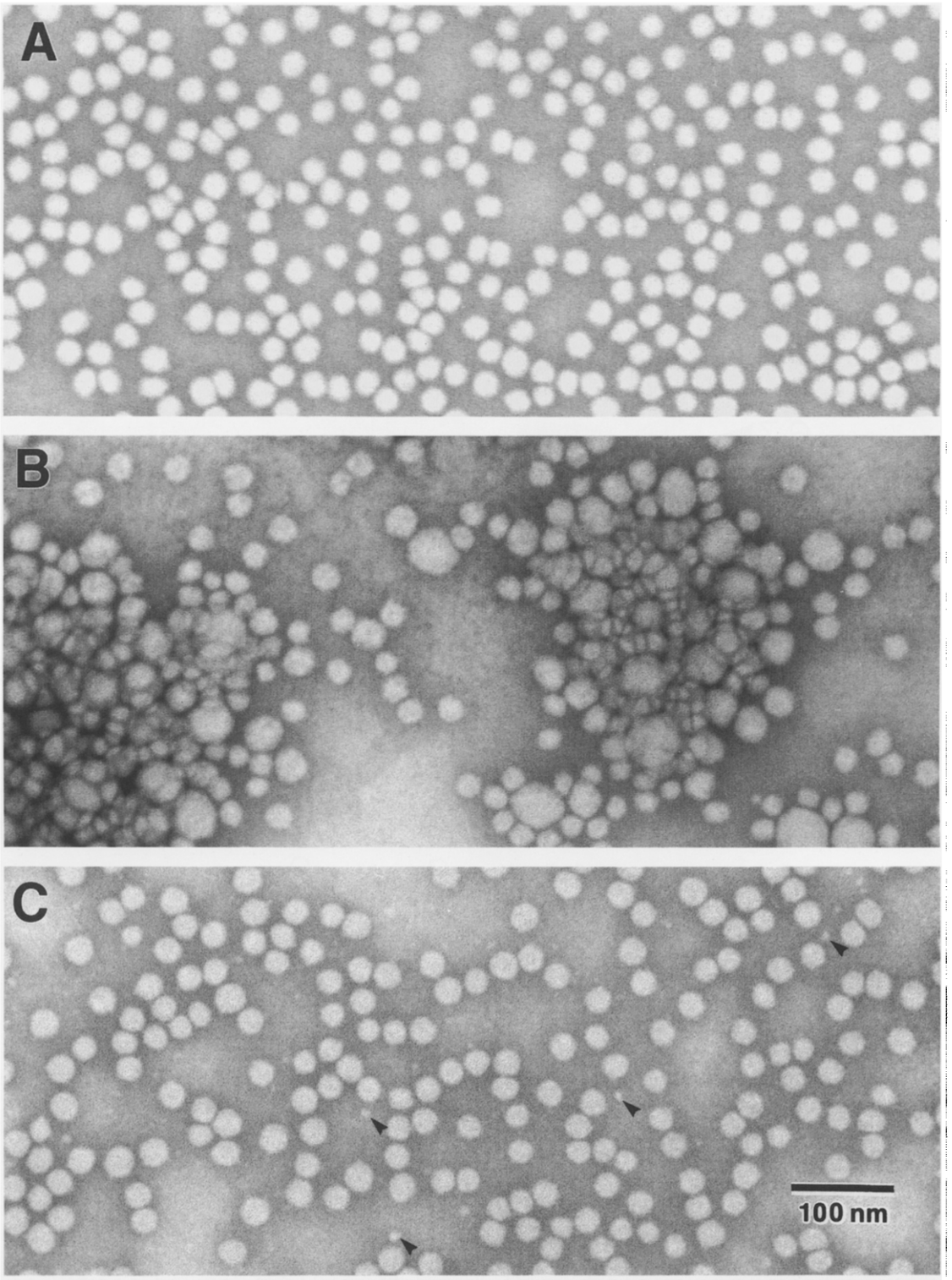

Fig. 4. Electron microscopy of lipoproteins. (A) Untreated LDL particles. (B) LDL after incubation with PL-C for 285 min ($A_{340\ nm}$ = 1.23). (C) Same incubation conditions as (B) plus 200 μg apoA-I. Arrows point to some of the oligomeric complexes of lipid-free apoA-I present in the sample.

onal phase transition [17,18]. To avoid disruption of the surface monolayer, the thermodynamically unstable lipid/water interface created by phospholipolysis must be stabilized. We have shown that exogenous amphipathic α-helical apolipoproteins prevent aggregation of LDL normally induced by PL-C. Apolipoproteins were shown to associate with LDL particles as a function of phospholipolysis and particles containing bound ex-

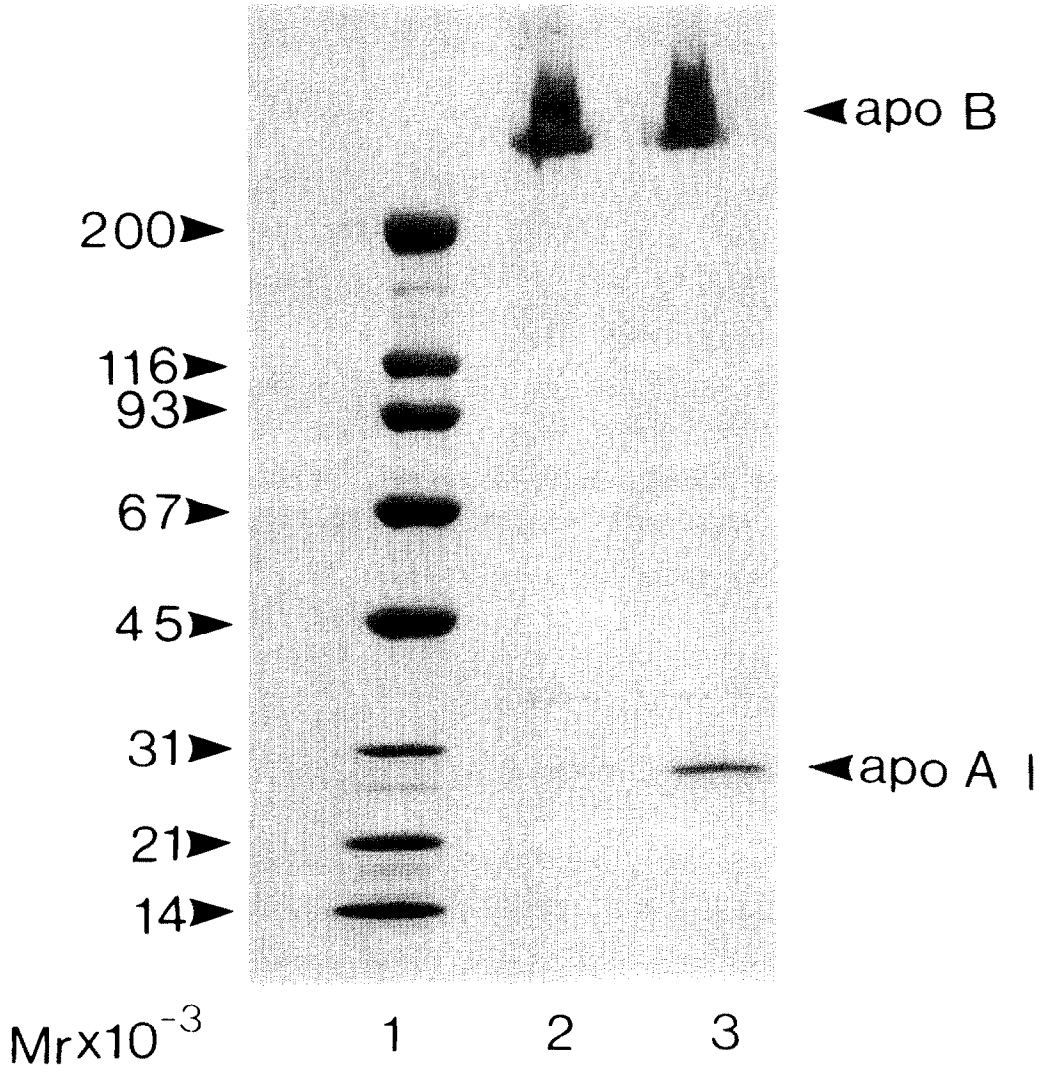

Fig. 5. Apolipoprotein association with PL-C treated LDL. 600 μg of LDL was treated with 1.5 units PL-C in the presence and absence of an equal amount of apoA-I at 37°C until the turbidity of the incubation without apolipoprotein reached an OD of 1.0 (340 nm). Reactions were stopped by adding 600 μl of 0.1 M EDTA. Samples were subjected to gel filtration and LDL containing fractions were subjected to SDS-PAGE. (Lane 1) Molecular markers; (Lane 2) control LDL incubation with apoA-I only; (Lane 3) PL-C treated LDL in the presence of apoA-I.

ogenous apolipoprotein were isolated. The nature of the interaction between these amphipathic apolipoproteins and the surface monolayer of LDL is not known but DAG appears to play a key role. Recently the tertiary structures of *Locusta migratoria* apoLp-III [19] and the LDL receptor binding domain of human apoE [20] were determined by X-ray crystallography, and it was shown that both contain elongated amphipathic α-helices linked by short loops. In the lipid-free state these apolipoproteins form helical bundles, with the hydrophobic face of their α-helices oriented inward and their hydrophilic faces exposed to the aqueous medium. In the case of apoLp-III, it has been proposed that, upon sensing lipoprotein surface hydrophobicity, the helical bundle opens permitting interaction of its hydrophobic domains with the surface [19].

During flight the amount of DAG associated with HDLp increases more than 3-fold creating the larger LDLp particle. Since the concentration of surface phospholipids does not increase during this transformation [5] it is thought that partitioning of DAG into the surface monolayer of the particle induces association of additional apoLp-III molecules. DAG molecules in the surface monolayer would intercalate between phospholipids, generating unstable hydrophobic gaps. Molecular modeling has shown that the hydrophobic face of an amphipathic α-helix from apoLp-III can fit between phospholipid head groups in the space created by intercalation of DAG [21]. In other experiments we have shown that facilitated DAG transfer from HDLp to LDL leads to creation of DAG-enriched LDL [1]. Beyond a threshold of DAG enrichment, however, LDL particles aggregate [2]. This aggregation was prevented when apoLp-III or nonB human VLDL apolipopro-

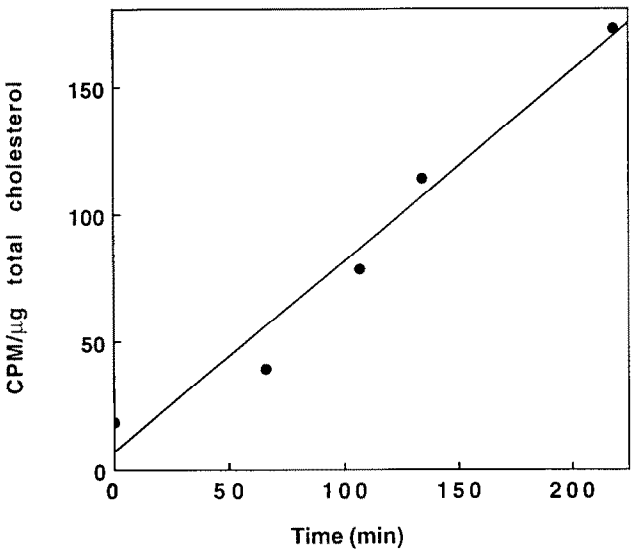

Fig. 6. Effect of phosphatidylcholine hydrolysis on association of [^{3}H]apoLp-III with LDL. 400 μg of LDL was treated with 1 unit PL-C in the presence of 100 μg of [^{3}H]apoLp-III (1200 cpm/μg) and 300 μg of unlabelled apoLp-III. Reaction aliquots were removed at the indicated times; LDL was re-isolated by density gradient ultracentrifugation and radioactivity and cholesterol content determined.

teins were included in the incubation. Instead, larger, DAG and apoLp-III enriched, VLDL-like particles were formed. In this reaction it is possible that DAG initially moves into the core but as the particle volume increases it partitions into the surface monolayer. At this point fusion/aggregation or apolipoprotein association occurs.

It has been reported that aggregation of LDL can be induced by mechanical disruption [22], sphingomyelinase [9], PL-C [8] or oxidation [23]. In each of these cases LDL particle aggregation has been correlated with the accumulation of cholesterol in macrophages. Since aggregated/fused LDL contain multiple apoBs and far more cholesterol than an individual native LDL, cellular uptake of aggregated LDL means cholesterol delivery can exceed cellular cholesterol excretion [24] and cellular cholesterol levels reach the threshold level necessary for acyl-CoA:cholesterol *O*-acyltransferase stimulation sooner and more extensively. Macrophage cholesteryl ester deposition leads to foam cell formation, a morphological hallmark of early atherosclerotic lesions. Sphingemyelinase activities are found in the arterial wall [25,26] and serum [10], and extracellular forms of phospholipase have been reported [11]. This fact, together with the finding that LDL aggregates have been isolated from arterial intima [27] suggests phospholipase induced LDL aggregation might occur in vivo. The present findings, which show that amphipathic apolipoproteins can prevent phospholipase-induced LDL aggregation suggest the balance between enzymatic activities specific for phospholipid hydrolysis and the availability of free apolipoproteins may be a determining factor in foam cell formation and atheroma.

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