Effects of phospholipase A₂ and its products on structural stability of human LDL: relevance to formation of LDL-derived lipid droplets

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Abstract
Hydrolysis and oxidation of LDL stimulate LDL entrapment in the arterial wall and promote inflammation and atherosclerosis via various mechanisms including lipoprotein fusion and lipid droplet formation. To determine the effects of FFA on these transitions, we hydrolyzed LDL by phospholipase A₂ (PLA₂), removed FFA by albumin, and analyzed structural stability of the modified lipoproteins. Earlier, we showed that heating induces LDL remodeling, rupture, and coalescence into lipid droplets resembling those found in atherosclerotic lesions. Here, we report how FFA affect these transitions. Circular dichroism showed that mild LDL lipolysis induces partial β-sheet unfolding in apolipoprotein B. Electron microscopy, turbidity, and differential scanning calorimetry showed that mild lipolysis promotes LDL coalescence into lipid droplets. FFA removal by albumin restores LDL stability but not the protein conformation. Consequently, FFA enhance LDL coalescence into lipid droplets. Similar effects of FFA were observed in minimally oxidized LDL, in LDL enriched with exogenous FFA, and in HDL and VLDL. Our results imply that FFA promote lipoprotein coalescence into lipid droplets and explain why LDL oxidation enhances such coalescence in vivo but hampers it in vitro. Such lipid droplet formation potentially contributes to the pro-atherogenic effects of FFA.

Supplementary key words lipoprotein fusion and rupture • free fatty acids • lipoprotein oxidation • albumin • thermal stability • atherosclerosis

In atherosclerosis, LDL-derived lipids are deposited in the subendothelium of the arterial wall. According to the “response to retention” hypothesis, atherogenesis is initiated upon LDL retention by the arterial proteoglycans and LDL modification by the resident hydrolases and oxidative agents (1–3). These modifications trigger a cascade of pro-inflammatory and pro-apoptotic responses that are caused, in part, by the toxic effects of the oxidized phospholipids and their hydrolytic products such as FFA and lyso-phosphatidylcholine (PC) (4, 5). Hydrolytic and oxidative modifications can also induce LDL aggregation, fusion, and coalescence into lipid droplets, which further enhance LDL retention in the arterial wall (6). LDL-derived small extracellular lipid droplets (30–400 nm) are prominent in early atherosclerotic lesions (7) and are observed in the experimental models of atherosclerosis [(8) and references therein]. Most of the lipids found in fibrous atherosclerotic plaques are present in such droplets [reviewed in (9)]. Moreover, fusion of modified LDL accelerates LDL uptake by arterial macrophages, eventually leading to foam cell formation and progression of atherosclerotic plaques containing large (400–6,000 nm) LDL-derived intracellular lipid droplets (7). Hence, the atherogenic potential of LDL is linked to their propensity to fuse and coalesce into lipid droplets.

Because nonmodified LDL do not fuse under physiologic conditions, modifications such as oxidation, lipolysis, and proteolysis are thought to be prerequisites for lipoprotein fusion [(1–4, 10) and references therein]. The effects of these modifications on LDL aggregation and fusion have been attributed to the packing defects in the particle surface (6, 8), which may result from an imbalance between this surface and the apolar core (12). A similar imbalance leading to lipoprotein fusion and rupture can result from other perturbations such as heating, chemical

Abbreviations: apo, apolipoprotein; CD, circular dichroism; DSC, differential scanning calorimetry; EM, electron microscopy; HSA, human serum albumin; Lp-PLA₂, lipoprotein-associated phospholipase A₂; moxLDL, minimally oxidized LDL; PC, phosphatidylcholine; PLA₂, phospholipase A₂; UV, ultraviolet.

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denaturation, detergents, etc. [reviewed in (13)]. For example, heating leads to irreversible remodeling of LDL into smaller and larger particles; the former resemble small dense LDL and the latter are apparent products of LDL fusion (14). Further heating leads to irreversible rupture of these particles and release of their core lipids that coalesce into droplets; the size and morphology of these droplets resemble the extracellular lipid droplets found in atherosclerotic lesions (7, 14). Hence, heating provides a useful tool to accelerate LDL remodeling and coalescence into lipid droplets and to monitor these transitions in real time.

Surprisingly, in LDL isolated from human plasma, oxidation progressively inhibits heat-induced remodeling and rupture (15). Consequently, contrary to the accepted notion, oxidation per se inhibits rather than promotes LDL remodeling. This prompted us to postulate that fusion and coalescence of oxidized LDL in the arterial wall are facilitated by other factors, such as the enhanced binding of oxidized LDL to the arterial proteoglycans, the imbalance between the FFA generation by lipases and removal by albumin, etc. (15). Here, we test the effects of PC hydrolysis by phospholipase A2 (PLA2) and removal of its products by albumin on heat-induced LDL remodeling, rupture, and lipid droplet formation.

Enzymes from the PLA2 family hydrolyze PCs at the sn-2 position to generate lyso-PC and FFA. Several types of secretory PLA2 (16, 17) and the lipoprotein-associated PLA2 (Lp-PLA2) that preferentially hydrolyses oxidized PCs in LDL (18) provide biomarkers of inflammation and atherosclerosis [reviewed in (19–22)]. Moreover, Lp-PLA2 has emerged as a causative agent of atherosclerosis and as a new therapeutic target (17, 23–27). Lp-PLA2 and phospholipase C (29) are reportedly enriched in the small dense LDL and/or in the electronegative LDL, which may promote fusion of these LDL and contribute to their enhanced pro-atherogenic properties (29).

We hypothesize that the pro-atherogenic properties of PLA2 result in part from the direct effects of its products on LDL fusion and rupture. This hypothesis is based on the effects of PLA2 and its products, lyso-PC and FFA (which promote positive and negative bilayer curvature, respectively), on specific steps in lipid bilayer fusion (30, 31). In addition, PLA2 enzymes can lyse various membranes, including cell membranes in erythrocytes as well as the membranes in various bacteria and viruses (32), which is important for the immune response. We speculate that the ability of PLA2 to promote membrane fusion and lysis may extend to lipoprotein fusion and rupture. This is suggested by studies from Hakala et al. (16) showing that LDL hydrolysis by PLA2 in the presence of arterial proteoglycans causes LDL fusion; in those studies, PLA2 were removed from LDL by using near-physiologic concentrations of albumin (20 mg/ml) in an essentially FFA-free state. Even though albumin is believed to remove most FFA produced upon lipolysis of plasma lipoproteins, excess FFA generated locally can partition into lipoproteins (33), particularly in the acidic environment of atherosclerotic lesions where albumin has impaired ability to remove FFA (34). Here, we test the effects of FFA retained in LDL on the heat-induced lipoprotein fusion and coalescence into lipid droplets. To do so, we compare the effects of PC hydrolysis in native and in oxidized LDL in the presence and in the absence of albumin. The results imply a potentially important role of FFA in the in vivo formation of lipoprotein-derived lipid droplets.

MATERIALS AND METHODS

Isolation of lipoproteins

Human lipoproteins from five healthy volunteers were used. Plasma was donated at a blood bank in compliance with the Institutional Review Board protocols and with written consent obtained from the volunteers. Single-donor lipoproteins were isolated from fresh EDTA-treated plasma by KBr density gradient ultracentrifugation in the density range 0.94–1.006 g/ml for VLDL, 1.019–1.063 g/ml for LDL, and 1.063–1.21 g/ml for HDL (35). Lipoproteins from each class migrated as a single band on the agarose gel and on the nondenaturing gel. Lipoprotein stock solutions were dialyzed against buffer A (10 mM Na phosphate buffer, 0.25 mM EDTA, 0.02% NaN3, pH 7.5), degassed, and stored in the dark at 4°C. The stock solutions were used within 2 weeks during which no protein degradation was detected by SDS PAGE and no changes in the net charge were observed on the agarose gel. Protein concentration was determined by a modified Lowry assay.

Preparation and characterization of lipoproteins hydrolyzed by PLA2

Lipoprotein solutions (3 mg/ml protein concentration) were dialyzed against buffer B (10 mM Tris, pH 7.5) and were incubated with porcine pancreatic PLA2 (Sigma) in buffer B containing 2 mM CaCl2 for 12 h at 37°C following established protocols (36). To obtain lipoproteins hydrolyzed to stage 1, 2, or 3, we used 0.05, 0.5, or 5 μg of PLA2, respectively. The reaction was stopped by adding EDTA to a final concentration of 15 mM. To remove FFA from the lipoproteins, 20 mg/ml of essentially fatty acid-free human serum albumin (HSA; Sigma) was included in some incubation mixtures (16); this albumin concentration corresponds to the average value found in the interstitial fluid of the arterial intima (37). The lipoproteins were resolated by ultracentrifugation. The complete removal of albumin from the lipoproteins with which it was coincubated was confirmed by SDS PAGE (see Fig.1B). Lipoproteins from the same plasma pool were subjected to identical incubation and resolation procedures but without PLA2 to assess the effects of spontaneous hydrolysis at 37°C, i. e., hydrolysis in the absence of exogenous PLA2; such hydrolysis results from the hydrolytic activity of apolipoprotein (apo)B (38, 39) and the LDL-associated Lp-PLA2. Such spontaneously hydrolyzed LDL (marked S) as well as those hydrolyzed to stages 1–3 by PLA2 (marked by the stage number) were dialyzed against buffer A for further studies.

LDL enrichment with exogenous oleic acid

Sodium oleate (>99% purity, from Sigma) was used. Native LDL (2 mg/ml protein) and a freshly prepared emulsion of oleic acid (8 mM) in 10 mM Na phosphate buffer, 250 mM EDTA, were coincubated at 37°C for 4 h or 12 h. Unbound oleic acid was removed by gel filtration using Superose 6 10/300 GL column by elution in buffer A at a flow rate of 0.5 ml/min. The final concentrations of oleic acid incorporated into LDL, which were determined by quantitative TLC analysis, were 1.5 mM after 4 h and 3 mM after 12 h of incubation, a significant enrichment as compared with unmodified LDL (0.065–0.01 mM).
Oxidation of LDL

LDL minimally oxidized by Cu^{2+} (moxLDL) were obtained following established protocols [(15) and references therein]. Briefly, LDL solution (0.1 mg/ml protein) was incubated with 5 μM CuSO₄ at 37°C in buffer B for 1 h. The reaction was quenched by adding EDTA to a final concentration of 250 mM, followed by cooling to 4°C and dialysis against buffer A. LDL oxidation under these conditions corresponds to the end of the lag phase during which the core antioxidants are consumed (as monitored by visible absorbance spectra of core carotenoids, such as those shown in supplementary Fig. II A) and the beginning of the propagation phase during which conjugated dienes are produced [as monitored by absorbance at 234 nm (15, 40)]. Mox-LDL prepared by this method showed no protein fragmentation by SDS PAGE, no significant changes in the apoB conformation by circular dichroism (CD) spectroscopy (15), and no changes in lipid composition by TLC (see Fig. 8C). Hydrolysis of moxLDL by PLA₂ to stage 1 (1-moxLDL) was done as described for native LDL in the absence or in the presence of 20 mg/ml FFA-free albumin to produce minimally oxidized and hydrolyzed FFA-free LDL (1-moxLDL-HSA). The LDL modified by these methods were reisolated by density gradient centrifugation, dialyzed against buffer A, and used for stability studies.

Gel electrophoresis

SDS PAGE was performed using a 4–20% gradient system. The gels were run at 150 V for 2 h and stained with Denvile Blue protein stain. Agarose gels were performed using a TITAN lipoprotein gel electrophoresis system. LDL samples containing 4 μg protein were loaded on the precast gels that were run using barbital-sodium barbital buffer at 60 V for 40 min and at 125 V for 7 min. The gels were dried at 70°C for 20 min, stained with 0.1% w/v Fat Red 7B stain in 95% methanol, destained in 75% methanol, and dried at 70°C.

Lipid analysis

Native and hydrolyzed LDL were analyzed by TLC for total lipid composition and by GC for FFA composition. The total amount of FFA was within the range reported for normal human plasma LDL (41). The lipids were extracted by the Folch method (42) with 2:1 chloroform:methanol and were dried under nitrogen. For TLC, known amounts of dry lipids were analyzed using hexane:ether:acetic acid (70:30:1) to separate apolar lipids, or hexane:ether:acetic acid (65:25:4:1) to separate lipoprotein moieties that reportedly show induced CD at these wavelengths [(14) and references therein]. Furthermore, heat-induced increase in the particle size due to fusion, rupture, and coalescence into lipid droplets was monitored by turbidity (that is proportional to the dynode voltage, V, recorded in CD experiments) or by right-angle light scattering [that was recorded by using total fluorescence accessory in the spectropolarimeter as described (45)]. The CD, turbidity, and light scattering melting data were recorded at the same wavelength from lipoprotein samples that were heated at a rate of 11°C/h. Far-UV CD data were normalized to protein concentration and expressed as molar residue ellipticity, [θ]_θ.

Differential scanning calorimetry

The heat capacity C_p(T) was recorded using an upgraded MC2 microcalorimeter (MicroCal, Amherst, MA) as described (14). The data were recorded from LDL solutions (2 mg/ml protein in buffer A) during heating from 5 to 110°C at a rate of 90°C/h under N₂ pressure of 40 psi. The buffer baselines were subtracted from the data. ORIGIN software was used for the data collection and analysis.

EM and adsorption and fluorescence spectroscopy

Lipoproteins were visualized by negative staining electron microscopy (EM) using a CM12 transmission electron microscope (Philips Electron Optics) as described (14, 15). PHOTOSHOP and EXCEL software were used for the analysis of particle size distribution; 200–250 particles were used for such an analysis. Absorption spectra of normal and hydrolyzed LDL (0.5 mg/ml protein in buffer A) were recorded at 25°C using Varian Cary-300 UV/Vis spectrophotometer. Fluorescence emission spectra were recorded of LDL solutions (0.05 mg/ml protein in buffer A) at 25°C using a Fluoromax-2 spectrofluorimeter. The spectra were recorded from 320–500 nm with excitation at 280 nm and 5 nm bandwidth for excitation and emission.

All experiments in this study were repeated 3–5 times to ensure reproducibility.

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and a positive peak at 195 nm characteristic of the mixture of α-helix and β-sheet in apoB (14, 46). LDL hydrolysis by PLA₂ led to a reduction in spectral intensity, indicating partial unfolding of the secondary structure. Removal of FFA by albumin led to further reduction in CD intensity and changes in spectral shape indicating additional unfolding. The secondary structure that was lost was assessed from the difference between the CD spectra of native LDL and that have been hydrolyzed to stage 1 and albumin-treated, (0)–(1·HSA) (Fig. 3, insert). This difference spectrum showed a negative peak at 218 nm and a positive peak at 197 nm, which is characteristic of the β-sheet. Similar CD changes were observed in the spontaneously hydrolyzed LDL (supplementary Fig. I A). Hence, mild LDL hydrolysis and FFA removal lead to a progressive unfolding of the β-sheet structure in apoB.

Near-UV/vis absorption spectra were recorded to test for possible chemical modifications produced by PLA₂ in the apolar core of LDL. The signal from carotenoids did not change upon hydrolysis to stages 1–3, suggesting an intact LDL core (supplementary Fig. II A). Furthermore, intrinsic Trp fluorescence spectra showed that LDL hydrolysis to stages 1–3 did not affect the wavelength of maximal

**RESULTS**

**Biochemical characterization of hydrolyzed LDL**

Agarose gel was used to assess the net charge on LDL hydrolyzed to various stages (Fig. 1A). Spontaneous hydrolysis at 37°C or hydrolysis by PLA₂ to stage 1 produced no changes in the net charge on LDL (lanes S and 1, Fig. 1A). However, hydrolysis to stage 3 induced an increase in the net negative charge on LDL, which resulted from the generation of large amounts of FFA (lane 3).

To test whether lipoprotein coincubation at 37°C with PLA₂ led to proteolysis, lipoproteins hydrolyzed to stages 1–3 were subjected to SDS PAGE. At 0.05 μg PLA₂ (lane 1-HSA, Fig. 1B), no apoB proteolysis in LDL was detected, yet at 0.5–5 μg PLA₂, apoB showed progressive fragmentation (lanes 2-HSA and 3-HSA, Fig. 1B). Because our main focus was on the effects of PC hydrolysis, most of the data reported here pertain to lipoproteins hydrolyzed to stage 1 to minimize the apo lipoprotein modifications.

TLC was used to assess total lipid composition in LDL that were either native or hydrolyzed to stages 1 to 3. Quantitative analysis clearly showed a progressive reduction in PC content with a concomitant increase in lyso-PC and FFA upon hydrolysis (Fig. 1C). The latter was further confirmed by GC, which was used to analyze fatty acid composition in native LDL and in LDL that have been hydrolyzed upon 12 h incubation at 37°C, either spontaneously or in the presence of 0.05 to 5 μg PLA₂ (0, S, 1, and 3, Fig. 2). An increase in FFA upon spontaneous hydrolysis was observed with further progressive increase upon increasing PLA₂ concentration (Fig. 2).

**Effects of mild hydrolysis by PLA₂ on the structural transitions in LDL**

The protein secondary structure in LDL was assessed by far-UV CD. Figure 3 shows CD spectra of native LDL (0) and of LDL that have been hydrolyzed to stage 1 (1) and treated by albumin to remove FFA (1-HSA). The spectrum of native LDL showed negative peaks at 208 and 218 nm and a positive peak at 195 nm characteristic of the mixture of α-helix and β-sheet in apoB (14, 46). LDL hydrolysis by PLA₂ led to a reduction in spectral intensity, indicating partial unfolding of the secondary structure. Removal of FFA by albumin led to further reduction in CD intensity and changes in spectral shape indicating additional unfolding. The secondary structure that was lost was assessed from the difference between the CD spectra of native LDL and of LDL that have been hydrolyzed to stage 1 and albumin-treated, (0)–(1·HSA) (Fig. 3, insert). This difference spectrum showed a negative peak at 218 nm and a positive peak at 197 nm, which is characteristic of the β-sheet. Similar CD changes were observed in the spontaneously hydrolyzed LDL (supplementary Fig. I A). Hence, mild LDL hydrolysis and FFA removal lead to a progressive unfolding of the β-sheet structure in apoB.

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**Fig. 1.** Changes in LDL moieties upon PC hydrolysis. LDL were hydrolyzed upon incubation for 12 h at 37°C either spontaneously (S) or in the presence of 0.05 (1), 0.5 (2) or 5 μg/ml PLA₂ (3). (0) stands for native nonmodified LDL, (1-HSA) indicates particles from which FFA have been removed by albumin. A: Net charge on LDL assessed by agarose gel electrophoresis. The migration pattern is very similar for stages 1 and 2. B: Protein modification assessed by SDS PAGE (4–20%, Denville blue stain). C: Lipid composition assessed by thin-layer chromatography. Error bars indicate the standard error or mean from five independent measurements.

**Fig. 2.** Gas chromatographic analysis of FFA in LDL. LDL were native (0) or hydrolyzed spontaneously (S) or in the presence of 0.05 (1) or 5 μg/ml PLA₂ (3). The data show an average of three independent measurements. Error bars indicate the range of deviations among individual measurements.
into lipid droplets. Importantly, Fig. 4F shows that this effect is partly abolished upon removal of the hydrolytic activity. Mild hydrolysis promotes heat-induced LDL rupture and formation of lipid droplets; and this effect is mainly due to FFA because preferential removal of FFA by albumin hampers LDL rupture and reduces the droplet size.

To better characterize the effects of hydrolytic products on the thermal transitions in LDL, we used differential scanning calorimetry (DSC). Figure 6 shows heat capacity data, \( C_p(T) \), recorded during LDL heating from 5–100°C at a rate of 90°C/h. In native LDL, three peaks corresponding to three structural transitions were observed in this (Fig. 6, black) and earlier studies [(14) and references therein]. The peak at near-physiologic temperatures reflects smectic-to-disorder phase transition in the lipid core, which is reversible [(47) and references therein]. Two high-temperature transitions are irreversible.

Fig. 4. Electron micrographs of negatively stained LDL showing combined effects of mild hydrolysis and heating. LDL were native (0) or hydrolyzed by PL\(\alpha\), to stage 1 (1) and albumin-treated (1-HSA). LDL solutions (2 mg/ml protein in buffer A) were heated to 100°C at a rate of 11°C/h. The heating data of native LDL and of LDL that have been hydrolyzed to stage 1 without albumin treatment showed much larger heat-induced increase in the amplitude of near-UV CD and turbidity, suggesting formation of larger lipid droplets (1, Fig. 5). This is consistent with the electron micrographs showing larger lipid droplets formed upon heating of LDL hydrolyzed to stage 1 (Fig. 4E). Spontaneously hydrolyzed LDL showed a similar trend (supplementary Fig. I B, C). Taken together, our EM, CD, turbidity, and light-scattering data clearly show that: i) mild hydrolysis promotes heat-induced LDL rupture and formation of large lipid droplets; and ii) this effect is mainly due to FFA because preferential removal of FFA by albumin hampers LDL rupture and reduces the droplet size.
The secondary structure of apoB in LDL after heating to 100°C was assessed by far-UV CD. The results of this and earlier studies of native LDL (14) showed irreversible thermal unfolding of the predominantly β-sheet conformation (supplementary Fig. IV A). Furthermore, LDL hydrolyzed to stage 1, in which the apoB β-sheets have been largely unfolded upon hydrolysis (Fig. 3), showed predominantly α-helical conformation before heating; these α-helices unfolded irreversibly upon heating to 100°C (supplementary Fig. IV B). These results suggest that the β-sheets in apoB are labile to perturbations such as heating or hydrolysis and unfold prior to the α-helices.

Effect of hydrolysis by hepatic lipase on LDL stability

To test whether FFA produced by lipases other than PLA₂ also reduce LDL stability, we used HL to hydrolyze LDL. Unlike PLA₂, HL hydrolyzes not only PCs in the lipoprotein surface but also triacylglycerides in the core with FFA produced in both reactions (48). Under conditions of our experiments, HL caused lipolysis but did not lead to apoB modification detectable by SDS PAGE (see supplementary data for detail). Such limited hydrolysis significantly reduced LDL stability that was assessed by turbidity in the heating experiments (supplementary Fig. V). Thus, similar to PLA₂, lipolysis by HL destabilizes LDL and promotes lipoprotein rupture.

Effect of enrichment with oleic acid on LDL stability

To test whether incorporation of exogenous FFA in the absence of lipolysis promotes lipoprotein rupture, LDL were enriched with oleic acid as described in the Methods. The agarose gel showed that such enrichment leads to an increase in the net negative charge on LDL (Fig. 7A). EM data showed that this enrichment has no detectable effect on the size and shape of the particle at an ambient temperature prior to heating (Fig. 7B). The heating data recorded by DSC showed that LDL enrichment with oleic acid to a final concentration up to 3 mM leads to a progressive low-temperature shift in the temperature of LDL fusion and rupture (Fig. 7C); the heating data recorded by turbidity showed a similar effect (Fig. 7D), indicating a large reduction in the temperature of LDL rupture and lipid droplet formation. Consequently, similar to FFA produced upon lipolysis, incorporation of exogenous FFA such as oleic acid destabilizes LDL and promotes heat-induced lipid droplet formation.

Effects of mild hydrolysis on LDL rupture assessed from the heating data in CD experiments. LDL were native (0) or hydrolyzed by PLA₂ to stage 1 (1) and albumin-treated (1-HSA). LDL solutions (2 mg/ml protein in buffer A) were heated at a rate of 11°C/h. The melting data were recorded at 280 nm by CD to monitor repacking of apolar lipids (A) and by turbidity (dyne/dvoltage) to monitor increase in the particle size upon rupture and coalescence into lipid droplets (B).

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**Effects of mild hydrolysis on LDL remodeling**

Combined effects of mild LDL oxidation and PC hydrolysis are particularly relevant because oxidized PCs in LDL...
form preferred in vivo substrates for Lp-PLA2 [reviewed in (25)]. To assess such combined effects on LDL stability, we used moxLDL. CD and turbidity melting data showed a small reduction in the amplitude of the rupture transition in moxLDL as compared with native LDL (Fig. 8, solid and dashed black lines), which is consistent with the observation that oxidation progressively hampers LDL remodeling and rupture in vitro (14). Hydrolysis of moxLDL to stage 1 (1-mox) led to a significant reduction in PC and increase in FFA and lyso-PC as assessed by TLC (Fig. 8C, D). Importantly, such hydrolysis led to a large increase in the amplitude of the thermal transition (1-mox-LDL, gray circles in Fig. 8), indicating formation of large lipid droplets which were also observed by EM (data not shown). This enhanced rupture and lipid droplet formation by 1-moxLDL was abolished upon FFA removal by albumin, as evident from the large reduction in the transition amplitude (1-mox-LDL·HSA, gray dotted line in Fig. 8). In summary, similar to nonoxidized LDL, i) hydrolysis of moxLDL to stage 1 greatly enhances lipoprotein rupture and coalescence into large lipid droplets; and ii) this enhancement is due to FFA and is abolished upon FFA removal.

Comparison with other plasma lipoproteins

To test the effect of PC hydrolysis on the heat-induced rupture of other lipoproteins, we analyzed human plasma HDL and VLDL that have been hydrolyzed by PLA2 and treated with albumin. Representative HDL data are shown in Fig. 9. The results reveal that, similar to LDL, HDL hydrolysis to stage 1 leads to a small but significant increase in FFA (Fig. 9A) and significantly increases the amplitude of the heat-induced rupture, indicating formation of larger lipid droplets (line 1, Fig. 9B); this amplitude decreases upon FFA removal by albumin (1-HSA, Fig. 9B), indicating a reduction in the droplet size. In VLDL, hydrolysis by using 0.05 μg/ml PLA2 promoted heat-induced rupture and coalescence into large lipid droplets at ambient temperature (supplementary Fig. VI B), leading to a rapid lipid phase separation that precluded further studies. Further comparisons with other lipoproteins are provided in the supplementary data (supplementary Figs. VA and VI A).
thermore, similar to LDL, VLDL rupture was hampered upon FFA removal by albumin (data not shown). Importantly, similar to LDL (Fig. 1B), hydrolysis of HDL or VLDL to stage 1 produced no apolipoprotein fragmentation or cross-linking detected by SDS PAGE; hence, the only lipoprotein modification observed at this stage was PC hydrolysis. We conclude that, similar to LDL, mild hydrolysis of PC by PLA₂ in HDL or VLDL produces FFA that promote lipoprotein rupture, whereas FFA removal by albumin hampers it.

DISCUSSION

The results reported here reveal that the heat-induced coalescence of LDL into lipid droplets is greatly enhanced upon FFA incorporation and is hampered upon FFA removal by albumin. The underlying mechanism for these effects probably relates to the lytic activity of FFA. Similar effects of FFA were observed in LDL that have been hydrolyzed spontaneously upon incubation at 37°C (supplementary Fig. I B, C), hydrolyzed enzymatically by PLA₂ (Figs. 5, 6) or by HL (supplementary Fig. V), enriched with exogenous oleic acid (Fig. 7), or oxidized (Fig. 8). Hence, the observed effects are not limited to one particular origin of FFA. Furthermore, these effects of FFA are not limited to LDL because similar effects were observed in HDL upon mild hydrolysis by PLA₂ (Fig. 9). Moreover, hydrolysis of VLDL by PLA₂ destabilized the lipoproteins to such an extent that they coalesced into lipid droplets at ambient temperatures without heating (supplementary Fig. VI). Consequently, the ability of FFA to promote lipoprotein rupture and coalescence into lipid droplets is a general phenomenon that is not limited to high temperatures but also applies to near-physiologic temperatures.

The results of our in vitro studies are relevant to physiologic conditions of elevated levels of FFA, which may result from the enhanced lipolysis by enzymes such as PLA₂ and/or impaired removal of the lipolytic products by albumin. For example, albumin’s ability to remove FFA is impaired under acidic conditions in advanced atherosclerotic plaques; under these conditions, LDL retain some of the FFA generated locally upon lipolysis (34). Another example is type-2 diabetes that is characterized by elevated levels of plasma FFA [(49) and references therein]. Our results suggest that these FFA are likely to promote formation of large VLDL- or LDL-derived lipid droplets in atherosclerotic plaques. Furthermore, our results help explain the earlier reports of enhanced lipid droplet formation from oxidized LDL in vivo, even though in vitro studies of isolated LDL showed that their remodeling and droplet formation was impaired upon oxidation (15). We propose that, because oxidized PCs on LDL are preferential substrates of Lp-PLA₂ (18), the hydrolytic products generated by Lp-PLA₂, particularly FFA, provide a driving force for lipid droplet formation by oxidized LDL in the arterial wall.

Finally, our results suggest that LDL remodeling and coalescence into lipid droplets is not significantly affected by the conformational changes in apoB such as irreversible unfolding of the β-sheet structure. Such progressive β-sheet unfolding was detected upon LDL hydrolysis to stage 1 followed by FFA removal (Fig. 3). This result is consistent with the far-UV CD spectra of hydrolyzed LDL reported by Greco et al. (11). Furthermore, LDL heating also leads to preferential unfolding of the β-sheet structure in apoB (supplementary Fig. IV A) (14). Moreover, preferential unfolding of the β-sheet structure in apoB was observed upon progressive oxidation of small VLDL (50). We conclude that various structural perturbations, including PC hydrolysis, oxidation, and thermal denaturation, lead to preferential unfolding of the β-sheet structure in apoB-containing lipoproteins. Hence, even though the amphipathic β-sheet rich domains are believed to irreversibly anchor apoB to the lipid surface and to be more strongly associated with this surface than the α-helix rich domains (51), the β-structure in apoB is more labile and tends to unfold more readily than the α-helical structure.

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REFERENCES

1. Williams, K. J., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. Arterioscler. Thromb. Vasc. Biol. 15: 551–561.
2. Camejo, G., E. Hurt-Camejo, O. Wiklund, and G. Bondjers. 1998. Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. Atherosclerosis. 139: 205–222.
3. Skålén, K., M. Gustafsson, E. K. Rydberg, L. M. Hultén, O. Wiklund, T. L. Innerarity, and J. Borén. 2002. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature. 417: 750–754.
4. Chisolm, G. M., and D. Steinberg. 2000. The oxidative modification hypothesis of atherogenesis: an overview. Free Radic. Biol. Med. 28: 1815–1826.
5. de Winder, M. P., and M. H. Hofker. 2000. Scavenging new insights into atherogenesis. J. Clin. Invest. 105: 1039–1041.
6. Öörni, K., M. O. Pentikäinen, M. Alle-Korpela, and P. T. Kovanen. 2000. Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions. J. Lipid Res. 41: 1703–1714.
7. Guyton, J. R., and K. F. Klemp. 1994. Development of the atherosclerotic core region. Chemical and ultrastructural analysis of microdissected atherosclerotic lesions from human aorta. Arterioscler. Thromb. 14: 1305–1314.
8. De Spirito, M. R. Brunelli, G. Mei, F. R. Bertani, G. Ciasca, G. Greco, M. Papi, G. Arcovito, F. Ursini, and T. Parasassi. 2006. Low density lipoprotein aged in plasma forms clusters resembling subendothelial droplets: aggregation via surface sites. Biophys. J. 90: 4293–4247.
9. Guyton, J. R. 2001. Phospholipid hydrolytic enzymes in a ‘cesspool’ of arterial intimal lipoproteins: a mechanism for atherogenic lipid accumulation. Arterioscler. Thromb. Vasc. Biol. 21: 884–886.
10. Pilihari, R., E. Hurt-Camejo, K. Öörni, and P. T. Kovanen. 2010. Proteolysis sensitizes LDL particles to phospholipolyse by secretory phospholipase A2 group V and secretory sphingomyelinase. J. Lipid Res. 51: 1801–1809.
11. Greco, G., G. Balogh, R. Brunelli, G. Costa, M. De Spirito, L. Lenzi, G. Mei, F. Ursini, and T. Parasassi. 2009. Generation in human plasma of misfolded, aggregation-prone electronegative low density lipoprotein. Biophys. J. 97: 629–635.
12. Liu, H., D. G. Scraba, and R. O. Ryan. 1993. Prevention of phospholipase-C induced aggregation of low density lipoprotein by amphipathic apolipoproteins. FEBS Lett. 316: 27–33.
13. Gursky, D. 2005. Apolipoprotein structure and dynamics. Curr. Opin. Lipidol. 16: 287–294.
