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Thermal stability of human plasma electronegative low-density lipoprotein: A paradoxical behavior of low-density lipoprotein aggregation

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ABSTRACT

Low-density lipoprotein (LDL) aggregation is central in triggering atherogenesis. A minor fraction of electronegative plasma LDL, termed LDL(−), plays a special role in atherogenesis. To better understand this role, we analyzed the kinetics of aggregation, fusion and disintegration of human LDL and its fractions, LDL(+) and LDL(−). Thermal denaturation of LDL was monitored by spectroscopy and electron microscopy. Initially, LDL(−) aggregated and fused faster than LDL(+), but later the order reversed. Most LDL(+) disintegrated and precipitated upon prolonged heating. In contrast, LDL(−) partially retained lipoprotein morphology and formed soluble aggregates. Biochemical analysis of all fractions showed no significant degradation of major lipids, mild phospholipid oxidation, and an increase in non-esterified fatty acid (NEFA) upon thermal denaturation. The main baseline difference between LDL subfractions was higher content of NEFA in LDL(−). Since NEFA promote lipoprotein fusion, increased NEFA content can explain rapid initial aggregation and fusion of LDL(−) but not its resistance to extensive disintegration. Partial hydrolysis of apoB upon heating was similar in LDL subfractions, suggesting that minor proteins importantly modulate LDL disintegration. Unlike LDL(+), LDL(−) contains small amounts of apoA-I and apoJ. Addition of exogenous apoA-I to LDL(+) hampered lipoprotein aggregation, fusion and precipitation, while depletion of endogenous apoJ had an opposite effect. Therefore, the initial rapid aggregation of LDL(−) is apparently counterbalanced by the stabilizing effects of minor proteins such as apoA-I and apoJ. These results help identify key determinants for LDL aggregation, fusion and coalescence into lipid droplets in vivo.

1. Introduction

The response-to-retention hypothesis states that the initiating event in the development of atherosclerotic lesions is the subendothelial retention of LDL, a process mediated by the binding of LDL to arterial proteoglycans (PGs) [1,2]. This binding is triggered by chemical modifications of LDL such as lipolysis, proteolysis or oxidation, which promote LDL aggregation [3,4]. Aggregated LDL particles contain multiple PG binding sites and bind to PGs in the arterial wall matrix with higher affinity than their native non-aggregated counterparts. LDL aggregation can evolve to irreversible LDL fusion (i.e. formation of larger lipoprotein-like particles) followed by lipoprotein disintegration and release of core lipids (or rupture) and coalescence into lipid vesicles and droplets that are too large to exit the subendothelial space of the arterial wall [3,5]. In this environment, macrophages internalize entrapped lipoproteins, leading to the formation of foam cells overloaded with cholesterol, a hallmark of atherosclerotic lesions.

Since LDL aggregation and fusion are important early steps in atherogenesis, extensive studies have been conducted to understand molecular mechanisms of these processes, with a particular focus on the action of lipases and proteases whose expression is increased in

Abbreviations: Apo, apolipoprotein; α-CT, α-chymotrypsin; CD, circular dichroism; CE, cholesterol ester; DAG, diacylglycerol; GAGs, glycosaminoglycans; LDL(−), electronegative LDL; LDL(+), non-electronegative LDL; LDL(total), total plasma LDL containing both LDL(+) and LDL(−); LPC, lyso-phosphatidylcholine; NGGE, non-denaturing polyacrylamide gradient gel electrophoresis; NEFA, non-esterified fatty acids; PC, phosphatidylcholine; PG, proteoglycans; SM, sphingomyelin; TEM, transmission electron microscopy; TG, triacylglycerol.

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atherosclerotic lesions [5–10]. However, other aspects, such as the relative susceptibility of specific LDL subfractions to aggregation and fusion, received relatively little attention [5,11,12]. Our focus here is on electronegative LDL, or LDL(−), which is a modified subfraction of plasma LDL with inflammatory and apoptotic properties [13]. The proportion of LDL(−) in plasma is increased under conditions of high risk of cardiovascular disease [14,15]. LDL(−) has increased propensity to aggregate; in fact, a minor sub-population of LDL(−) is aggregated in plasma [16,17]. Moreover, LDL(−) can promote in vitro aggregation of monomeric, non-electronegative native LDL particles, termed LDL(+) [17–19]. Together, these studies suggest that, although LDL(−) constitutes only 3–5% of total plasma LDL, it may importantly contribute to atherogenesis by promoting LDL aggregation and favoring subendothelial retention of LDL. An unique feature of LDL(−) is a relatively high content of non-apoB proteins [20]. The role of these proteins in the aggregation behavior of LDL(−) is unknown, but some of them, such as apoJ and apoA-I, can prevent aggregation, fusion and rupture of total LDL [21,22]. In the current work, we analyzed the role of apoJ and apoA-I in aggregation of LDL subclasses.

Thermal analysis is widely used to determine structural stability and susceptibility to aggregation of proteins and lipoproteins [23]. Our previous thermal denaturation studies revealed that stability of lipoproteins is determined by kinetic barriers, and suggested that similar barriers modulate lipoprotein remodeling and fusion in vivo [11,24–26]. Importantly, thermal denaturation of lipoproteins such as LDL mimics key aspects of their aggregation, fusion and rupture in vivo, and the products of the heat-induced LDL fusion and rupture are similar to LDL-derived extracellular deposits in atherosclerotic lesions ([5] and references therein). This prompted us to propose that perturbations of a lipoprotein assembly by various means (mechanical, thermal, enzymatic, oxidative etc.) produce a finite number of similar structural responses such as aggregation, fusion and rupture. This concept is illustrated in the Supplemental Fig. S1 showing gel filtration profiles of LDL that have been subjected to lipolysis by sphingomyelinase or phospholipase C or incubation at 37 °C, pH 6 to mimic in vivo conditions in atherosclerotic plaques, as well as to vortexing or heating. Similar responses of LDL assembly to these perturbations suggest that the general trends revealed in thermal denaturation studies are applicable to LDL remodeling at ambient conditions in vivo. Hence, LDL are exposed to high temperatures as a means to accelerate LDL remodeling and quantify its kinetics.

In the current work, we use thermal denaturation as a tool to determine structural stability and susceptibility to aggregation, fusion and disintegration of LDL(−) and LDL(+). The results reveal that, unlike LDL(+) or LDL(total), which form large insoluble particles upon complete thermal denaturation, LDL(−) shows faster initial aggregation but much less extensive rupture and hence, stays longer in solution in the aggregated form. We propose an explanation for these surprising findings based on the distinct biochemical composition of LDL(−), and discuss their implications for the role of LDL(−) in atherogenesis.

2. Material and methods

2.1. Lipoprotein isolation

Plasma of healthy normolipemic volunteers was obtained in the Lipid Laboratory of Hospital de Sant Pau with their written informed consent upon approval by the institutional ethics committee. Total LDL was isolated from pooled plasma by sequential ultracentrifugation (density range 1.019–1.050 g/mL) using KBr gradients at 4 °C. All density solutions contained 1 mM EDTA and 2 μM BHT. LDL were dialyzed against buffer A (10 mM Tris, 1 mM EDTA, pH 7.4) and stored at −80 °C in 10% sucrose solution until use.

Total LDL was filtered using 0.2 μm filter and subfractioned into LDL(+) and LDL(−) by stepwise anion-exchange chromatography using HiPrep Q HP 16/10 column (GE Healthcare) [27]. LDL(total), LDL(+) and LDL(−) were then dialyzed against standard phosphate buffer (20 mM Na phosphate, pH 7.0) and used for further experiments unless otherwise stated. The protein concentration in LDL subfractions was measured by using a modified colorimetric Lowry assay. LDL concentration is expressed as mg protein/mL, unless otherwise indicated. ApoJ-containing LDL (LDL/J+) and apoJ-depleted LDL (LDL/J−) were obtained by affinity chromatography in NHS-HiTrap columns to which an apoJ-specific antibody was attached as described [21]. For biochemical heat-induced changes, LDL samples (0.35 mg protein/mL) in standard phosphate buffer were incubated at 82 °C for 15, 30 or 90 min (end-point). A subset of samples was centrifuged at 14,000 rpm for 10 min at 4 °C to determine the composition of soluble and insoluble particles.

2.2. Chemical characterization of LDL subfractions

Major lipid and protein composition of LDL subfractions was measured by commercial methods adapted to a Cobas 6000/c501 autoanalyzer. Total cholesterol, triglycerides and apolipoprotein (apo) B reagents were from Roche Diagnostics. Phospholipids and non-esterified fatty acids (NEFA) reagents were from Wako Chemicals. Phosphatidylcholine (PC) and sphingomyelin (SM) content was evaluated by normal-phase HPLC in a System Gold chromatograph (Beckman) equipped with a photodiode array detector, as described [28]. Dipalmitoyl-glycero-phosphodymethyl ethanolamine (DGPE, ref. P0399 Sigma) was used as an internal standard. The peak areas corresponding to PC and SM were quantified at 205 nm. The ratio of the absorbance at 205 nm to 234 nm was used as an index of PC oxidation [29].

Non-denaturing gradient (2–16%) gel electrophoresis (NGGE) was used to assess particle size. NGGE were run at 100 V for 4–6 h with 1% Sudan black pre-stained LDL subfractions, as described [30]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE, 10% acrylamide) was used to assess apoB degradation and the presence of minor apolipoproteins in LDL. SDS gels were run at 100 V for 1.5–2 h, and were stained with Coomassie blue. ApoB, apoA-I and apoJ in LDL subfractions were detected by Western blotting after protein transfer from SDS PAGE to nitrocellulose. Proteins were transferred at 30 V for 1 h, incubated in blocking buffer (50 mM Tris, 500 mM NaCl (TBS), pH 7.4 containing 0.1% casein) for 30 min, washed 3 times with TTBS (TBS containing 0.1% Tween 20) and probed with polyclonal antibody anti-apoB (dilution 1/5000, Acris Ab), anti-apoA-I (dilution 1/1000, Acris Ab) and anti-apoJ (dilution 1/1000, Novus Biologicals) for 2 h, followed by horseradish peroxidase-conjugated anti-goat secondary antibody (dilution 1/5000, Jackson Immuno Research). Nitrocellulose was revealed with Immun-Star developing kit (BioRad).

2.3. Circular dichroism spectroscopy

CD data were recorded by using an AVIV 400 or an AVIV 62DS spectropolarimeter following published protocols [11] with minor modifications. Briefly, to compare the secondary structure in monomeric and in minimally aggregated LDL subfractions, far-UV CD spectra were recorded in the wavelength range 190–250 nm from LDL samples containing 0.1 mg protein/mL in standard buffer (20 mM Na phosphate, pH 7.0). Far-UV CD data were normalized to protein concentration and expressed in units of molar residue ellipticity, [Θ]. Since aggregated LDL are expected to cause spectral distortions in far-UV [31], no quantitative secondary structural analysis based on far-UV CD spectra was performed. ORIGIN software was used for CD data analysis and display.

2.4. Thermal denaturation studies

Structural stability of LDL subfractions was assessed in the heating experiments by simultaneously monitoring near-UV CD and turbidity (dynode voltage) at 320 nm; for methodological details see references [11,23,32]. Previously we showed that heating causes a large increase