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Estradiol Binding Prevents ApoB-100 Misfolding in Electronegative LDL(−)†

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ABSTRACT: Seeking for a modified lipoprotein present in plasma that could account for the atherogenic effect of high cholesterol, several years ago electronegative LDL(−) was identified. The peculiar feature of LDL(−) is an apoprotein misfolding that triggers the formation of aggregates, perfectly fitting in size the subendothelial droplets observed in early phases of atherogenesis. Apoprotein misfolding was therefore proposed as a possible atherogenic modification. LDL(−) can be spontaneously produced *in vitro* by plasma incubation through phospholipid hydrolysis catalyzed by the activity of endogenous phospholipases. As a consequence, apoprotein is misfolded. 17β-Estradiol (E2), a specific ligand to apoB-100, was used to unravel the relationship between negative charge of the lipoprotein and apoprotein structural/conformational shift. Although E2 addition to plasma does not prevent LDL(−) generation nor phospholipase activity, it deeply stabilizes apoB-100 structure, thus preventing its structural and conformational shift. Apoprotein stabilization extends to lipids. Indeed, while a loosening of lipid packing is observed together with apoprotein misfolding, conversely, when E2 stabilizes apoprotein, lipid structure is preserved. Finally, even in the presence of LDL(−), the E2-stabilized LDL is resistant to aggregation, unambiguously demonstrating that misfolding, but not negative charge, primes aggregation. In conclusion, electronegative charge and misfolding are independent and distinct features of LDL(−), and apoprotein misfolding rather than the increase in the negative charge emerges both as a valid biomarker and as an appealing pharmacological and nutritional target.

Human plasma contains small amounts of modified low-density lipoprotein (LDL)[1] that is toxic to cultured endothelial cells and induces events compatible with a cell response to injury (*1*−*3*). Being isolated from the bulk of LDL by anion-exchange chromatography as a more anionic particle, this modified LDL was named electronegative LDL, LDL(−). Many reports suggested a relationship between an increase in LDL(−) in plasma and increased cardiovascular disease risk (*4*−*8*). The key feature of LDL(−) is the misfolding of the apoprotein, apoB-100, that drives LDL aggregation and formation of amyloid-like fibrils (*9*−*12*).

Although LDL(−) can be produced in vitro from native LDL through various manipulations (*13*−*15*), we found that incubation of plasma at physiological temperature is the simplest and less perturbing procedure for a continuous production of LDL(−). Evidence was obtained about a secretory calcium-dependent phospholipase A2 (sPLA2) as the enzymatic activity that, in plasma, accounts for the production of LDL(−) (*16*).

However, a relevant missing information is about the relationship between the negative charge and misfolding, i.e., whether the two events are related or do occur independently.

We here addressed this issue by using the hormone 17β-estradiol (E2), a ligand specific for apoB-100 whose binding renders the whole particle more compact and resistant to oxidative modifications (*17*, *18*).

The results of our study show that, during plasma incubation, E2 does not prevent the phospholipase-dependent conversion of LDL into LDL(−) but completely blocks apoprotein misfolding. Therefore, electronegativity is not necessarily linked to misfolding, while apoB-100 emerges as a dynamic structure sensing the interactions with the lipid surface and with specific ligands.

EXPERIMENTAL PROCEDURES

Unless specified, all reagents were from Sigma-Aldrich (Milan, Italy).

LDL Isolation. LDL was isolated from venous blood following routine procedures (*10*), either from freshly collected plasma (LDL-F) or from plasma previously incubated at 37 °C for 20 h (LDL-I). Blood donors were healthy, normolipemic, and normal fasting volunteers, falling into the age range spanning from 30 to 56 years. LDL concentration was determined as the apoprotein concentration by using a molecular weight of 500000 and by the Bradford reagent. In total LDL, subclasses were analyzed by using anion-exchange chromatography in an AKTA-FPLC system (GE Healthcare, Amersham Biosciences, Piscataway, NJ) using a MonoQ 5/50 GL column and a multistep gradient from 0 to 0.3 M NaCl. Percent amount of subclasses was calculated from the elution profiles by integrating the absorbance of each eluted peak

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[1]Abbreviations: E2, 17β-estradiol; Laurdan, 2-dimethylamino-6-lauroylnaphthalene; LDL, low-density lipoprotein; LDL(−), electronegative LDL; LDL-F, LDL isolated from fresh plasma; LDL-I, LDL isolated from incubated plasma; PLA2, phospholipase A2.

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using the Origin software (Microcal Software Inc., Northampton, MA).

Lipid Analysis. Lipids were extracted from LDL by adding 3 mL of $CHCl_3$−MeOH (1:2) to 0.8 mL of LDL samples. After vortexing, 0.8 mL of a 0.88% KCl solution plus 3 mL of $CHCl_3$ was added, the samples were vortexed again and centrifuged, and the upper aqueous phase was removed and discarded. The organic phase was dried under a gentle stream of nitrogen and redissolved in 0.5 mL of $CHCl_3$−MeOH (1:1); samples were stored at −20 °C until undergoing analysis. All solvents contained 10 mg/L butylated hydroxytoluene. TLC analysis was performed on silica gel TLC plates (Merck Kieselgel 60). Lipid classes were separated in chloroform−methanol−30% ammonia (65:25:5 v/v). After elution, lipids were visualized by 8-anilinonaphthalene-1-sulfonate and identified using authentic standards.

LDL Incubation with PLA2. LDL at a concentration of 0.5 μM apoprotein (final volume: 2 mL) were incubated with 1 ng/μL PLA2 from *Naja naja* venom (Sigma Aldrich, Milan, Italy) for 30 min at 37 °C in the presence of 5 mM $CaCl_2$. The reaction was stopped by adding 10 mM EDTA. Samples aliquots were immediately used for lipid extraction and for circular dichroism measurements.

Circular Dichroism. CD spectra from the different LDL samples (with apoprotein concentrations of 0.1 μM) were recorded on a JASCO spectropolarimeter, model J710 (Tokyo, Japan), using a 0.1 cm quartz cuvette. The cell holder compartment was maintained at 37.0 ± 0.1 °C. Four spectra were averaged for each measurement.

Fluorescence Measurements. Tryptophan fluorescence lifetime was measured in LDL by using the harmonic response technique, at 0.1 μM protein concentration. The excitation source was a 300 ± 6 nm laser diode, and the emission was collected through a WG330 cutoff filter. The phase and modulation data were collected using the K2 fluorometer (ISS Inc., Champaign, IL) and the accompanying software. The fluorometer cell holder was kept at 37.0 ± 0.1 °C using a circulating water bath. The data analysis was performed by the Globals Unlimited software (Laboratory for Fluorescence Dynamics, UCI, Irvine, CA) using a Marquardt algorithm. The goodness of fit was evaluated on the basis of the value of the χ^2.

The value of 2-dimethylamino-6-lauroylnaphthalene (Laurdan; Molecular Probes Inc., Eugene, OR) generalized polarization (GP) (*19*) was measured in a GREG 200 fluorometer (ISS Inc.) fitted with a xenon arc lamp and photon counting electronics (PX01; ISS Inc.). LDL samples (0.1 μM) were equilibrated at 37 °C in the fluorometer cell holder, and blank spectra were acquired for subtraction later on. The LDL was then labeled by adding to 3 mL samples 0.3 μL of a 1.34 mM solution of Laurdan in dimethyl sulfoxide (final concentrations: 0.4 μM Laurdan, 0.01% (vol %) DMSO) and incubated for 7 min. Emission spectra were then acquired from 400 to 500 nm, using 380 nm excitation and 8 nm bandwidths. The cell holder was thermostatically maintained at 37.0 ± 0.1 °C using a circulating water bath. Following subtraction of the blank, the Laurdan GP value was calculated from the emission spectra according to

$$GP = (I_{420} - I_{480})/(I_{420} + I_{480}) \quad (1)$$

where I_{420} and I_{480} are the emission intensities at 420 and 480 nm, respectively.

Light Scattering. The light scattered from 1 mg/mL LDL protein was monitored using a commercial light scattering ALV spectrometer setup (ALV, Langen, Germany), consisting in a CGS-5000 rotating arm goniometer, a photomultiplier tube (EMI, Ruislip, U.K.), an ALV 5000 multitau digital correlator operating at a sampling time of 200 ns, and an Innova 70 argon ion laser (Coherent, Santa Clara, CA) operating at 488 nm and 100 mW. The scattering cell was immersed in a refractive index matching fluid (toluene) kept at 37 ± 0.1 °C. Light scattering data were collected simultaneously from a scattering volume of ~100 μm^3, at an angle of 90°, and analyzed using a specially developed software.

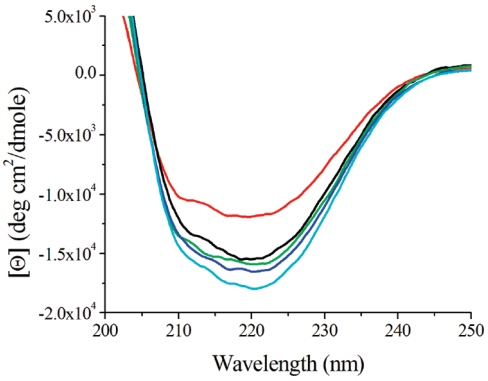

FIGURE 1: Effect on apoB-100 structure of E2 addition to plasma. CD spectra of LDL-F (black), LDL-I (red), and LDL-I in the presence of different E2 concentrations during plasma incubation. E2 concentration: 0.1 μM (green); 0.25 μM (blue); 0.5 μM (cyan).

RESULTS

E2 Addition to Plasma Stabilizes ApoB-100 Secondary Structure and Conformation. The crucial feature of LDL isolated from human plasma and incubated overnight at 37 °C (LDL-I) is the marked decrease of CD absorbance relative to LDL isolated from fresh plasma (LDL-F). This was attributed to the formation of LDL(−) where the apoB-100 is misfolded (*10*, *16*). In the presence of E2 added to plasma, the structural shift of apoB-100 was fully prevented (Figure 1). In agreement with previous findings (*17*, *18*) E2 also led to a small dose-dependent increase of the CD absorbance.

We next examined variations in the apoprotein conformation in the different LDL samples through the analysis of tryptophan fluorescence lifetimes. The overall decay of the 37 tryptophan residues in apoB-100 is rather complex, being described by a continuous distribution of two lifetime components (Figure 2A) (*17*). Both plasma incubation and E2 affected the profile of tryptophan lifetime distribution. Plasma incubation in the absence of E2 resulted in a shift of the longer component toward shorter values, which was accompanied by a marked narrowing in width (Figure 2B). This effect is compatible with a loosening of the protein tridimensional scaffolding, since (1) the aqueous solvent is expected to quench tryptophan fluorescence, resulting in lower lifetime values, and (2) the exposure of tryptophans to the solvent increases their mobility, resulting in a more homogeneous distribution. The presence of E2 during plasma incubation prevented the above modifications in the center and width of the longer lifetime component, eventually also with a small increase in its fraction (Figure 2C).

E2 Addition to Plasma Does Not Prevent LDL(−) Generation. We tested whether the structural and conformational effect of E2 on apoB-100 could be associated to a decreased formation of LDL(−). We previously showed that in LDL

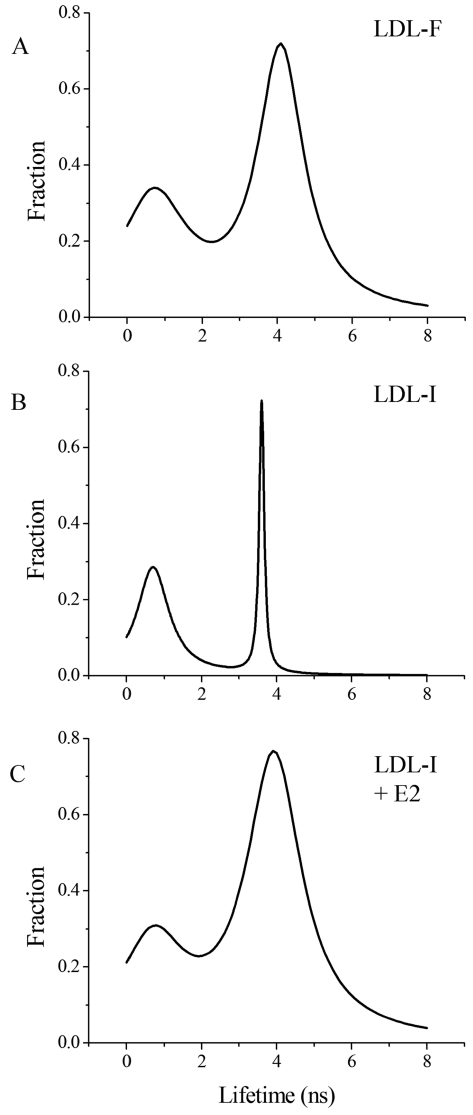

FIGURE 2: Effect on apoB-100 conformation of E2 addition to plasma. Representative examples of tryptophan fluorescence lifetime distributions in LDL-F (A), LDL-I (B), and in LDL-I isolated from plasma to which 0.1 μM E2 was added (C).

isolated from preincubated plasma the percentage of LDL(−) rises up to about 60%, due to the activity of secretory calcium-dependent phospholipases A2 (sPLA2) (*16*). When E2 was added to plasma, LDL(−) was still produced, although in a slightly lesser extent (Figure 3A). The option that E2 could inhibit the formation of lysophospholipids in LDL by affecting sPLA2 activity was easily ruled out by a lipid analysis. Indeed, in the presence of 0.5 μM E2 the lysophospholipid production was the same (Figure 3B).

E2 Stabilizes the Apoprotein Structure Regardless of a Massive Lipid Perturbation by Exogenous Phospholipase. The stabilizing effect of E2 on apoprotein structure was further investigated through a model of massive lipid perturbation. In vitro phospholipid hydrolysis by cobra venom calcium-dependent PLA2 was previously shown to produce LDL(−) with misfolded apoB-100 (*15*). By using this model, the structural effect of E2 on LDL apoprotein was further confirmed, even in the presence of major lipid modifications. After a short incubation of 30 min at 37 °C, PLA2 converted practically all phosphatidylcholines (PC) into lysophosphatidylcholine (LPC) (Figure 4A). This was unaffected by E2, an almost complete lysis of PC being detected even at a high E2 concentration of 1 μM. As expected, the structure of apoB-100 was profoundly altered by PC lysis into LPC but only in the absence of E2. Impressively instead, in the presence of E2 the CD spectrum perfectly overlapped that of untreated LDL-F (Figure 4B).

We concluded that, irrespective of the massive lipid perturbation operated by the cobra venom PLA2, E2 had a direct and noticeable stabilizing effect on apoB-100 structure.

Apoprotein Stabilization Assists Lipid Packing. We further questioned whether the structural stabilization operated by E2 on apoB-100 could, conversely, affect lipid supramolecular structure. For the purpose, we explored lipid molecular dynamics in LDL by using the generalized polarization (GP) function of Laurdan, a probe sensitive to changes in lipid packing (*19*). Laurdan GP value is directly related to lipid packing, being lower, for instance, in the presence of lysophospholipids. Indeed, and in agreement with our previous findings (*16*), a looser lipid packing, as a GP decrease, was monitored in LDL-I. Instead, the decrease of Laurdan GP was prevented, in a concentration-dependent manner, when plasma was incubated in the presence of E2 (Figure 5). At 0.5 μM E2, the modification of lipid packing was completely prevented. Consistently with the notion of an interaction between the apoprotein and lipids in LDL, we could detect a lipid loosening only in parallel to apoprotein misfolding, and conversely lipid structure was preserved when the apoprotein was stabilized by its ligand E2.

E2 in Plasma Prevents LDL Aggregation. Finally, being the apoprotein misfolding with its α-to-β structural shift crucial for LDL aggregation, we tested whether the particle stabilization could also affect aggregation. In agreement with our previous light scattering results (*10*), LDL-F did not aggregate while LDL-I aggregated after a lag phase of about 20 h. When the plasma incubation was performed in the presence of E2, the scattering intensity of LDL-I did not change (Figure 6), indicating that misfolding primes aggregation regardless of the negative charge.

DISCUSSION

The epidemiological evidence that an increased level of LDL positively correlates with the risk of cardiovascular disease implies that LDL must be somehow modified in order to prime the pathological process. In vitro modification of LDL by different manipulations, usually massive peroxidation, indeed yields particles able to reproduce some biological events typical of the early phases of atherogenesis. While a clear and unquestioned evidence of oxidatively modified LDL in plasma is missing, LDL(−) was originally detected in plasma as a lipoprotein minimally different from the bulk LDL. In addition, its concentration positively correlates with cardiovascular disease markers. The misfolding of apoB-100 later emerged as the most peculiar feature differentiating LDL(−) from native LDL, with an α-to-β structural shift and a conformational shift (*11*). This drives a domino process of aggregation that propagates misfolding over all LDL, thus triggering further aggregation with the formation of amyloid-like structures (*10*, *12*). Remarkably, the formation of LDL(−) prone to a typical amyloidogenic aggregation can be induced by a process as simple as the plasma incubation at 37 °C and was found dependent on phosphatidylcholine hydrolysis by an endogenous secretory phospholipase (*16*).

This set of information defined the forthcoming question on the relationship between the negative charge and the structural/conformational shift of the apoprotein. To address this issue, we

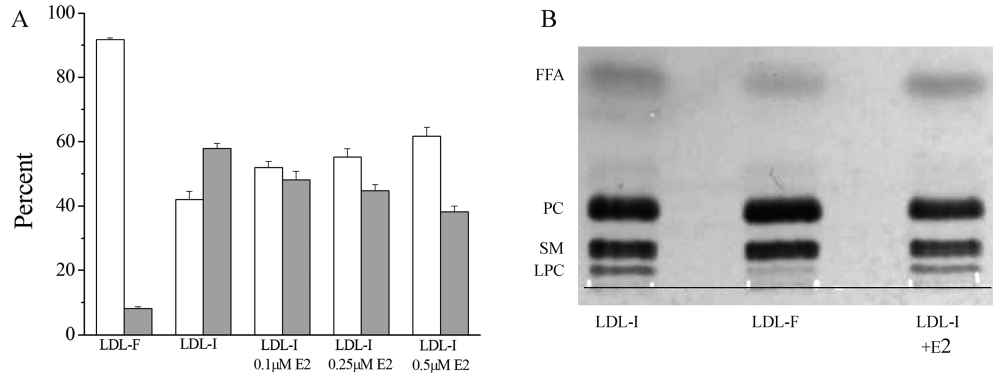

FIGURE 3: E2 does not affect the increase in LDL(−) during plasma incubation. (A) Percent amount of native LDL (white) and of LDL(−) (gray) in LDL purified from fresh plasma (LDL-F) from plasma preincubated at 37 °C for 20 h (LDL-I) also in the presence of different E2 concentrations. Average of three independent determinations with standard error. Significance of data in the presence of E2 relative to LDL-I: $p < 0.05$. (B) Chromatogram of lipids from different LDL samples. The E2 concentration added in plasma was 0.5 μM. Key: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; FFA, free fatty acids.

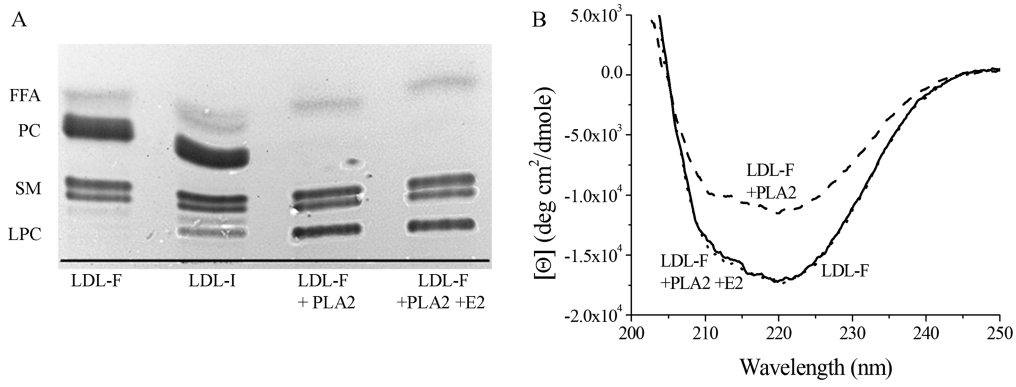

FIGURE 4: E2 stabilizes the apoprotein structure regardless of the action of PLA2. (A) Chromatograms of lipid extracted from LDL-F, after the action of cobra venom PLA2, and in this last sample in the presence of 1 μM E2. Key: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; FFA, free fatty acids. (B) CD spectra of LDL-F (continuous), LDL-F after the action of cobra venom PLA2 (dash), and in this last sample in the presence of 1 μM E2 (dot).

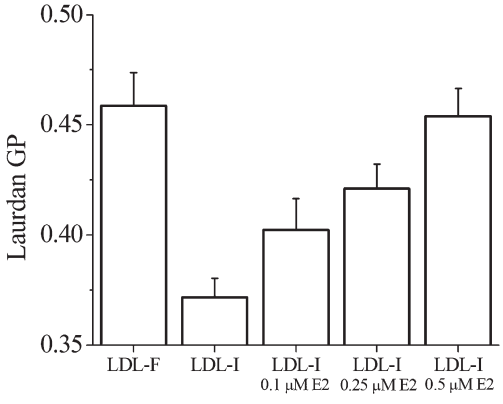

FIGURE 5: Consequences of apoprotein stabilization on LDL lipid packing. Laurdan GP values in the different LDL samples, isolated from fresh (LDL-F) or from preincubated plasma (LDL-I), this last also in the presence of different E2 concentrations. Average of three independent experiments with standard deviation.

resorted to check whether a specific ligand to apoB-100, known to stabilize the structure, was also competent for preventing the formation of more negative particles. Indeed, when E2 is added to isolated LDL, an increased ellipticity is observed, apparently due to E2 binding to a single and highly specific site on the apoprotein (*17*, *18*) which renders it definitely more stable.

Here we found that the presence of E2 in plasma does not prevent LDL conversion into LDL(−) and does not affect phospholipase activity. The minor, although significant, decrease in LDL(−) formation in the presence of E2 could be attributed to a decreased substrate accessibility for the phospholipase, i.e., to an increased lipid packing, as that detected by Laurdan GP (also discussed below). This effect is in agreement with the reported inverse dependence of PLA2 activity with lipid order (*20*). Nevertheless, E2 in plasma actually fully prevents misfolding, a particularly robust effect since the native apoprotein folding is conserved even after the exhaustive hydrolysis of phosphatidylcholine by means of cobra venom PLA2. Thus, as a first conclusion, we broke the aphorism that negative charge and misfolding must be connected.

The fact that LDL(−) are not necessarily misfolded, thus amyloidogenic, weakens the value of its chromatographic quantitation as a cardiovascular risk biomarker, unless also the structure is analyzed.

The reason for the increased negative charge remains obscure. Asatryan (*15*) suggested that the formation of LDL(−) could originate from phospholipid hydrolysis either through an increased free fatty acid concentration or after the induction of conformational changes in apoB-100 that would expose different residues. Our evidence definitively rules out structural and conformational changes in apoprotein, while the option that free fatty acids released by phospholipases could play a role still holds.

Remarkably, the effect of E2 is not limited to a structural stabilization, resulting also in a gain in ellipticity. This suggests

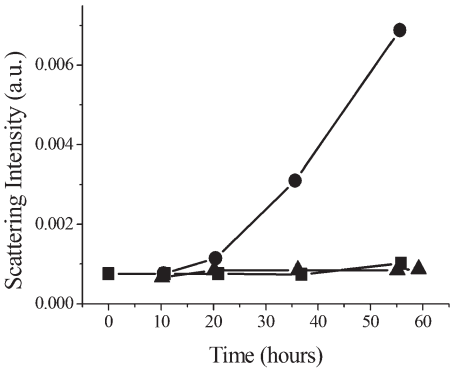

FIGURE 6: E2 in plasma prevents LDL aggregation. Light scattering intensity versus time of LDL purified from fresh plasma (■) and from plasma that was preincubated at 37 °C for 20 h (●), this last also in the presence of 0.1 μM E2 (▲).

the interesting possible notion of coexisting interconverting structures of the apoprotein also in its "native" state, well fitting the function of accommodating changing amounts of lipids.

Prevention of apoprotein misfolding by E2 also prevents a lipid loosening after the increased lysophosphatidylcholine fraction. Due to its low relative concentration, we can rule out a direct effect of E2 on LDL lipid structure, through its eventual partitioning into the lipid phase. Actually, and as previously discussed (*17*, *21*), Laurdan GP can detect changes in packing for modifications of lipid composition in the order of several mole percent, while in our conditions we have at most 0.02 mol % E2 relative to LDL outer layer lipids. Therefore, the left option is that the preserved lipid packing when apoB-100 is stabilized descends from a lipid−protein structural interplay at work in LDL.

From the observed stabilization of apoB-100 structure by E2, any physiological reasoning about an effect of E2 in vivo is premature. In addition, although the binding constant of E2 for its site in apoB-100 is rather high ($k = (1.7 \pm 0.2) \times 10^6$) (*18*), several E2 binding proteins exist in plasma, and this prevents any rigorous attempt to draw conclusions on the actual LDL saturation. Using a similar experimental approach of incubating E2 in plasma and at similar concentrations, an increased LDL resistance to oxidative modification was already shown (*22*). A quantitative determination of E2 bound to LDL gave a molecular ratio as low as 1 E2 to 500 LDL. In agreement with these authors, we should conclude that E2 binding to its site in LDL drives such a stable structural and conformational shift to the apoprotein, maintained even after E2 is released. E2 effect should, therefore, be due to a transient binding to LDL in a chaperone-like fashion and, reasonably, during 20 h incubation, this hormone can traffic between different LDL particles and also between other proteins. As a final consideration, we note that in our experimental conditions E2 is added to plasma; then LDL are isolated by two centrifugations and exhaustively dialyzed twice in a 1:1000 volume ratio. Very little, if any, E2 can remain bound. All of this argues in favor of a stable conformational shift of apoB-100 that persists also when E2 is removed. Nevertheless, the fine mechanism can only be proposed and further dedicated studies are needed to unravel this impressive and intriguing evidence.

In conclusion, the present data demonstrate that electronegativity and misfolding are independent and distinct features of LDL(−), that specific ligands such as E2 stabilize the apoprotein structure, and that apoprotein and lipid structural organization are closely interconnected.

Misfolding, over the electronegative charge, emerges as a crucial modification of LDL, leading to aggregated particles perfectly fitting the size of the subendothelial droplets observed in pathological samples (*10*, *23*). In the absence of conclusive data on the mechanism for the onset of vascular diseases, we propose the apoprotein misfolding as a relevant atherogenic character in modified LDL (*9*, *10*, *12*); therefore, its possible prevention by protein ligands comes out as an appealing pharmacological and nutritional target.

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