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Complement Protein C1q Recognizes Enzymatically Modified Low-Density Lipoprotein through Unesterified Fatty Acids Generated by Cholesterol Esterase†

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ABSTRACT: We previously reported that enzymatically modified low-density lipoprotein (E-LDL) particles obtained by LDL treatment with trypsin and then cholesterol esterase are recognized by C1q and activate the C1 complex of complement. The objective of this study was to identify the E-LDL component(s) recognized by C1q. In addition to trypsin, plasmin, thrombin, tryptase, and matrix metalloprotease-2 each yielded E-LDL particles with high C1-activating efficiency, and the C1 activation extent was strictly dependent on cholesterol esterase treatment in all cases. When incorporated into vesicles, the lipid fraction of E-LDL, but not of native LDL, triggered C1 activation, and activation correlated with the amount of unesterified cholesterol generated by cholesterol esterase. Whereas treatment of E-LDL particles with human serum albumin reduced their fatty acid content, both cholesterol and unesterified fatty acids were decreased by methyl-β-cyclodextrin, both treatments resulting in dose-dependent inhibition of the C1-activating ability of the particles. Incorporation of linoleic acid into phosphatidylcholine-containing model vesicles enabled them to interact with the C1q globular domain and to trigger C1 activation, and cholesterol enhanced both processes by facilitating incorporation of the fatty acid into the vesicles. Direct evidence that C1q binds E-LDL through its globular domains was obtained by electron microscopy. This study demonstrates that C1 binding to E-LDL particles involves recognition by the C1q globular domain of the unesterified fatty acids generated by cholesterol esterase. The potential implications of these findings in atherogenesis are discussed.

Atherosclerosis is considered to be a chronic inflammatory process (*1*), and it is widely accepted that the arterial inflammation that precedes plaque development is caused by accumulation of low-density lipoproteins (LDL)[1] in the extracellular matrix of the blood vessels (*2*). LDL particles undergo various modifications, including enzymatic degradation, aggregation, and oxidation. Indeed, the arterial intima has been shown to contain a number of oxidative agents and hydrolytic enzymes, including several proteases (chymase, tryptase, plasmin, matrix metalloproteinase), sphingomyelinase, phospholipase A_2, and cholesterol esterase (*3*−*9*). These enzymes are thought to have a role in transforming native LDL particles into the extracellular lipid droplets and vesicles found in the intima during the early steps of atherogenesis. Lipid particles enriched in unesterified cholesterol have been isolated from the human arterial intima (*10*), and these were found to be chemically and structurally similar to the LDL derivative obtained *in vitro* by sequential treatment with trypsin and then with cholesterol esterase (*11*). This enzymatically modified form of LDL was termed E-LDL (*3*). Evidence for the presence in atherosclerotic lesions of particles resembling E-LDL has also been provided using immunohistochemistry (*12*).

A number of studies also support the hypothesis that oxidative, rather than enzymatic, modifications are responsible for the generation of LDL derivatives endowed with atherogenic properties (*13*−*15*). It has been proposed that these two types of alteration possibly do not compete but rather complement each other, with E-LDL being involved in the early steps of atherosclerosis and oxidation playing a role in disease progression (*16*).

The complement system, a major component of innate defense against pathogens, is also emerging as an important factor in atherosclerosis. The terminal C5b-9 complement complex was shown to be present in atherosclerotic lesions (*17*, *18*). The complement-activating properties of LDL derivatives were initially described by Seifert et al. (*19*, *20*), who demonstrated that a specific cholesterol-containing lipid particle present in human atherosclerotic lesions activates this system to completion. Additional evidence that LDL deposited in the subendothelium is enzymatically converted to a complement activator was provided by Torzewski et al. (*12*). The ability of the various LDL forms to activate complement was investigated in detail by Bhakdi and coworkers, who reported that, unlike native LDL and oxidized LDL, E-LDL has the ability to activate complement both in a CRP-dependent manner and directly (*21*).

Triggering of the classical complement pathway results from binding of the C1 complex, via its recognition subunit C1q, to immune and nonimmune activators and leads to activation of its associated proteases C1r and C1s. We recently provided the first experimental evidence that E-LDL efficiently activates C1 under

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[1]Abbreviations: ApoB-100, apolipoprotein B-100; CEase, cholesterol esterase; C1q GR, C1q globular region; E-LDL, enzymatically modified low-density lipoprotein; HSA, human serum albumin; LA, linoleic acid; LDL, low-density lipoprotein; MBCD, methyl-β-cyclodextrin; PC, phosphatidylcholine.

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conditions close to the physiological situation and that C1q binds E-LDL particles with high affinity (*22*). The objectives of the present study were to analyze the modifications required to generate E-LDL particles endowed with the ability to activate C1 and to identify the molecular component(s) of E-LDL recognized by C1q.

EXPERIMENTAL PROCEDURES

Reagents. Human plasmin, chymase, tryptase, kallikrein, thrombin, matrix metalloproteinase-2, bovine trypsin, soybean trypsin inhibitor, sphingomyelinase from *Bacillus cereus*, phospholipase A_2 from porcine pancreas, cholesterol, cholesteryl docosahexaenoate, cholesteryl linoleate, cholesteryl palmitate, cholesteryl arachidonate, cholesteryl oleate, fatty acids, fatty acid-free human serum albumin, and methyl-β-cyclodextrin were obtained from Sigma. LDL and apolipoprotein B-100 were from Calbiochem. Cholesterol esterase from *Candida cylindracea* was purchased from Saikagaku Corp. C1 inhibitor was purified from human plasma as described previously (*23*, *24*).

C1 Subcomponents. The C1q recognition subunit of C1 and the proenzyme form of C1s−C1r−C1r−C1s were purified from human plasma using previously described methods (*23*, *25*, *26*). The C1q globular domain was obtained by collagenase digestion as described previously (*27*). The concentrations of purified C1q and C1s−C1r−C1r−C1s were determined spectrophotometrically using values of A (1%, 1 cm) at 280 nm of 6.8 and 13.5, and M_r values of 459300 and 330000, respectively. The homogeneity of the purified proteins was assessed by SDS−PAGE analysis under reducing and nonreducing conditions.

LDL Purification. Human LDL was isolated from plasma of healthy volunteers by a two-step gradient ultracentrifugation method (*28*), dialyzed extensively against 20 mM Hepes, 150 mM NaCl, and 2 mM $CaCl_2$, pH 7.0 (Hepes buffer), and stored under nitrogen at 0 °C for a maximum of 3 weeks. The amounts of LDL are expressed in terms of protein. Protein concentration was determined using the QuantiPro BCA assay kit from Sigma.

Generation of E-LDL Particles. Enzymatically modified LDL (E-LDL) was prepared by sequential treatment of native LDL with a protease and with cholesterol esterase (CEase). Different proteolytic enzymes (plasmin, matrix metalloproteinase-2, tryptase, chymase, kallikrein, thrombin) were tested, in addition to trypsin which was used routinely. LDL (1 mg/mL) was first treated with 20 μg/mL protease in the Hepes buffer at 37 °C for 2 h and then with 20−320 milliunits/mL CEase at 37 °C for different periods as indicated. Prior to CEase treatment, trypsin, plasmin, and kallikrein were inhibited with 80 μg/mL soybean trypsin inhibitor; chymase, thrombin, and tryptase were incubated with 5 mM diisopropyl phosphorofluoridate for 30 min at 37 °C; matrix metalloproteinase-2 was blocked by addition of 5 mM EDTA. EDTA and diisopropyl phosphorofluoridate were removed by dialysis prior to measurement of the C1-activating ability of the corresponding E-LDL preparations.

In some experiments, LDL (1 mg/mL) was treated with 0.1 unit/mL sphingomyelinase from *B. cereus* in 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 5 mM $MgCl_2$, pH 7.4, at 37 °C for 18 h or with 60 milliunits/mL phospholipase A_2 from porcine pancreas at 37 °C for 18 h in the presence of 2% (w/v) BSA in 10 mM Hepes, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 140 mM NaCl, pH 7.4 (*29*).

Characterization of LDL Derivatives. Degradation of the ApoB-100 moiety of LDL after protease treatment was monitored by SDS−glycerol−polyacrylamide gel electrophoresis (*30*). The morphology and size distribution of the E-LDL particles were visualized by electron microscopy using the negative staining technique (*31*). Briefly, samples (3−4 μL) were applied to carbon-coated grids, then an equal amount of stain (either 1% sodium silicotungstate, pH 7.4, or 2% uranyl acetate, pH 4.5) was added, and the grids were allowed to dry in air. Observation was performed on a Philips CM12 transmission electron microscope, and images were recorded on films at a magnification of 45000×.

Lipid Composition Analysis. The lipid fractions from LDL and E-LDL samples were extracted using the procedure of Bligh and Dyer (*32*). Unesterified cholesterol, cholesteryl esters, and unesterified fatty acids were fractionated by high-performance thin-layer chromatography on HPTLC silica gel 60 (Merck) using hexane/ether/acetic acid/water (80:18.5:1.2:0.3 v/v) as a solvent (*29*). Unesterified cholesterol and cholesteryl esters were also analyzed by reverse-phase high-pressure liquid chromatography (*33*) on a 3.9 × 150 mm NovaPak C18 column (Waters) using an isocratic acetonitrile/2-propanol/water (44/54/2 v/v) solvent system and detection at 210 nm. The amounts of cholesterol and of the individual cholesteryl esters were determined from calibration curves obtained with the corresponding commercial standards. Fractions corresponding to unknown compounds were collected and analyzed by mass spectrometry using chemical ionization with ammonia (*33*).

E-LDL Modifications. E-LDL was incubated with varying concentrations of methyl-β-cyclodextrin at 37 °C for 1 h (*29*). The E-LDL content in unesterified fatty acids was decreased as described by Suriyaphol et al. (*34*). Briefly, fatty acid-free albumin at a final concentration of 2%, 4%, or 10% (w/v) was added to 1 mL of 1 mg/mL E-LDL, and samples were incubated for 1 h at 37 °C. Each mixture was then submitted to ultracentrifugation (45000 rpm, 10 h at 10 °C) to isolate the lipoprotein fraction by flotation.

Lipid Vesicles. After extraction of the lipid fraction of native LDL, trypsin-treated LDL, and E-LDL (*32*), the solvent was evaporated under nitrogen, and the lipids were dispersed in 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 1 mM $CaCl_2$, pH 7.4. The mixtures were sonicated for 20 min under nitrogen in a bath sonicator and then passed through a 0.45 μm filter.

Vesicles containing phosphatidylcholine (PC) alone, PC + cholesterol, PC + fatty acids, or PC + cholesterol + fatty acids were prepared by dissolving the corresponding lipids at the indicated ratios in chloroform/methanol (2:1) and then following the same procedure as described above.

C1 Activation Assay. The ability of E-LDL and lipid vesicles to induce C1 activation was measured using an *in vitro* assay as described previously (*22*). Briefly, C1 was reconstituted from purified C1q and proenzyme C1s−C1r−C1r−C1s. The complex (0.25 μM) was incubated in 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 1 mM $CaCl_2$, pH 7.4, for 90 min at 37 °C in the presence of 1 μM C1 inhibitor and various amounts of different activators, as indicated. The extent of activation was measured by SDS−PAGE followed by Western blot analysis using an anti-C1s antibody.

Cosedimentation Analyses. Lipid vesicles containing PC alone, PC:cholesterol (1:2), PC:linoleic acid (1:2), or PC:cholesterol:linoleic acid (1:2:2) mixtures (each 300 μg) were incubated with the C1q globular domain (10 μg) in 100 μL of 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 1 mM $CaCl_2$,

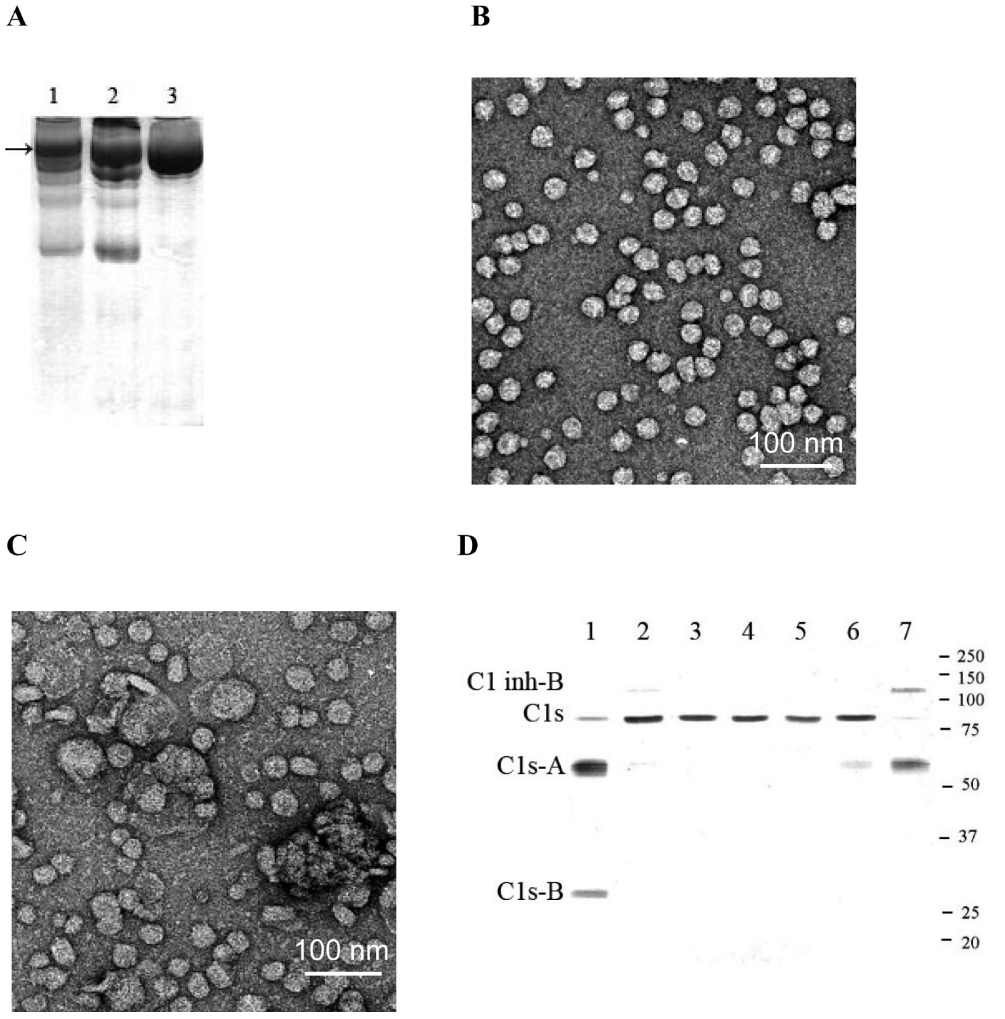

FIGURE 1: Structural and functional characterization of LDL and E-LDL particles. (A) SDS−glycerol PAGE analysis of LDL; lane 1, commercial ApoB-100; lane 2, commercial LDL; lane 3, LDL purified by gradient ultracentrifugation. The arrow indicates the position of ApoB-100. (B) Electron micrograph of negatively stained native LDL. (C) Electron micrograph of negatively stained E-LDL obtained by sequential treatment of native LDL (1 mg/mL) with trypsin (20 μg/mL, 2 h at 27 °C) and then with CEase (320 milliunits/mL, 2 h at 37 °C). (D) Western blot analysis of the C1-activating ability of various LDL and E-LDL samples. Proenzyme C1 (0.25 μM) was reconstituted from C1q and the C1s−C1r−C1r−C1s tetramer and incubated for 90 min at 37 C alone (lane 1) or in the presence of 1 μM C1 inhibitor (lane 2), C1 inhibitor + native LDL (lane 3), C1 inhibitor + LDL preincubated at 37 °C for 2 h (lane 4), C1 inhibitor + CEase-treated LDL (lane 5), C1 inhibitor + trypsin-treated LDL (lane 6), and C1 inhibitor + E-LDL (lane 7). The concentration of all LDL and E-LDL samples was 0.5 mg/mL. Each sample was then submitted to SDS−PAGE analysis under reducing condition, and C1s activation was assessed by Western blot using a polyclonal anti-C1s antibody. C1 activation in the presence of C1 inhibitor leads to the formation of covalent C1 inhibitor−C1s B chain complexes. See Experimental Procedures for more experimental details.

pH 7.4, for 30 min at 22 °C. Samples were centrifuged at 300000*g* for 30 min at 4 °C, allowing separation of the supernatant from the lipid-containing pellet. The GR contents of the supernatant and pellet fractions were determined by SDS−PAGE analysis followed by Western blotting, as described previously (*35*).

RESULTS

In a previous study (*22*) we had obtained indirect evidence that the commercial human LDL preparation used to generate E-LDL in some experiments was possibly oxidized or degraded to some extent. In the present work, all experiments were carried out using purified LDL samples prepared in-house and stored at 0 °C under nitrogen for no longer than 3 weeks. As shown by SDS−glycerol PAGE analysis of the purified LDL preparation (Figure 1A), no significant degradation of ApoB-100 was observed, in contrast to LDL and ApoB-100 preparations obtained from a commercial source. Purified LDL usually contained traces of a low molecular weight protein with an electrophoretic mobility corresponding to that of apolipoprotein E (see Figure 2A). Observation by electron microscopy after negative staining revealed LDL particles relatively homogeneous in size, with an average diameter of about 25 nm (Figure 1B).

Unless otherwise stated, E-LDL particles were obtained by sequential treatment with trypsin and then CEase. As illustrated in Figure 1C, these modifications generated a heterogeneous mixture, containing fused and aggregated particles with a diameter up to 60 nm. A few multilamellar structures (not visible on Figure 1C) were present in E-LDL preparations resulting from overnight treatment with CEase.

A C1 activation assay was used in most cases to probe the interaction between E-LDL and the C1q component of C1, as illustrated in Figure 1D. In keeping with our previous observations (*22*), native LDL and trypsin-treated LDL did not activate C1 to a significant extent. In the same way, CEase had no effect without prior treatment of LDL with trypsin, suggesting that it was ineffective under these conditions (Figure 1D, lane 5).

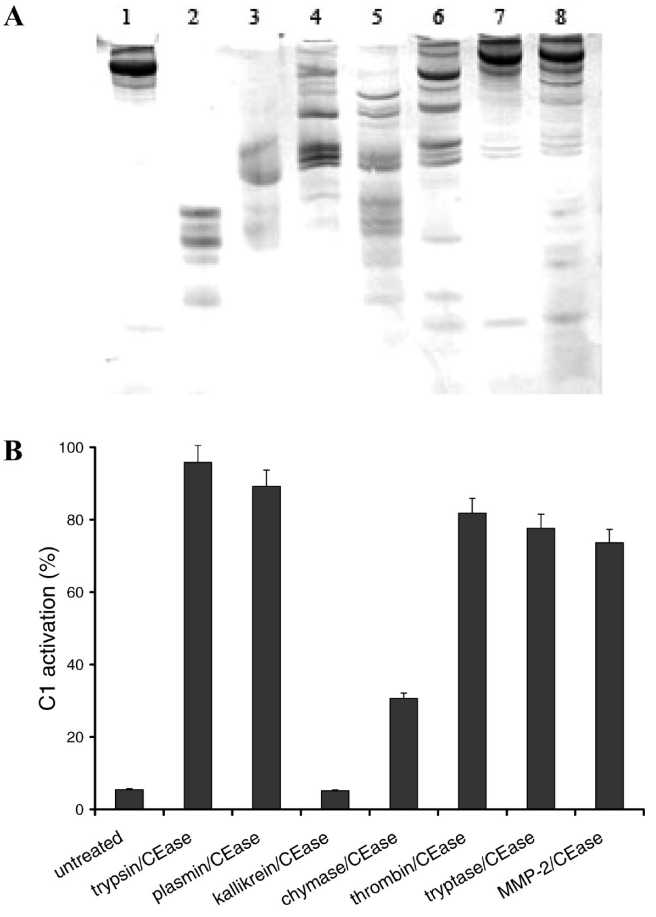

FIGURE 2: Generation of E-LDL particles using selected physiologically relevant proteases. (A) SDS−glycerol PAGE analysis of LDL treatment with various proteases. Native LDL was incubated alone (lane 1) or treated with trypsin (lane 2), plasmin (lane 3), kallikrein (lane 4), chymase (lane 5), thrombin (lane 6), tryptase (lane 7), or matrix metalloprotease-2 (lane 8) and analyzed by SDS−glycerol PAGE under reducing conditions as described under Experimental Procedures. (B) The LDL samples cleaved with the various proteases as described in (A) were then treated with 320 milliunits/mL CEase for 18 h at 37 °C, and their C1-activating ability was assessed by SDS−PAGE analysis as illustrated in Figure 1D and described under Experimental Procedures. The concentration of all samples was 0.5 mg/mL. The data shown represent the mean ± SD of three independent experiments.

In contrast, LDL submitted to trypsin cleavage and then to CEase treatment yielded >90% C1 activation after incubation for 90 min at 37 °C in the presence of excess C1 inhibitor, as shown by the generation of the A and B chains characteristic of activated C1s, the B chain forming a covalent complex with C1 inhibitor (Figure 1D, lane 7).

Generation of E-LDL Particles Using Physiologically Relevant Proteases. We first investigated whether, in addition to trypsin, other proteases known to be present in atherosclerotic lesions could be used in conjunction with CEase to generate E-LDL particles with the ability to activate C1. As shown by SDS−glycerol PAGE analysis (Figure 2A), trypsin, plasmin, kallikrein, chymase, and thrombin all split the ApoB-100 component of LDL to significant extents, whereas tryptase and matrix metalloproteinase-2 had milder effects. After neutralization of their respective protease activity, all samples were subsequently incubated with CEase and then tested for their ability to activate C1. Samples treated initially with plasmin, thrombin, tryptase, and matrix metalloproteinase-2 each displayed high

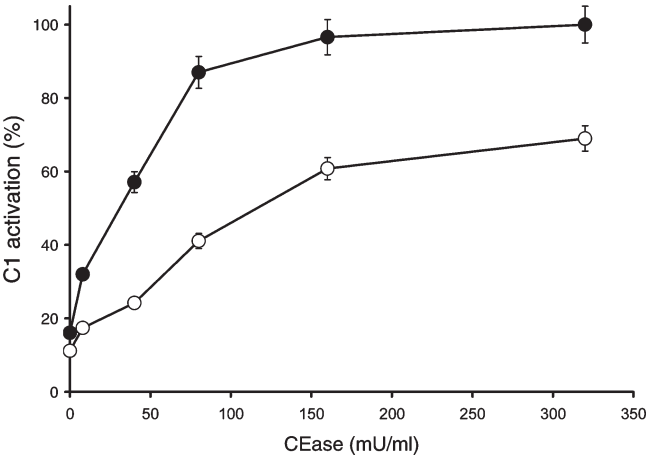

FIGURE 3: Effect of CEase treatment on the ability of E-LDL to induce C1 activation. LDL (1 mg/mL) was first treated with 20 μg/mL trypsin for 2 h at 37 °C and then incubated with varying concentrations of CEase for either 2 h (open circle) or 18 h (closed circle) at 37 °C. The C1 activation ability of the resulting particles was determined as described under Experimental Procedures. The concentration of the samples in the assay was 0.5 mg/mL. The data shown represent the mean ± SD of three independent experiments.

C1-activating ability, comparable to that obtained using trypsin (Figure 2B). In contrast, chymase only induced about 30% activation, and kallikrein had no activating effect. Thus, although initial treatment of LDL with a protease was clearly a prerequisite to generate C1-activating particles, there was no strict correlation between the extent of ApoB-100 degradation and the resulting C1 activation, as best illustrated by the examples of kallikrein and tryptase (Figure 2).

In addition to CEase, other lipases, such as phospholipase A_2 and sphingomyelinase, have been found in the arterial intima (*36*, *37*). We also tested the ability of these enzymes to generate a C1-activating derivative upon incubation with native LDL. Treatment of LDL with either phospholipase A_2 or sphingomyelinase using conditions described under Experimental Procedures did not result in C1 activation (data not shown).

C1-Activating Ability of E-LDL Particles Is Dependent on CEase Treatment. To assess the importance of CEase action in the generation of E-LDL particles endowed with C1-activating ability, native LDL was first submitted to trypsin cleavage and then incubated with varying concentrations of CEase for either 2 or 18 h at 37 °C. As shown in Figure 3, increasing the CEase concentration readily increased the C1-activating ability of the particles under both conditions, resulting in complete C1 activation after incubation for 18 h with 320 milliunits/mL CEase. In a control experiment, no C1 activation was observed when using CEase specifically blocked with diisopropyl phosphorofluoridate (data not shown). Thus, although this treatment was inefficient when applied directly to native LDL (see Figure 1D, lane 5), modification of trypsin-treated LDL with CEase was clearly a determinant step for generating particles exhibiting C1-activating ability.

C1 Recognizes the Lipid Component of E-LDL. To investigate whether recognition by C1q involved the lipid component or the protein moiety of E-LDL, the lipid fractions of native LDL, trypsin-treated LDL, and E-LDL were extracted and used to prepare lipid vesicles which were then tested for their ability to activate C1. As shown in Figure 4A, vesicles containing the lipid fraction of unmodified or trypsin-treated LDL did not yield C1 activation. In contrast, vesicles arising from LDL

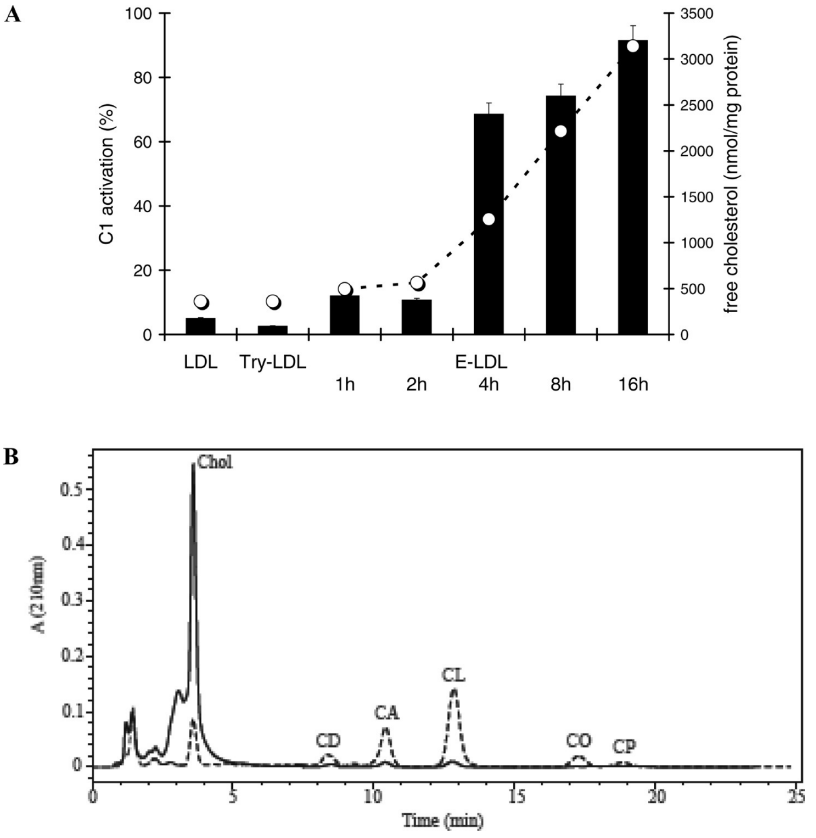

FIGURE 4: C1 activation by E-LDL-derived vesicles and correlation with the amount of unesterified cholesterol generated. (A) LDL (1 mg/mL) was treated with 20 μg/mL trypsin for 2 h at 37 °C and then with 320 milliunits/mL CEase for the indicated periods at 37 °C. The lipid fraction from 50 μg of each sample was extracted and incorporated into vesicles as described under Experimental Procedures. Each vesicle was tested for its C1-activating ability (black bars) as described under Experimental Procedures. The data shown represent the mean ± SD of three independent experiments. The cholesterol content of each lipid fraction (open circles) was determined by reverse-phase HPLC, as illustrated in (B). Key: LDL, unmodified LDL; Try-LDL, trypsin-treated LDL. (B) Reverse-phase HPLC analysis of the lipid fractions of native LDL (dotted line) and of the E-LDL sample obtained after treatment with CEase for 16 h at 37 °C (bold line). Analysis was performed as described under Experimental Procedures, and peaks were detected from absorbance at 210 nm. Unesterified fatty acids were not recovered from the column under the conditions used. The data shown are representative of five independent experiments. Key: Chol, cholesterol; CD, cholesteryl docosahexaenoate; CA, cholesteryl arachidonate; CL, cholesteryl linoleate; CO, cholesteryl oleate; CP, cholesteryl palmitate.

samples first treated with trypsin and then incubated with CEase for different periods progressively developed C1-activating ability, reaching about 90% activation after 16 h. In contrast, the protein fraction left from this sample after lipid extraction did not induce significant C1 activation, and the same negative result was obtained using unmodified or trypsin-treated ApoB-100 (data not shown).

As shown by reverse-phase HPLC (Figure 4B), the lipid fraction of native LDL contained a small amount (398 ± 50 nmol/mg of protein) of unesterified cholesterol, as well as the cholesteryl esters characteristic of LDL (22:6, 20:4, 18:2, 18:1, and 16:0 in average amounts of 21 ± 0.8, 649 ± 109, 824 ± 165, 345 ± 62, and 612 ± 89 nmol/mg of protein, respectively). Overnight treatment with CEase led to almost complete disappearance of the cholesteryl ester peaks, with a concomitant increase of unesterified cholesterol to 3140 ± 160 nmol/mg of protein. Detailed analysis of the lipid fraction of the E-LDL particles generated during progressive incubation with CEase revealed a good correlation between the amount of unesterified cholesterol generated and the extent of C1 activation (Figure 4A). It was concluded from these results that C1q recognized one or more lipid components of E-LDL, the correlation between C1 activation and unesterified cholesterol strongly suggesting that cholesterol itself, or fatty acids, or both of these molecules arising from CEase treatment could be involved in the recognition process.

E-LDL-Induced C1 Activation Is Inhibited by Treatment with MBCD or Human Serum Albumin. To further investigate the nature of the lipid component(s) recognized by C1q, E-LDL was treated with specific reagents with a view to selectively remove cholesterol and unesterified fatty acids. Treatment of the particles with increasing concentrations of methyl-β-cyclodextrin, a reagent known to extract unesterified cholesterol from membranes, progressively abolished their C1-activating ability (Figure 5A). However, parallel analysis of the E-LDL particles by thin-layer chromatography provided clear evidence that MBCD not only depleted cholesterol but also removed unesterified fatty acids, yielding in each case over 70% depletion at an MBCD concentration of 2.25 mM (see Supporting Information). In the next experiment, E-LDL was treated with fatty acid-free human serum albumin (Figure 5B). Again, preincubation with increasing albumin concentrations decreased the C1 activation ability of the particles in a dose-dependent fashion, resulting in >60% inhibition when E-LDL was treated with 10% (w/v) albumin. This value is much higher than the expected concentration of fatty acid-free albumin in human plasma, precluding significant interference with the C1 activation process. Analysis by thin-layer chromatography revealed that the C1-activating ability of the samples roughly correlated with the extent of unesterified fatty acid removal, with 2%, 4%, and 10% HSA yielding 35.4%, 67.1%, and 76.5% depletion, respectively.

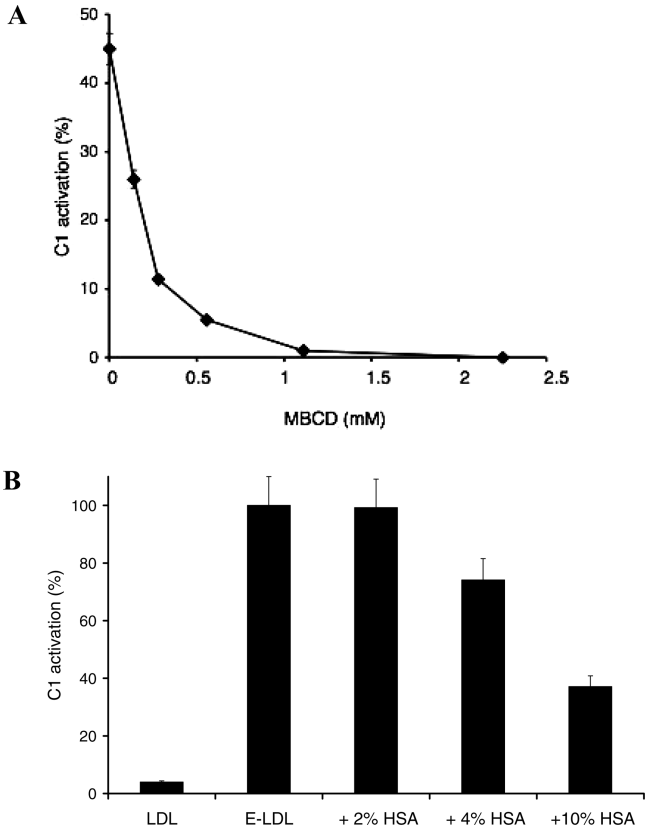

FIGURE 5: Inhibition of E-LDL-induced C1 activation by methyl-β-cyclodextrin and human serum albumin. (A) E-LDL particles (0.125 mg/mL) were treated with increasing concentrations of methyl-β-cyclodextrin (MBCD), and their C1-activating ability was measured as described under Experimental Procedures. (B) E-LDL particles (0.5 mg/mL) were incubated with increasing concentrations of fatty acid-free human serum albumin, and their C1-activating ability was measured as described under Experimental Procedures. Key: LDL, native LDL. The data shown in (A) and (B) represent the mean ± SD of three independent experiments.

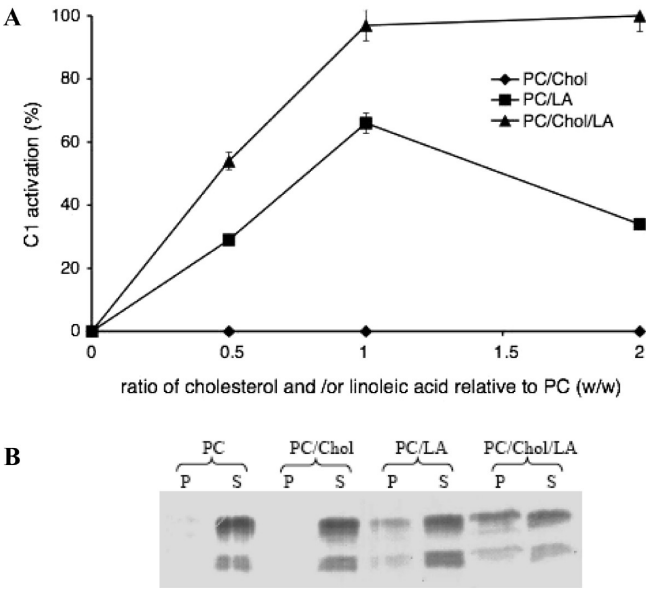

FIGURE 6: (A) C1 activation by cholesterol- and linoleic acid-containing vesicles. Vesicles were prepared from PC alone, PC + cholesterol, PC + linoleic acid, or PC + cholesterol + linoleic acid and tested for their C1-activating ability as described under Experimental Procedures. The values on the *x*-axis represent the w/w ratios of cholesterol alone, linoleic acid alone, or both cholesterol and linoleic acid relative to PC. For the ternary mixtures, a value of 2 corresponds to a cholesterol:linoleic acid: PC ratio of 2:2:1. The data shown represent the mean ± SD of three independent experiments. (B) Cosedimentation analysis of the interaction between C1q GR and vesicles prepared from PC, cholesterol, and linoleic acid. Vesicles were prepared from PC alone, cholesterol:PC (2:1 w/w), linoleic acid:PC (2:1 w/w), and cholesterol:linoleic acid:PC (2:2:1 w/w). Each vesicle was allowed to interact with the C1q GR. After ultracentrifugation, the pellet and supernatant fractions were separated, and their relative C1q GR content was assessed by SDS−PAGE analysis as described under Experimental Procedures. The data shown are representative of three independent experiments. Key: Chol, cholesterol; LA, linoleic acid; P, pellet; S, supernatant.

Cholesterol was not removed under these conditions. Taken together, these data provided strong indication that the unesterified fatty acids generated upon CEase treatment were responsible for C1 binding to E-LDL particles.

C1q Binds to Linoleic Acid-Containing Vesicles through Its Globular Domain. To verify the above hypothesis, lipid vesicles were prepared from PC and increasing amounts of unesterified cholesterol and/or linoleic acid and then tested for their C1 activation potential (Figure 6A). Vesicles containing PC alone or increasing cholesterol:PC ratios did not induce C1 activation. Addition of increasing amounts of linoleic acid initially increased the C1-activating ability of the vesicles, yielding about 65% activation at a 1:1 linoleic acid:PC ratio, and then had the opposite effect at a 2:1 ratio. In contrast, addition of both linoleic acid and cholesterol readily and dose-dependently increased the ability of the vesicles to activate C1, yielding complete activation at a 2:2:1 linoleic acid:cholesterol: PC ratio.

To further investigate this question, lipid vesicles containing PC + cholesterol, PC + linoleic acid, or PC + cholesterol + linoleic acid were prepared at a 2:1 cholesterol and/or linoleic acid ratio relative to PC. After incubation of these vesicles with the C1q globular domain (GR), interaction was assessed by cosedimentation analysis, from the relative amount of C1q GR associated with the vesicles in the ultracentrifugation pellet (Figure 6B). No significant binding to vesicles prepared from PC alone or PC + cholesterol was observed, the whole C1q GR population being found in the supernatants in both cases. Using vesicles prepared from PC and linoleic acid, about 20% of the C1q GR molecules were found in the pellet fraction, indicating slight but significant binding. This value increased to 46% when, in addition to PC, both cholesterol and linoleic acid were present. Analysis of the pellets by thin-layer chromatography revealed that incorporation of linoleic acid into the vesicles was much more efficient in the presence of cholesterol. Thus, when added alone, only 13.8% of the linoleic acid molecules were incorporated into the vesicles, this value increasing to 52.1% when cholesterol was present. It was concluded from these observations that linoleic acid was the only ligand recognized by C1q, the enhancing effect of cholesterol being due by its ability to facilitate incorporation of the fatty acid into the vesicles. In addition, it was clear from the cosedimentation experiments that interaction was mediated by the C1q GR moiety.

Electron Microscopy Analysis of E-LDL-Bound C1q Molecules. Negative staining electron microscopy was next used to visualize C1q binding to E-LDL particles (Figure 7). Some C1q molecules not bound to E-LDL particles, exhibiting the typical bouquet-like shape with six globular domains attached to a stalk, could be recognized (see a representative example in Figure 7D). Bound C1q molecules were clearly

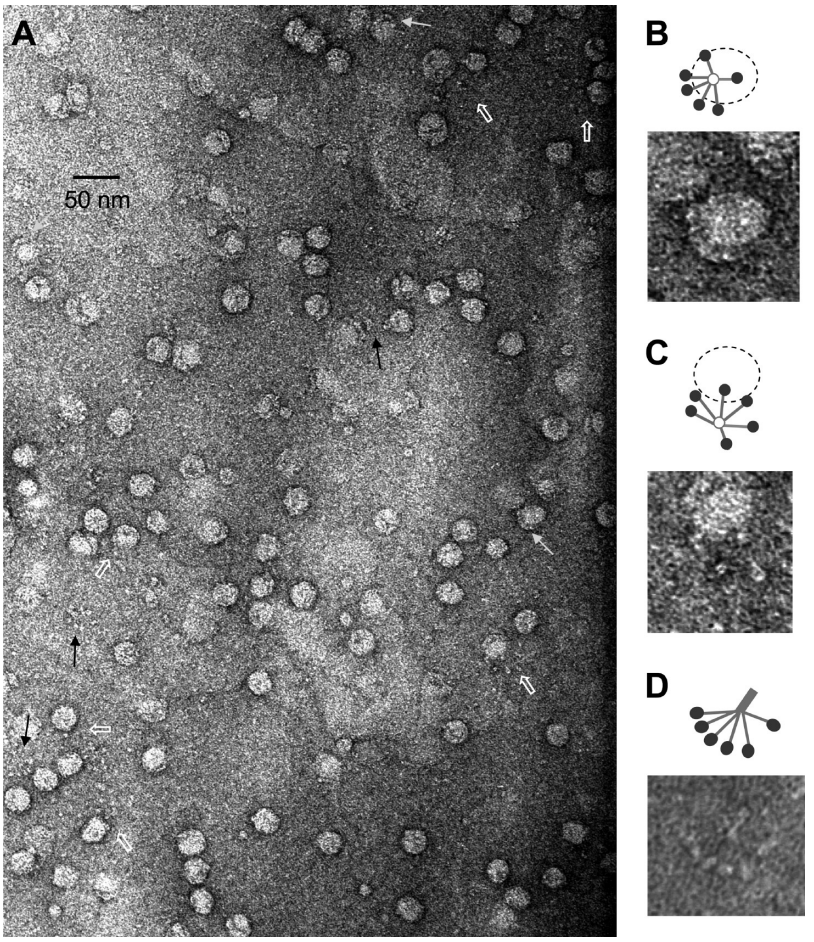

FIGURE 7: Electron micrographs of E-LDL-bound C1q molecules. E-LDL (1 mg/mL) was preincubated with C1q (1 mg/mL) for 30 min at 37 °C in 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 1 mM $CaCl_2$, pH 7.4. Samples were diluted 100-fold, applied to the grid, and stained with 2% uranyl acetate. (A) Overall view. White arrows indicate examples of bound C1q molecules with their globular heads following the curvature of the E-LDL particles (see detailed view in B). Open white arrows indicate examples of C1q molecules bound through only a few heads (see detailed view in C). Black arrows indicate examples of C1q molecules lying close to a particle, possibly detached during sample preparation, or molecules not bound to a particle. (D) Representative example of a free C1q molecule.

observed to interact with E-LDL particles through their globular heads. In some cases, most of the globular heads of C1q were found to follow the curvature of the particles, as illustrated in Figure 7B. Other C1q molecules bound to the particles through only a few heads (Figure 7C). We also observed C1q molecules not bound to E-LDL particles but concentrating in large non-vesicle structures, possibly corresponding to lipids released from the enzymatic treatment.

DISCUSSION

The major objectives of this study were to analyze at a molecular level the mechanisms that allow E-LDL particles to bind and trigger activation of the C1 complex and, more particularly, to identify the E-LDL ligand(s) recognized by its C1q subunit. The various approaches used in this study all concur in the conclusion that C1 binding to E-LDL involves recognition by C1q of the unesterified fatty acids generated upon CEase treatment. This conclusion is supported by the following observations: (i) The extent of E-LDL-induced C1 activation was shown to be highly dependent on CEase treatment. (ii) C1 activation was triggered by vesicles prepared from the lipid fraction of E-LDL particles and correlated with the amount of unesterified cholesterol generated by CEase. (iii) E-LDL-induced C1 activation could be inhibited by depletion of unesterified fatty acids. (iv) Incorporation of linoleic acid into PC-containing model vesicles enabled them to both interact with C1q GR and trigger C1 activation.

Generation of E-LDL particles endowed with C1-activating ability required sequential treatment of native LDL with a protease and then with CEase. Although trypsin was used routinely for this purpose, several proteolytic enzymes (plasmin, tryptase, thrombin, matrix metalloprotease-2) known to be present in the atherosclerotic lesions (*6*, *7*, *38*, *39*) were also able to generate E-LDL particles with high C1-activating ability. This is in agreement with a previous report by Bhakdi et al. (*21*) showing that proteinase K, cathepsin H, and plasmin each generate E-LDL particles with similar complement-activating properties. Considering that CEase itself is also present in the arterial intima (*11*, *40*, *41*), these observations lend credit to the hypothesis that E-LDL-like particles endowed with the ability to trigger C1 activation are indeed generated *in vivo*. Consistent with this hypothesis, it should be emphasized that, as shown by electron microscopy, extensive hydrolysis of cholesteryl esters by CEase generated large, irregularly shaped, liposome-like particles, structurally similar to those previously described by Chao et al. (*11*) and strikingly reminiscent of the unesterified cholesterol-rich lipid particles observed in atherosclerotic lesions (*10*, *42*−*44*). It is also interesting to note that treatment of native LDL particles with sphingomyelinase or phospholipase A_2,

two lipolytic enzymes also found in the arterial intima (*4*, *5*, *8*), did not elicit C1 activation. Thus, in contrast to cholesteryl ester cleavage, hydrolysis of the phosphatidylcholine and sphingomyelin molecules located in the outer shell of LDL has no impact on C1 binding.

Prior treatment of LDL with a protease was clearly a prerequisite to allow efficient hydrolysis of cholesteryl esters, providing strong indication that proteolysis of ApoB-100 facilitates access of these molecules to CEase. Nevertheless, there was no strict correlation between the overall extent of ApoB-100 degradation, as judged from SDS−PAGE analysis, and the C1-activating ability of the resulting E-LDL particle. However, it should be emphasized that SDS−PAGE only gives a rough account of the extent of LDL degradation by a protease. In addition, the ability of CEase to hydrolyze cholesterol esters likely varies depending on the protease used. Although this apparent discrepancy remains to be fully resolved, the most plausible hypothesis is that proteolytic cleavage of LDL disorganizes ApoB-100, as suggested previously (*11*). Since ApoB-100 covers a large area of the LDL particle (*45*), this would allow CEase to gain access to the underlying hydrophobic core containing cholesteryl esters and triglycerides, resulting in the generation of large amounts of unesterified cholesterol and fatty acid molecules, thereby allowing recognition of the latter by the C1q moiety of C1.

Considering the large number of unesterified cholesterol molecules generated upon CEase treatment, and in view of previous reports indicating that model liposomes containing a high concentration of unesterified cholesterol trigger complement activation in a C1q-dependent manner (*46*, *47*), it was tempting to speculate that cholesterol could be involved in the recognition process. Our data clearly indicate that although cholesterol increases the ability of linoleic acid-containing vesicles to bind C1q GR and activate C1, this is an indirect effect due to its ability to facilitate incorporation of the fatty acid into the vesicles (Figure 6). These findings are in agreement with previous reports showing that fatty acids incorporate into PC-containing vesicles (*48*) and that mixtures of cholesterol and palmitic acid form nonphospholipid liposomes (*49*). Taking these elements into consideration, a likely explanation for the biphasic C1-activating effect yielded by vesicles formed from PC + linoleic acid (Figure 6A) is that fatty acid molecules do not fully incorporate into the vesicles, thereby competing with the vesicles for interaction with C1, hence the inhibition of C1 activation observed at high linoleic acid:PC ratios. In contrast, the presence of cholesterol increases the capacity of the vesicles to incorporate linoleic acid, hence the dose-dependent relationship observed under these conditions. That cholesterol is not recognized by C1q is indeed consistent with our current view of the structure of the LDL particle, since, in addition to ApoB-100 and phospholipids, the outer shell is known to contain unesterified cholesterol molecules (*50*). Thus, binding to cholesterol would allow C1q to recognize native LDL particles, leading to inappropriate complement activation.

The precise mechanism by which C1q recognizes its E-LDL ligands remains to be fully established at a molecular level. Nevertheless, as shown by our cosedimentation and electron microscopy analyses, C1q binding is clearly mediated by its globular domain, which provides strong indication that, in addition to its numerous ligands already identified (*51*), this domain also recognizes unesterified fatty acids and therefore likely possesses a binding site for their carboxyl group. This is in line with the known ability of C1q to bind a variety of polyanionic molecules, including DNA and heparin (*52*), and consistent with the fact that, due to the generation of multiple unesterified fatty acid molecules on their surface, E-LDL particles turn into polyanions. There is increasing evidence that fatty acids have the ability to trigger cell apoptosis (*53*, *54*), and it has been shown that exposure of endothelial cells to E-LDL results in programmed cell death (*55*). In view of these observations, it is temping to hypothesize that, through its ability to bind fatty acids on E-LDL particles, C1q may prevent or control their proapoptotic effect. Thus, in addition to its established role in the sensing and clearance of apoptotic cells (*35*, *56*), C1q may also regulate fatty acid-induced apoptosis. Considering the possible implication of endothelial cell apoptosis in the atherogenesis process (*55*, *57*), such a property would bear important biological implications.

This study provides further evidence that E-LDL is an additional example of an altered self-component recognized by C1q, further establishing the role of this protein as a major sensor of these elements. It remains to be fully established whether E-LDL-mediated C1 activation contributes to the atherosclerosis inflammatory reaction or results in the removal of E-LDL particles.

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SUPPORTING INFORMATION AVAILABLE

A graph illustrating removal of unesterified cholesterol and fatty acids upon treatment of E-LDL particles with methyl-β-cyclodextrin. This material is available free of charge via the Internet at http://pubs.acs.org.

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