Atherosclerosis: another protein misfolding disease?

Fulvio Ursini, Kelvin J.A. Davies, Matilde Maiorino, Tiziana Parasassi and Alex Sevanian

The secondary structure and conformation of apo-B 100 in low-density lipoproteins (LDL) are imposed by lipid–protein interactions and dynamics, and affected by the introduction or removal of lipids during the course of lipoprotein metabolism. Following an alteration of the water–lipid interface as a result of, for example, oxidation of lipids, the supramolecular structure becomes destabilized and apoB can misfold. These events have been observed in LDL$^-$, a fraction of oxidatively modified LDL isolated *in vivo*. This modified lipoprotein possesses several atherogenic properties and represents an *in vivo* counterpart of *in vitro* modified LDL that is implicated in atherosclerosis. The misfolding of apoB, its aggregation, resistance to proteolysis, and cytotoxicity are common motifs shared by LDL$^-$ and amyloidogenic proteins. Based on these analogies, we propose that atherogenesis could be considered as a disease produced by the accumulation of cytotoxic and pro-inflammatory misfolded lipoproteins.

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Fulvio Ursini*
Matilde Maiorino
Dept of Biological Chemistry, University of Padova, Viale G. Colombo 3, 35121, Padova, Italy.
*e-mail: ursini@mail.bio.unipd.it

Kelvin J.A. Davies
Ethel Percy Andrus Gerontology Center, The University of Southern California, 3715 McClintock Avenue, Los Angeles, California 90089-0191, USA.

Tiziana Parasassi
Institute of Neurobiology & Molecular Medicine, National Research Council, Viale Marx 15-43, 00137 Roma, Italy.

Alex Sevanian
Dept of Molecular Pharmacology & Toxicology, University of Southern California, 1985 Zonal Avenue, Los Angeles, California 90033, USA.

Proper folding into the correct secondary structure and conformation is indispensable for the biological function of proteins and is achieved during, or immediately following protein synthesis [1]. Cells respond to accumulating unfolded proteins by increasing the expression of chaperones that assist in protein folding. This process works in synergy with proteolytic systems that detect and degrade unfolded or damaged proteins. The detection of unfolded proteins, referred to as the unfolded-protein response, occurs in the endoplasmic reticulum (ER) [2,3], and is mediated by a unique set of transcription factors [4]. If unfolding takes place outside the ER, or such proteins accumulate rapidly from extrinsic sources, they will not be effectively sensed by the ER. If these proteins are not efficiently degraded by scavenger mechanisms, they can result in cell injury [5]. Unfolding of a protein, when not reverted in the energy-consuming process of refolding, can lead either to frank denaturation or, when the condition is compatible with the formation of hydrogen bonds, to misfolding.

A sporadic, inherited or acquired functional inefficiency in removing misfolded proteins is associated with severe degenerative syndromes such as Alzheimer's, Parkinson's and Creutzfeld–Jacob disease, as well as the more commonly encountered type 2 diabetes [6]. During the various pathological conditions associated with misfolded proteins, one or more proteins convert from a native soluble form to aggregates originally described as 'amyloid'. These fibrillar aggregates, which can be either extracellular or intracellular, are cytotoxic and result in alteration of cellular function. Although the initiating event might be distinct in different diseases, a general pattern is seen where loss of native protein stability (usually an α-helix domain) is followed by a misfolding that leads to a relative increase in β-sheet structure. The β structure is particularly stable and 'seeds' further polymerization into largely intertwining β-strand structures. The resulting, stable, proteolytically resistant and cytotoxic fibrils are hallmarks of several degenerative diseases [7]. Recent results *in vitro* indicate that fibrillar aggregates of proteins not connected with specific diseases can be inherently cytotoxic, suggesting a common mechanism for all protein misfolding diseases [8].

Proper initial folding takes place when hydrophobic interactions among amino-acid side-chains participate in the initial formation of a 'molten globule' [1,7]. In this process, facilitation of folding provides a first defense against the formation of insoluble aggregates that contain inappropriate proportions of β-structure. When optimal interactions between lipophilic and amphipathic components are altered, for example by mild denaturation, whereby hydrogen bonds are preserved, an α-helix to β-sheet transition can take place [7]. This conformational shift (misfolding) can be primed by such factors as mutations, inappropriate post-translational modifications and oxidation, all of which contribute to the intrinsic instability of the protein [9].

Following oxidative modifications, loss of α structure and the exposure of hydrophobic domains (denaturation) represent a driving force for intracellular degradation. However, when hydrogen bonds among the backbone of polypeptide chains give rise to a β structure, the misfolded protein becomes more stable [7]. This gives rise to proteolytically resistant [10] polymerized β-amyloid fibrils [11]. The aggregates of misfolded proteins also inactivate proteasomes [12] and lysosomes [13]. The only residual defense system to the type of damage brought about by fibrils of misfolded proteins is a recently identified intracellular metalloprotease. This protease, originally named 'insulin-degrading enzyme', appears to specifically eliminate peptides with amyloidogenic potential and thus provides the last defense against accumulation of misfolded proteins [14].

Resistance to proteolysis has been also observed for oxidized low-density lipoprotein (LDL) [15,16]. During the process of atherosclerosis, the accumulation of intracellular apoB-100 is proportional to the extent of LDL oxidation. The apoprotein from oxidized LDL accumulates in high-molecular-weight insoluble fractions, is resistant to proteolysis; the ubiquitin–proteasome pathway becomes inhibited [16], and lysosomal activity is reduced together with disruption of these organelles [17].

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We propose that atherogenesis emanates from the vascular accumulation of modified LDL, where apoB is misfolded and the particles aggregate into a cytotoxic, pro-inflammatory and protease-resistant entity. In this respect, atherosclerosis presents interesting analogies to the other well-known chronic diseases that are based on the accumulation of misfolded proteins.

Protein–lipid interaction and structure of Apo-B

LDL is produced in plasma from very (V)LDL. Apo B-100 is present as a single copy per LDL particle and is not exchangeable with other lipoproteins, as is the case for other apoproteins. A full-length apoB-100 (4536 amino acids) is present in LDL derived from VLDL produced in the liver. Computerized homology alignments and characterization of secondary structure for amphipathic domains indicate that apoB-100 has a supramolecular structure where domains with α or β structure are arranged in the following order from the N to the C-terminus: α1–β1–α2–β2–α3 [18]. The amphipathic domains associate with lipids. The α1 domain is homologous to microsomal triglyceride transfer protein, and during synthesis, a lipid pocket is formed in the N-terminal region of apoB that drives particle expansion by progressive interactions with β1 amphipathic domains [19].

Lipid domains on the LDL surface interact rather specifically with regions of apo-B that have an affinity for particular phospholipids, and these interactions impart secondary and tertiary structure to the protein [20]. The notion that a β structure is an important irreversible lipid-associating domain is supported by spectroscopic evidence for preferential binding with phosphatidylcholine. By contrast, dynamic interactions with other surface phospholipids is predominantly via α-helical-rich domains [20]. The dynamic interaction with lipids ensures the secondary structure and conformation of apoB that adjust to changes in particle composition during metabolism. Accordingly, when shrinkage or expansion of particles is obtained by changing the temperature below or above the phase transition of core lipids, reversible increases in β-structure and decreases in α-helical structure are observed [21]. Secondary structural modifications appear to accommodate surface and particle shape changes during the utilization of LDL lipids. The α-helix component (associated with surface lipids) increases when LDL particle size is reduced through the action of lipoprotein lipase [18]. β-domains also undergo conformational changes during conversion of VLDL to LDL [22], although to a smaller extent than the less stable α-helix. Besides accommodating changes in triglyceride or cholesteryl ester content, conformationally responsive regions in apoB-100 affect the surface charge that influences electrostatic interactions with the cell surface or extracellular matrix.

The lipid–protein interactions mediate correct protein folding, which in turn affect the oxidative stability of lipids. This has been described for phospholipid–cholesterol interactions at the plasma membrane and LDL surface, where the peculiar packing of surface lipids – determined by the high-cholesterol content and the interaction with proteins – contributes to oxidative resistance [23]. A relationship between structure and oxidative resistance has also been observed in core lipids of LDL by analyzing thermally induced transitions, leading to shifts between cooperative structural order and disorder [24]. During the process of cooling, the reversible decrease in α-helical and β-sheet conformation in apoB-100 is evident below the phase transition of the lipoprotein core and corresponds to the ordering of core lipids [25]. These transitions are most apparent at the phase transition temperature and also influence oxidative stability [24].

Temperature changes, limited proteolysis, increased ionic strength of the medium, oxidation or addition of detergents are expected to induce a loss of apoB secondary structure and conformational changes. Under these conditions a loss of α-helical structure occurs while the relative percentage of the more stable β-structure increases. This can be regarded as a misfolding event and is accompanied by coalescence of lipids, particle aggregation and eventual fusion into large droplets and multi-membranous material [26]. The latter represent the structural features of oxidized LDL that is entrapped within the subendothelial matrix [27].

The structure of LDL determines binding affinities to receptors that mediate regulated *versus* unregulated uptake, and thereby an altered structure is expected to affect the metabolic fate of LDL. In general, a loss of apoB-100 structure is thought to account for diminished uptake by normal LDL receptors and increased uptake by alternate receptors [28] where the potential for intracellular accumulation and adverse effects is higher.

LDL modification in the degenerative disease: atherosclerosis

Atherosclerosis is a progressive disease of the large arteries where lipids and proteins derived from circulating LDL accumulate both within cells and in the extracellular space. The early phase of the disease exhibits the hallmarks of a 'response to injury' that manifests in endothelial cells and adjacent tissues. It is now widely held that injury from high cholesterol levels is actually a result of cytotoxic forms of LDL [28]. The ensuing apoptosis of endothelial cells and expression of inflammatory cytokines activates the recruitment and proliferation of inflammatory cells while massive lipid peroxidation of LDL takes place in the subendothelial space [29].

Structurally modified LDL is taken up by resident macrophages through various scavenger receptors, however, modified LDL also accumulate as

extracellular droplets produced by vesicle fusion and aggregation to proteoglycans. This process can be perpetuated by the accumulation of activated inflammatory cells that oxidatively modify LDL [29]. Analogous to the effects of Alzheimer's disease β-amyloid, the misfolded proteins of modified LDL could be sensed as a 'foreign body' and induce the production of reactive oxygen species [30].

LDL oxidation and Apo-B structure: a common link to an oxidative origin for degenerative diseases

Several candidate mechanisms have been proposed for the oxidative modification of LDL in plasma and the arterial wall. In most instances, modification is promoted by inflammatory cells, following an early response to injury brought about by endothelial cells. The oxidizing species are identified as reactive oxygen and reactive nitrogen species [31]. These reactive products can directly attack either the protein or lipid components of LDL. In some instances, a preferential reactivity has been described for proteins indicating that apoB-100 modification can occur with minimal oxidation of lipids [32]. Cell-mediated oxidation of LDL is perhaps the most likely mechanism for modification that produces atherogenic particles, and the activities of NADPH oxidase, myeloperoxidase and nitric-oxide synthase have the chemical potential for modifying LDL lipids and apo-B, thus producing an atherogenic lipoprotein.

The conventional view of LDL oxidative modification to atherogenic particles maintains that extensive degradation of polyunsaturated fatty acids yields decomposition products such as reactive aldehydes that in turn modify specific amino-acid residues (notably lysine) [28]. More recently, evidence for direct oxidation of the protein indicates that preferred oxidation of specific amino-acid residues (particularly tyrosine, phenylalanine, cysteine, tryptophan and methionine) occurs through the action of hemoproteins such as myeloperoxidase myoglobin or hemoglobin. Indeed, proteins might be as susceptible to oxidation as lipids [34,35], but the precise contribution of lipid *versus* protein oxidation in terms of modification of LDL to atherogenic particles remains unclear. Identification of specific agents responsible for this modification will be a difficult matter to resolve given that, as discussed above, changes in lipids are likely to evoke a change in apoB-100, and vice versa.

An interesting link between apoB structure and conformation and oxidative stability of LDL has been recently observed using 17-β-estradiol. This steroid binds with high affinity to a specific binding site in LDL and this increases resistance to oxidation, apparently by stabilizing the dynamic secondary structure [36].

Apo-B is misfolded in LDL⁻ isolated from plasma

An *in vivo* modified LDL, referred to as LDL^- by virtue of its increased negative surface charge, has been identified and characterized by several groups. This lipoprotein is associated with the small dense LDL fraction that is considered to be atherogenic [37]. Accordingly, it is cytotoxic to vascular cells and induces pro-inflammatory cytokine production analogous to the atherogenic forms of minimally modified LDL [37]. LDL^- is present in plasma at concentrations ranging from 0.1 to 5% of LDL, but increases remarkably under different conditions that can produce modification of LDL structure. An increased percentage of LDL^- has been observed in hypercholesterolemia [38,39], in type 2 diabetes [40,41], in uremia [42], following exhaustive physical exercise [43] and in post-prandial lipemia [44]. *In vitro* particles similar to LDL^- have been produced by incubating LDL in the presence of endothelial cells [45], hemoproteins [32], myelopereoxidase [46] or lipase [47].

It is worth noting that oxidative modifications are not necessarily the only way to produce the same population of distinct electronegative particles. Nevertheless, LDL^- isolated from plasma is enriched with lipid oxidation products, although the amounts are low and not readily measured by conventional methods [48]. Particularly relevant to the effect on particle structure is the increased content of lipid hydroperoxides (ranging from 0.3 to 1.7 nmol/mg cholestrol), which is sufficient to profoundly alter lipid packing and water penetration into the surface monolayer. The general polarization of the amphipathic probe Laurdan indicates that the water–lipid boundary of LDL^- particles is altered, allowing deeper water penetration [49]. This is expected to influence apo-B secondary structure. Indeed, circular dichroism spectra for LDL^- show an almost complete loss of α-structure whereas β-structure appears more conserved [49], as expected because of its greater stability. This loss of secondary structure is similar to the features of copper-oxidized LDL, although in the latter case, lipid peroxidation is massive and the apoB is usually fragmented. Alterations at the water–lipid boundary, and the loss of secondary structure, enable new interactions at hydrophobic domains that account for a general mechanism for protein unfolding. Tryptophan fluorescence lifetime measurements show a predominant component at a very long lifetime suggesting that tryptophan residues have relocated into an extremely lipophilic environment. This feature appears to be unique for LDL^- as shorter tryptophan fluorescence lifetimes are observed for copper-oxidized LDL [36]. Thus, following oxidation *in vitro*, secondary structure is lost and the protein unfolds into a conformation where tryptophan residues are more exposed to water. The difference between copper-oxidized LDL and LDL^- is also apparent by electron microscopy, where heavily oxidized LDL appears as coarse aggregates of disintegrated particles, while LDL^- forms long branched polymer aggregates (Fig. 1).

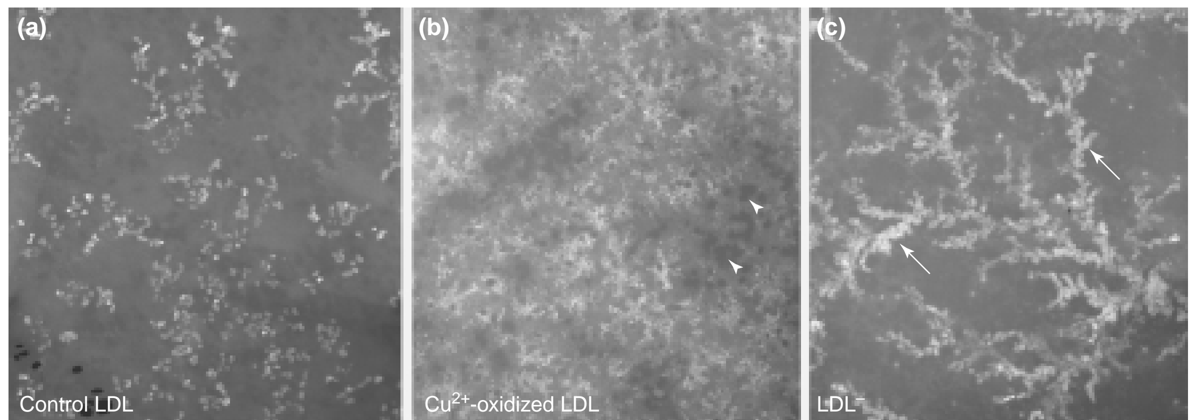

Fig. 1. Low-density lipoproteins (LDL), Cu^{2+}-oxidized LDL, and LDL^-. Samples were deposited onto coated copper electron microscopy grids and stained with osmium tetroxide. The visual fields shown were selected based on the representative appearances of each LDL preparation and differences in the number of LDL particles in the field vary as a result of the extent of aggregation. (a) Control LDL (freshly isolated total LDL) was more evenly distributed over the entire grid, whereas (b) Cu^{2+}-oxidized LDL (freshly isolated LDL using 10-μM $CuSO_4$ for 6 h at 37°C) and (c) LDL^- (isolated by anion exchange HPLC as described in Ref. [48]) were distributed in specific areas as aggregates, while other areas were devoid of particles. Cu^{2+}-oxidized LDL appears as a disintegrated mass of lipid and protein (dark staining, arrowheads), while LDL^- exists as long bead-like aggregates with intact particles (arrows). Magnification, 20 000×.

Aside from the different conformations attained by misfolded apo-B, a peculiar feature of LDL^- that distinguishes it from *in vitro* oxidized LDL is that changes in apoB-100 take place in the absence of substantial lipid peroxidation. Indeed, evidence for increased lipid hydroperoxide content requires very sensitive methodologies, such as single-photon counting. To account for the small increase for lipid hydroperoxides along with a major alteration in protein structure, one might postulate that lipid hydroperoxides are partially removed, reduced or otherwise processed, whereas protein alteration is irreversible. The mode of LDL oxidation that yields LDL^- must be smoother, more limited and more specific than *in vitro* methods for modification, and implies that biological processing is required as the particle is modified. This is expected in plasma where oxidative processes are gradual and limited because of the strongly antioxidant nature of the environment.

Besides oxidation, modification of LDL structure might also be attained through detergent-like effects. Recent studies indicate that LDL^- represents a population of particles enriched with non-esterified fatty acids (NEFA) [43]. Enrichment with NEFA beyond a critical threshold could disrupt the organization of particle surface lipids and their interaction with apoB while enhancing the oxidative susceptibility of lipids [50].

If LDL particles become more than minimally modified, they would be rapidly cleared by the scavenger receptors of the reticulo-endothelium system. In the sub-endothelial space, minimally modified particles such as LDL^- are likely to become massively oxidized [28,47,50].

Concluding remarks

Having lost the supramolecular structure that maintains particle stability, *in vivo* modified LDL^- is highly prone to oxidation and aggregation in the subendothelial space where it has a cytotoxic and pro-inflammatory effect [51]. The loss of α-helix and the prevalence of β-sheet structure in apoB-100 might also impart resistance to proteolysis, thus contributing to pathogenic events. These effects are analogous to β-amyloid and possibly many other misfolded proteins [8], and represent a degenerative-inflammatory process in the arterial wall.

From the available data we propose that following an initial event leading to destabilization of LDL structure and conformation, the misfolded particle becomes prone to aggregation and oxidation, is sensed as 'non-self' and becomes resistant to available repair and proteolytic mechanisms. The response to injury brought about by misfolded LDL becomes the driving event for atherogenesis, but could evolve into different disease states for different tissues.

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