LDL phospholipid hydrolysis produces modified electronegative particles with an unfolded apoB-100 protein

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Abstract  Electronegative low density lipoprotein (LDL−) formation that structurally resembles LDL− isolated from plasma was evaluated after LDL treatment with snake venom phospholipase A2 (PLA2). PLA2 treatment of LDL increased its electrophoretic mobility in proportion to the amount of LDL− formed without evidence of lipid peroxidation. These changes dose-dependently correlated with the degree of phospholipid hydrolysis. Strong immunoreactivity of LDL− subfraction from plasma and PLA2-treated LDL (PLA2-LDL) to amyloid oligomer-specific antibody was observed. Higher β-strand structural content and unfolding proportionate to the loss of α-helical structure of apolipoprotein B-100 (apoB-100) of LDL− isolated from both native and PLA2-LDLs was demonstrated by circular dichroism (CD) spectropolarimetry. These structural changes resembled the characteristics of some oxidatively modified LDLs and soluble oligomeric aggregates of amyloidogenic proteins. PLA2-LDL was also more susceptible to nitration by peroxynitrite, likely because of exposure of otherwise inaccessible hydrophilic and hydrophobic domains arising from apoB-100 unfolding. This was also demonstrated for plasma LDL−. In contrast, PLA2-LDL was more resistant to copper-mediated oxidation that was reversed upon the addition of small amounts of unsaturated fatty acids. The observed similarities between PLA2-LDL−-derived LDL− and plasma LDL− implicate a role for secretory PLA2 in producing modified LDL− that is facilitated by unfolding of apoB-100.

Human plasma contains an electronegative LDL subfraction (LDL−) that possesses atherogenic properties and is associated with increased cardiovascular disease risk (1, 2). Increased levels of LDL− have been observed in hypercholesterolemic subjects (3), type 2 diabetics (4, 5), uremic subjects (6), after exhaustive physical exercise (7, 8), and during postprandial lipemia (9). LDL− is associated with small, dense LDLs and is enriched in lipid peroxides (1, 10). Modification of the protein component of LDL−, apolipoprotein B-100 (apoB-100), with the loss of secondary structure and increased unfolded character, has also been described (11). There are several mechanisms described for LDL− formation, each yielding a particle resembling circulating LDL−. Particles similar to LDL− can be produced in vitro by transition metals or hemoproteins (6, 10), enzymatically by myeloperoxidase (12), lipooxygenase, and lipase (13, 14), and by incubating LDL in the presence of vascular cells (15), particularly under shear stress (16). All of these reactions occur with concomitant lipid peroxidation in LDL particles with levels at times greatly exceeding those found in plasma LDL−. The requirement for lipid peroxidation for the formation of LDL− particles remains in question, because various LDL− particles can contain different amounts of lipid species, notably free fatty acid, oxidized phospholipids, and lysophospholipids, depending on the origin or mechanism of LDL− formation.

Secretory phospholipase A2 (sPLA2) is released into the circulation under inflammatory conditions, where it hy-
hydrolyzes lipoprotein phospholipids at the sn-2 position, releasing free unsaturated fatty acids and generating lysophospholipids. Growing evidence suggests that increased sPLA₂ activity may contribute to atherosclerosis (17, 18). Different members of the sPLA₂ family have been found in plasma (17) and extracellularly in the arterial intima and atherosclerotic lesions (19–21); however, direct reactions with plasma or intimal lipoproteins in vivo have been difficult to assess. In vitro hydrolysis of the surface phospholipids on LDL using nonmammalian low molecular weight PLAs produces physicochemical changes leading to alterations in particle structure as well as the biological function of LDL (17, 18). Spin resonance spectroscopy studies revealed increased surface free cholesterol and sphingomyelin content accompanied by decreased particle fluidity (22). Hydrolysis of 50% of the phospholipids in LDL leads to the formation of smaller, denser particles with increased ability to bind extracellular matrix proteoglycans (23). A related feature of PLAT-treated particles involves the conformational alteration of the protein, apoB-100, that imparts a greater tendency to aggregate (24). It has also been shown that reaction of PLAT with lipoproteins can occur in the presence of plasma (17) and that storage and minimal LDL oxidation induce the ability of PLAT to degrade surface lipids (25).

The aforementioned properties of PLAT-treated LDL suggest a resemblance to in vivo circulating LDL⁻. It was of interest to study whether PLAT treatment of LDL may lead to LDL⁻ formation occurring without evidence of lipid peroxidation.

MATERIALS AND METHODS

Materials

Naja naja venom PLAT was obtained from Sigma Chemical Co. (St. Louis, MO). Amyloid β (Ab) was a generous gift from Dr. Ralph Langen (University of Southern California, Los Angeles, CA). Monoclonal nitrotyrosine antibody was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). An antibody to oligomeric aggregates was produced earlier as described (26). Bovine serum albumin (fatty acid free) and all other reagents were of analytical grade from Sigma.

LDL isolation and treatment with PLAT

LDL was isolated from expired human plasma obtained from the Blood Bank (University of Southern California Hospital). After ultracentrifugation on a density gradient of sodium bromide (27), LDL (d = 1.019–1.063) was collected, desalted using centrifugal filtering devices with a 30 kDa cutoff (Amicon), and equilibrated in 20 mM Tris-HCl, pH 7.8. Aliquots of LDL were incubated for 1 h at 37°C with different amounts of snake venom PLAT in Tris-HCl buffer (pH 7.8) containing 5 mM CaCl₂ in the absence or presence of 0.1% BSA. The enzyme appeared as a single band at ~14 kDa on SDS-PAGE stained by Sypro Ruby, and there was no proteolytic activity associated with it (e.g., LDL⁻apoB-100 after treatment with PLAT was intact; data not shown). In our assays, 1 ng of PLAT generated 1.4 μmol of free fatty acids per minute from 1 μg of LDL protein. Appropriate controls were incubated without PLAT under identical conditions. Thereafter, reactions were stopped by the addition of 10 mM EDTA (final concentration), and samples were cooled on ice and centrifuged using Amicon filters to remove PLAT and salts. Aliquots were taken from reaction mixtures for the measurement of free fatty acid content using a NEFA kit (WAKO). Protein was measured by a modified Lowry method using the ECL kit (Pierce, Rockford, IL). Oligomer staining blots were blocked in 10% dry milk and TBS containing 0.001% Tween. Blots using secondary antibodies only were also used to assess the specificity of antibody staining. Nitrotyrosine and oligomer-specific blots were quantified by densitometry using Scion software (Scion Corp.).

Circular dichroism

Circular dichroism (CD) analysis of chloride anion-free LDL samples (reconstituted in phosphate buffer, pH 7.4) was performed using 100 μg/ml LDL protein. CD spectra were recorded on a Jasco-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at room temperature using a 0.1 cm quartz cuvette in the region of 190–250 nm. At least 10 spectra for each sample were averaged, and blank measurements were subtracted. Secondary structures were analyzed using CDNN CD spectra deconvolution software.

Statistical analysis

At least three separate experiments were performed for each study, all samples being analyzed in triplicate and standard deviations calculated where appropriate. Significance was determined using Student’s t-test and set at P < 0.05.
RESULTS

LDL treatment with PLA₂ results in LDL⁻ formation

LDL treatment with snake venom PLA₂ for 1 h resulted in a dose-dependent increase in the net electronegative charge of the LDL particle (Fig. 1A). In the absence of BSA, the REM of particles was increased from 1 in control to 1.1 after incubation with 5 ng/ml PLA₂ and to 1.4 after treatment with 50 ng/ml PLA₂. The presence of 0.1% BSA slightly inhibited the REM increase (e.g., 1.25 versus 1.4 for treatment with 50 ng/ml PLA₂). Notably, PLA₂-treated LDL displayed smearing on agarose gels tailing down to the starting point (Fig. 1A), indicative of a heterogeneous population of particles with different REMs and possible aggregation. Consistent with the REM data, there was a dose-dependent increase in the LDL⁻ subfraction after incubation with PLA₂ in the presence and absence of 0.1% BSA (Fig. 1B). LDL⁻ levels increased from 1.41 ± 0.96% in controls to 4.3 ± 0.006% and 13.1 ± 4.3% after exposure to 5 and 50 ng/ml PLA₂, respectively. LDL⁻ levels were decreased in the presence of 0.1% BSA, being lower by 12% and 40% after treatment with 5 and 50 ng/ml PLA₂, respectively. The PLA₂ inhibitor quinacrine (100 μM) inhibited LDL⁻ formation by 50% (data not shown), confirming that this LDL⁻ production was attributable to LDL lipolysis. There was no change in the levels of lipid peroxides measured by leukomethylene blue assay before and after incubation with PLA₂. In all samples, lipid peroxides were below the detection limit. Increases in LDL⁻ and REM of PLA₂-treated LDL correlated well with the release of free fatty acids (Fig. 1C).

PLA₂-treated LDL forms specific aggregates and is enriched in β-sheet structures

To study the effect of PLA₂ treatment on the conformational characteristics of LDL-apoB-100, subfractions of PLA₂-treated and control LDL were separated on anion-exchange resin and analyzed by dot blot using oligomer-specific antibody. This antibody specifically recognizes a wide variety of soluble oligomers of amyloidogenic proteins and peptides and is null to fibrillar Aβ as described previously (26). PLA₂-treated LDL and its LDL⁻ subfraction showed significantly increased reactivity to this antibody (Fig. 2A). Isolated LDL⁻ from plasma LDL, accounting for ~1–2% of total LDL (tLDL), also showed very strong positive reactivity to oligomer-specific antibody in contrast to blots of the tLDL, which were negative. LDL⁻ subfractions from native and PLA₂-treated LDL were comparable in terms of reaction to the antibody relative to that of Aβ protein that was used as a positive control. Interestingly, mechanically aggregated LDL (by vortexing LDL for 1 min) had little or no reactivity to the oligomer-specific antibody.

Conformational changes led to increased β-sheet structure formation in PLA₂-treated LDL, confirmed using CD spectropolarimetry. Although all spectra showed mostly the predominance of α-helical structure, there were differences between CD spectra of tLDL, nLDL, and particularly LDL⁻ subfractions (Fig. 2B, C). Consistent with dot blot results, CD spectra showed changes in the regions for both α-helical and β-strand structures. LDL⁻ recovered from native LDL showed a significant decrease in α-helical structure, whereas β-strand structural content increased (Fig. 2B, Fig. 3A). The CD spectra for the LDL⁻ derived from PLA₂-treated LDL was similar to that of LDL⁻ isolated from native plasma-derived LDL (Fig. 2C). nLDL subfractions from both control and PLA₂-treated LDLs had slightly higher α-helical structure content, with little or no reactivity toward the oligomer-specific antibody (data not shown) and correspondingly lower β-strand content than total LDL (Figs. 2B, 3A, B). Analysis of these data using CDNN software showed that LDL⁻ isolated from native LDL or PLA₂-treated LDL was enriched in β-structure content (antiparallel, parallel, β-turn) and particularly in the content of random coils (reaching 45% and 38% versus 4.7% and 12% for total LDL from control and PLA₂-treated LDLs, respectively) (Fig. 3A, B). The shift from α-helical to β-conformations or random coil...
structures suggests increased unfolding of the protein. Furthermore, comparative analysis of CD spectra of LDL particles oxidized by copper or hemoglobin or modified enzymatically by PLA₂ and containing 10% LDL levels also demonstrated protein unfolding with increases for all β-sheet structures (Table 1).

**PLA₂-treated LDL and LDL⁻ are more susceptible to nitration than native LDL**

We then studied whether the conformational changes in PLA₂-treated LDL affected the ability of LDL to be nitrated after exposure to peroxynitrite. The increased nitration was hypothesized to be attributable to exposure of tyrosine residues in lipolyzed LDL-apoB-100 to an environment favorable for nitration. Indeed, incubation of LDLs at 0.2 mg/ml with 50 μM peroxynitrite (1 mol peroxynitrite/1 mol LDL-tyrosine) resulted in significantly higher nitration levels for PLA₂-treated LDL compared with native LDL from plasma (Fig. 4A). Densitometric analyses showed that nitration levels increased by ~34% and 45%, respectively, for treatments using 5 and 50 ng/ml concentrations of PLA₂. LDL⁻ from native LDL displayed a stronger tendency for nitration, representing ~187% of control LDL (Fig. 4A). Simultaneously, levels of lipid peroxidation products measured as MDA were significantly lower for PLA₂-treated LDL, showing an inverse relationship to the nitrotyrosine levels (Fig. 4B). The respective MDA levels in LDL decreased by 30% and 50% after treatment with 5 and 50 ng/ml PLA₂. In contrast, MDA levels in plasma-derived LDL were increased; however, this LDL usually contains substantially more lipid peroxides (1) that can trigger even higher levels of peroxidation after the reaction with peroxynitrite. Finally, BSA at 0.1% inhibited both the nitration and the formation of reactive aldehydes by peroxynitrite for both native- and PLA₂-treated LDL (data not shown).

**PLA₂-treated LDL is resistant to copper-mediated oxidation**

One of the most prominent features of plasma LDL⁻ is its increased oxidizability (1). The conformational and compositional modifications found in LDL that becomes converted to LDL⁻ after PLA₂ treatment were anticipated to increase oxidative susceptibility. The increased oxidative susceptibility of LDL to transition metal-catalyzed ox-
oxidation has been previously shown to be proportional to the content of LDL− particles (10). When PLA2-treated LDL was subjected to copper-mediated oxidation, there was an increase in LDL− levels (Fig. 1); however, the oxidation lag period for copper-mediated oxidation was significantly delayed (Fig. 5A) compared with control LDL. The decrease in LDL oxidizability and altered kinetics of oxidation corresponded to the amount of PLA2 used to treat LDL. The lag period increased from ~52 min in controls to ~74 min for treatment with 5 ng/ml PLA2 and ~84 min using 50 ng/ml PLA2. The presence of 0.1% BSA, added during the reactions with PLA2 did not significantly affect the oxidation kinetics of control or PLA2-treated LDLs (54, 78, and 85 min, respectively, for control, 5, and 50 ng/ml PLA2).

The decreased oxidizability of PLA2-treated LDL may be due to the loss of phospholipid-apoB-100 interaction and “depletion” of unsaturated fatty acids (substrates for the initiation of copper-mediated LDL oxidation) from the outer LDL surface, and association of these fatty acids with the lipid core resulting in production of micellar structures. LDL samples were therefore enriched with a mixture of unsaturated fatty acids [oleic, linoleic, and arachidonic acids (20:4:0:10)], followed by the removal of nonassociated fatty acids by ultracentrifugation in the presence of 1% BSA. Levels of free fatty acids increased in both control and PLA2-treated LDL particles, as shown in Table 2; however, for PLA2-LDL, significantly more fatty acids were associated with the particles than in control LDL samples (~2- to 3-fold increase). This fatty acid enrichment decreased the lag time of PLA2-treated LDL (Fig. 5B), shifting it from 84 min (without fatty acid supplementation) to 65 and 44 min using supplementation with 0.04 and 0.2 mM free fatty acid, respectively (Fig. 5C). In contrast, the lag time of control LDL was not substantially decreased using the same supplementation procedure.

### DISCUSSION

In this study, we show that by treating LDL with low molecular weight PLA2, LDL− is formed in significant amounts without evidence of increased lipid peroxidation. The PLA2-LDL shares basic properties with LDL− recovered from plasma. A notable similarity between LDL− isolated from plasma and PLA2-treated LDL involves conformational modification of protein with the formation of specific oligomeric aggregates and enrichment in β-strand structures while α-helical structure is decreased and the apoB-100 is unfolded into a random coil (Figs. 2, 3). Similar to LDL−, the unfolding of apoB-100 makes PLA2-LDL more susceptible to nitration by peroxynitrite. However, unlike LDL− from plasma, treatment with PLA2 produces LDL that is more resistant to copper-mediated oxidation.

### TABLE 1. Secondary structure content of native and modified LDLs

<table>
<thead>
<tr>
<th>LDL</th>
<th>α-Helix</th>
<th>Anti-Parallel</th>
<th>Parallel</th>
<th>β-Turn</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>93.2 ± 4.45</td>
<td>0.0 ± 0.0</td>
<td>1.25 ± 0.6</td>
<td>6.2 ± 1.3</td>
<td>4.7 ± 3.0</td>
</tr>
<tr>
<td>PLA2-LDL</td>
<td>86.2 ± 1.3a</td>
<td>0.2 ± 0.0</td>
<td>1.9 ± 0.14a</td>
<td>8.0 ± 0.3a</td>
<td>12.4 ± 0.85a</td>
</tr>
<tr>
<td>CuLDL</td>
<td>45.7 ± 10.4b</td>
<td>4.4 ± 2.4b</td>
<td>6.9 ± 2.0b</td>
<td>14.4 ± 1.5a</td>
<td>30.0 ± 7.3b</td>
</tr>
<tr>
<td>HbLDL</td>
<td>33.5 ± 6.0b</td>
<td>7.8 ± 2.8b</td>
<td>9.6 ± 1.4b</td>
<td>16.4 ± 1.2a</td>
<td>38.5 ± 3.0a</td>
</tr>
</tbody>
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CuLDL, LDL mildly oxidized with 10 µM copper for 3 h; HbLDL, hemoglobin-oxidized LDL, prepared as described earlier (6); PLA2, phospholipase A2. Apolipoprotein B-100 from all modified LDLs that bear significantly higher proportions (>10%) of electron-dense low density lipoprotein (LDL−) possess lower α-helical and higher β-strand structures. Circular dichroism spectra of total LDLs were taken and analyzed using CDNN software. Average values from three different experiments ± SD are reported. Values are expressed as percentage of total structures identified in the samples.

*P < 0.005 compared with the corresponding data for native LDL.

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**Fig. 4.** Peroxynitrite-induced nitrotyrosine (A) and malondialdehyde (MDA; B) formation in control and PLA2-treated LDL samples. After incubation with 50 µM peroxynitrite (1 mol peroxynitrite/1 mol tyrosine) for 30 min at room temperature, LDL samples were spotted on polyvinylidene difluoride membranes and blotted against nitrotyrosine (NT) antibody. A: Densitometry results of experiments performed at least in triplicate. B: MDA in the same samples was measured using thiobarbituric reactive substances (TBARS) assay. *P < 0.05, **P < 0.005 compared with tLDL data. Error bars represent SD of averages of at least 3 individual experiments.
conformational changes to LDL produce misfolding of apoB-100, which in turn impart bolic process acting on LDL (or an LDL precursor) to/H9262 (H9262) and absence (bold lines) of 0.1% BSA was incubated with 10 series of experiments before oxidation, control and PLA 2-treated conformational changes in LDL (31). PLA 2-treated LDL ing treatment with low molecular weight PLA2 producing matic modifications of LDL have been described, includ-120 Journal of Lipid Research Volume 46, 2005ics (B). A: Control and PLA2-treated LDL in the presence (dashed lines) and absence (bold lines) of 0.1% BSA was incubated with 10 μM Cu2+, and conjugated dienes were measured at 234 nm. B: In a series of experiments before oxidation, control and PLA2-treated LDL were enriched with free fatty acids at 0 mM (bold thick lines), 0.04 mM (dashed lines), and 0.2 mM (thin lines) as described in Materials and Methods. C: Calculated lag periods of oxidation kinetics after fatty acid enrichment of LDL samples. OD, optical density. Open bars, control LDL; closed bars, PLA2-treated LDL.

Most mechanisms that describe the formation of LDL resembling plasma LDL involve the oxidative modification of particles (6, 10). However, it has been suggested that plasma LDL may arise from a yet undescribed metabolic process acting on apoB-100 (or an LDL precursor) to produce misfolding of apoB-100, which in turn imparts conformational changes to LDL-like particles (30). Enzymatic modifications of LDL have been described, including treatment with low molecular weight PLA2 producing conformational changes in LDL (31). PLA2-treated LDL has been previously shown by electron microscopy to appear as smaller, denser particles that have a tendency to aggregate (23, 24). Generation of small, dense LDL particles by sPLA2 (types IIA, III, and X) has also been demonstrated (17, 18, 32). LDL particles modified by PLA2 as well as by oxidation have greater extracellular matrix retention and can contribute to foam cell formation (18, 32, 33). These properties are also characteristic of plasma LDL (10). Consistent with these observations, PLA2-treated LDL showed an increase in REM proportional to the LDL content. The REM and LDL content were dose-dependently correlated with the degree of phospholipid hydrolysis (Fig. 1).

LDL may be formed by reactions that are independent of lipid peroxidation. This could involve the association of particles with released negatively charged fatty acids after lipolysis. The accumulating negative charge may in turn mask positively charged residues. Benitez et al. (8) showed that the formation of LDL arises from free fatty acid association with LDL. However, this may only partially account for the increased electronegativity of lipolyzed LDL. Phospholipid hydrolysis can also produce conformational changes in apoB-100 that would expose different hydrophobic and hydrophilic residues and could account for the increased REM. ApoB-100 appears to undergo conformational changes during LDL lipolysis in the presence of albumin that preferentially binds fatty acids and lysophospholipids released by PLA2, preventing their association with LDL. However, our findings show that in the presence of 0.1% albumin, PLA2-treated LDL displayed a decrease in REM that was accompanied by significantly lower amounts of LDL formation (Fig. 1). This could simply be accounted for by lesser amounts of fatty acids associat-26). However, this is different from the mechanically inding with LDL particles than would occur in the absence of albumin, thus leading to a slightly decreased negative charge. In this case, the contribution of free fatty acids to the net negative charge of particles is not excluded.

The oligomer-specific antibody recognizes soluble oligomeric aggregates of some amyloidogenic proteins regardless of their amino acid sequences, suggesting a common structural feature among them (26). Therefore, the increased reactivity of LDL derived from plasma and PLA2-LDL to this antibody represents a more specific conformational modification of apoB-100 with the formation of oligomeric aggregates that resemble amyloidogenic proteins. Previously, the ability to aggregate has been demonstrated for PLA2-treated LDL and plasma LDL (23, 24, 26). However, this is different from the mechanically induced aggregation of LDL that probably does not induce the formation of the structural feature recognized by oligomer-specific antibody (Fig. 2A). Interestingly, soluble oligomers appear to be the most toxic species of amyloids,
thus implicating stronger biological activity to PLA₂-LDL and LDL⁻ subfractions. Whether the atherogenic properties described for plasma LDL⁻ (27) and PLA₂-LDL (18, 32, 33) are related to the observed specific structural conformations needs further investigation. Furthermore, conformational modification of lipolyzed LDL resulted in significant conversion of apoB-100 to the β-sheet conformation (Fig. 2A), as demonstrated by CD (Figs. 2B, C, 3). This appears to be a general characteristic of modified LDLS that have >10% of their particles converted to LDL⁻ (Table 1). LDLS modified by hemoglobin and by mild oxidation with copper, as well as PLA₂-LDL, showed increased β-strand content proportional to the extent of apoB-100 unfolding (Table 1). Therefore, disruption of the apoB-100-lipid interaction in LDL by means of either peroxidation or compositional alteration may lead to structural reorganization of the particle. A 50% reduction in phospholipid content of LDL results in a 25% decrease in surface area, producing smaller and denser particles (23). This would produce altered folding of apoB-100 sufficient to accommodate a more compact structure, enabling greater interactions with the lipids remaining on the surface as well as the particle core. This is consistent with the pentapartite computer-generated model for LDL (34) that designates α-helices as flexible surface lipid binding domains (allowing for expansion or contraction of particles) and β-strands that tightly associate with the neutral lipid core as well as amphipathic domains.

LDL treatment with PLA₂ leads to unfolding of apoB-100 and possible exposure of new regions to oxidative or otherwise modifying reactions. One consequence of this could be the exposure of tyrosine residues to an environment more conducive to nitration. A comparable presentation of hydrophobic domains of apoB-100 after its unfolding has been shown by analysis of tryptophan fluorescence lifetimes in LDL⁻ (11). Comparison of fluorescence lifetimes between nLDL and LDL⁻ showed that tryptophan residues of LDL⁻ were buried in more hydrophobic domains. PLA₂-LDL as well as plasma-derived LDL⁻ demonstrated increased susceptibility to nitration by peroxynitrite (Fig. 4A). Interestingly, an inverse correlation between protein nitration and lipid peroxidation levels was found in LDL after PLA₂ treatment (Fig. 4B). Lipid oxidation has been shown to accompany nitration of LDL by peroxynitrite (35); however, lipolysis of LDL limits the lipid substrate available for peroxidation, slowing the catalysis of lipid oxidation by peroxynitrite. This is in good agreement with the results of copper-mediated LDL oxidation of liposome-hydrolyzed particles (Fig. 5).

LDL⁻ represents a modified LDL that can be generated by various reactions that in virtually all cases produce particles highly susceptible to lipid peroxidation. This is considered to be a prominent characteristic of atherogenic LDL. For example, LDL treated with lipoprotein lipase and phospholipase is more prone to lipoxigenase-induced oxidation (36). LDL⁻ isolated from plasma and mixtures enriched with LDL⁻ also have substantially increased sensitivity to copper- or heme-mediated oxidation (10). The unfolding of apoB-100 also appears to make LDL more susceptible to oxidation (30). However, unlike LDL⁻ from plasma, treatment with PLA₂ produces LDL that is more resistant to copper-mediated oxidation, as illustrated by the significantly longer oxidation lag period (Fig. 5) and by the formation of less MDA content compared with that of control LDL after treatment with peroxynitrite (Fig. 4B). The LDL⁻ produced by PLA₂ could be uniquely more oxidation resistant as a result of lower peroxide content and fewer unsaturated fatty acids, two important components of catalytic initiation and the propagation of lipid peroxidation. However, in the absence of albumin, free fatty acids would accumulate within LDL particles, producing micellar structures that are more resistant to copper-mediated oxidation than the monolayer structure of LDL. Indeed, increased oxidative resistance has been shown for LDL enriched with free fatty acids (8, 37, 38). Benitez et al. (8) reported that adding fatty acids in excess of 50 mol/mol LDL protein strongly inhibited oxidation. In our PLA₂-LDL reactions, generation of even lesser amounts of free fatty acids (30 mol/mol LDL using 5 ng/ml PLA₂) already resulted in an increased lag of copper oxidation of LDL (Figs. 1C, 4A). It is plausible that intact oxidizable phospholipids bearing unsaturated fatty acids and arranged in the monolayer configurations found in LDL are required to induce lipid peroxidation. This is consistent with long-established theories for lipid peroxidation in ordered systems (39) and with the remarkable difference in susceptibility to copper-mediated oxidation of liposomes or micelles compared with LDL. Addition of more unsaturated fatty acids to PLA₂-treated LDL in the presence of albumin reversed the oxidative resistance (Fig. 5C). This may be accounted for by substantially more unsaturated fatty acid retention on PLA₂-LDL after LDL separation from albumin (Table 2). PLA₂-LDL possesses greater negative surface charge that is in part explained by apoB-100 conformational changes.

In conclusion, LDL hydrolysis by PLA₂ generates LDL⁻ particles with structural similarities to circulating LDL⁻ as well as other modified LDLS containing increased proportions of LDL⁻. A potential role for secretory PLA₂ is implicated during the formation of atherogenic LDL particles. Because the resultant modified LDL is less susceptible to oxidation, our findings suggest that initial modification of LDL by sPLA₂ can be protective in terms of oxidative/inflammatory/atherogenic effects. However, when PLA₂-treated LDL particles acquire small amounts of unsaturated fatty acids, they are exposed to catalyst such as lipoxigenase (36), or undergo lipid exchange or other interactions with lipoproteins, the particles assume enhanced susceptibility to oxidation as described for LDL⁻ particles in general.

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