Aggregation of LDL with chondroitin-4-sulfate makes LDL oxidizable in the presence of water-soluble antioxidants

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Abstract The content of plasma and arterial interstitial fluid in water-soluble antioxidants makes it unlikely for low-density lipoprotein (LDL) to oxidize by the oxidation mechanisms most frequently discussed. By aggregation of LDL in the presence of chondroitin-4-sulfate (C-4-S), but not with chondroitin-6-sulfate or sphingomyelinase, a complex arises which can oxidize in the presence of 20 μM ascorbate and 300 μM urate. This oxidation sensitivity even persists after the gel-filtration of an LDL/C-4-S/Cu^{2+} complex, indicating entrapment of Cu^{2+} within. This corresponds well to the known ability of C-4-S to bind copper ions and is a potential mechanism by which LDL oxidation in the arterial intima is facilitated after prolonged retention by the extracellular matrix.

Key words: Atherosclerosis; Lipid peroxidation; Antioxidant; Extracellular matrix; Glycosaminoglycan; Chondroitin-4-sulfate

1. Introduction

The oxidation of low-density lipoprotein (LDL) is supposed to be a crucial event in atherogenesis (for a review see [1]) but it remains unclear to date by which mechanisms and by which oxidants lipoproteins are oxidized in the arterial wall in vivo.

Recently, it has clearly been shown that lipoproteins do not oxidize [2] in the presence of water-soluble antioxidants, such as ascorbate (AscH) and urate (UA), which are present in the subendothelial lining fluid at similar concentrations as in plasma (20–100 μM AscH and 300–400 μM UA) [3]. However, several lines of evidence indicate a large extent of lipid peroxidation in atherogenesis [4], hence the question arises by which mechanism(s) LDL could be oxidized indeed in an antioxidant-rich environment. One such possibility for lipid peroxidation in the arterial wall is by aggregation, under which circumstances metal ions found in atherosclerotic deposits [5], such as Cu^{2+} and Fe^{3+}, might become trapped within aggregates, in addition to the prolonged retention of such aggregates, which itself is likely to favor oxidation. Aggregation of LDL has been shown to arise from shear stress (e.g. by vortexing or shaking) and is enhanced by many factors, such as glycosaminoglycans and proteoglycans [6], or by enzymes, e.g. sphingomyelinase (SMase) [7]. Moreover, aggregates of LDL with glycosaminoglycans and proteoglycans were previously found to oxidize more easily than isolated LDL [8,9]. However, it has never been investigated whether and how such aggregates can oxidize in the presence of water-soluble antioxidants.

Copper ions have been used frequently as a model to induce oxidation of LDL but their relevance for oxidation in vivo has been questioned. Nevertheless, there is increasing evidence that Cu^{2+} is found in atherosclerotic plaque either in free form [10], colocalizing with iron and ceroid [11] or bound to protein, such as ceruloplasmin. Such ceruloplasmin-bound Cu^{2+} may be redox-active by itself [12], even in the presence of antioxidants [13], or become liberated by the low pH at inflammatory sites [5] or through the action of nitric oxide [14].

By aggregation of LDL not only the ratio of surface to volume of the lipoproteins is decreased unfavorably for aqueous antioxidants in order to prevent oxidation, also Cu^{2+} ions eventually trapped within the aggregates might start redox-cycling, a process which is thought to be triggered by reduction of the metal by α-tocopherol [15,16]. It might be enhanced if there is no interference by aqueous antioxidants, which may lead to the regeneration of α-tocopherol by AscH, for example.

12/15-Lipoxygenase (12/15-LOX) has been shown to be involved in the formation of atherosclerotic lesions [17], and recently it has been demonstrated in an apoE-knockout mouse model that the expression of 12/15-LOX might contribute considerably to the formation of atherosclerotic plaques [18]. It has been shown that 'seeding' of LDL with hydroperoxides produced by incubation with 12/15-LOX leads to enhanced sensitivity of LDL to Cu^{2+}-induced oxidation [19].

Therefore we wanted to test the hypothesis that aggregation of LDL with glycosaminoglycans can lead to facilitated oxidation even in the presence of high concentrations of water-soluble antioxidants, which would otherwise prevent LDL oxidation.

2. Materials and methods

All materials were of analytical grade or better, and from Sigma or Merck (Vienna, Austria), unless specified otherwise. Chondroitin-4-

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Abbreviations: LDL, low-density lipoprotein; AscH, ascorbate, ascorbic acid; UA, urate, uric acid; SMase, sphingomyelinase; 12/15-LOX, 12/15-lipoxygenase; EDTA, ethylenediaminetetraacetic acid; LL-CL, low-level chemiluminescence; C-4-S, chondroitin-4-sulfate; C-6-S, chondroitin-6-sulfate

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sulfate (C-4-S) and chondroitin-6-sulfate (C-6-S) were from Sigma (C-4-S from bovine trachea, 70%, containing 30% C-6-S; C-6-S was from shark cartilage, 90%, 10% C-4-S) and used as obtained from the manufacturer. AscH was from Loba (Fischamend, Austria), 12/15-LOX from rabbit reticulocytes was a kind gift of Prof. Dr. Hartmut Kühn, Charité, Berlin, Germany.

Experiments shown are representative of at least two independent experiments.

2.1. Isolation of human LDL

LDL was prepared from pooled ethylenediaminetetraacetic acid (EDTA) plasma of healthy volunteers of both sexes by ultracentrifugation in a single-step discontinuous density gradient in a Beckman NVT65 rotor, as described [20]. Prior to use, LDL was stored in an evacuated glass vial under argon at 4°C for a maximum of 2 weeks. LDL concentration was determined from its total cholesterol content, using the CHOD-PAP enzymatic test kit (Boehringer-Mannheim, Germany), assuming a molar mass of LDL of 2.5 MDa and a cholesterol content of 32.2 wt%.

2.2. Monitoring of LDL oxidation by low-level chemiluminescence (LL-CL)

Prior to oxidation of LDL, EDTA and KBr were removed by gel-filtration as described [21]; briefly, 1 ml of LDL solution was loaded onto a Bio-Rad Econo-Pac 10DG column (Bio-Rad, Richmond, CA, USA), and eluted with 3 ml PBS (phosphate-buffered saline: 160 mM NaCl, 10 mM Na-phosphate, pH 7.4; containing 2 mM $CaCl_2$and 4 mM $MgCl_2$), collecting LDL in the last ml of eluent. Oxidation was performed (after addition of antioxidants, glycosaminoglycans and enzymes, if applicable) at 37°C by addition of a 100 μM solution of $CuSO_4$to a 0.3 μM solution of LDL in PBS, to give a final concentration of 4.3 μM Cu^{2+}.

LL-CL was measured in a Lucy 1 luminometer (Anthos Labtech Instruments, Salzburg, Austria) equipped with a photon counting photomultiplier (sensitivity ranging from 300 to 700 nm). Integration time for each data point was set to 90 s. The assays were performed at 37°C, in a white microplate. The source of LL-CL is the decay to the ground state of triplet carbonyl compounds produced by recombination of lipid peroxyl radicals to non-radical products [22]. It was shown that the lag time of LL-CL closely corresponds to the lag in the formation of conjugated dienes, and that the intensity of LL-CL is proportional to the square of the oxidation rate. Its advantage over spectrophotometric detection of conjugated dienes is that its performance is not impeded by the presence of UV-absorbing compounds in the medium. Experiments were performed at least twice.

2.3. Aggregation of LDL

LDL was aggregated in the presence of C-4-S or C-6-S in the presence of Ca^{2+} and Mg^{2+} (2 mM and 4 mM, required for the activity of 12/15-LOX and SMase, respectively) and/or vortexing, using the shake mode of the luminometer. Aggregation by SMase (*Bacillus cereus*) was performed essentially as described in [7], except that the buffer was PBS (plus 2 mM $CaCl_2$, 4 mM $MgCl_2$).

The aggregation of lipoproteins was monitored photometrically measuring the increase of turbidity at 680 nm.

3. Results

3.1. Oxidation of LDL aggregated by shear forces and by the action of SMase

LDL (0.3 μM) was aggregated either by vigorous shaking in the luminometer (1 min) or by preincubation with SMase (up to 50 mU, 30 min, 37°C) in the presence of 20 μM AscH and 300 μM UA before the addition of Cu^{2+}. Fig. 1 shows that compared to the antioxidant-free control, the presence of AscH and UA considerably prolonged the lag phase of LDL oxidation, about 20-fold. Aggregation by shaking did not significantly increase the oxidizability of LDL in the presence of AscH and UA. Neither was a prooxidant effect observed after enzymatic aggregation with SMase, at high concentrations of SMase the enzyme even prolonged the lag phase. Interestingly, inclusion of 20 μM AscH and 300 μM UA increased LL-CL intensity by about 60% (corresponding to about 30% increase in oxidation rate, as LL-CL is proportional to the square of the oxidation rate).

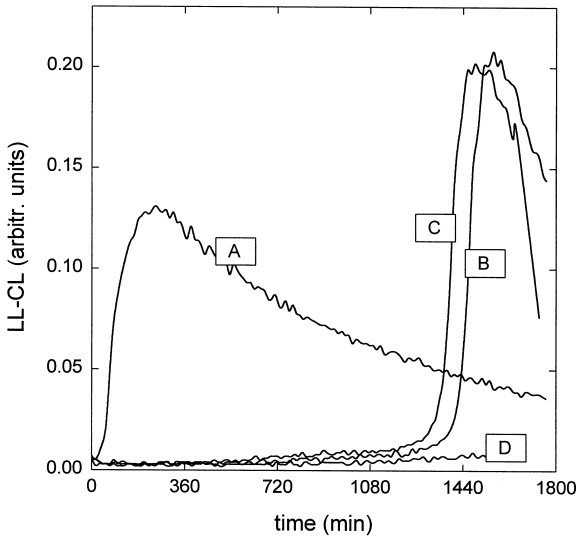

Fig. 1. LDL oxidation after aggregation by shear forces and SMase. LDL was incubated at 0.3 μM, oxidation was induced by addition of 4.5 μM Cu^{2+}. (A) Control, (B) 20 μM AscH+300 μM UA, (C) like (B), but shaken for 1 min, (D) like (B), but after 30 min preincubation with 50 mU SMase.

3.2. Effect of aggregation by glycosaminoglycans, C-4-S, and C-6-S

LDL (0.3 μM) was oxidized by incubation with 4.8 μM Cu^{2+} after addition of C-6-S (Fig. 2A) or C-4-S (Fig. 2B) at 0.25–1 mg/ml, in the presence of 20 μM AscH and 300 μM UA. A significant decrease of the lag time before onset of rapid oxidation compared to the antioxidant-containing control (trace B) was observed, strongly depending on the concentration of C-4-S. C-6-S was considerably less effective than C-4-S and did not exhibit a pronounced concentration dependence like C-4-S.

To make sure that aggregation is responsible for the observed effects, LDL (3–4 mg/ml), C-4-S (1.3 mg/mg LDL) and Cu^{2+} (6.4 μM/mg LDL) were thoroughly mixed and briefly vortexed. The mixture was gel-filtrated as described in Section 2.2 and diluted to give 0.3 μM LDL (leading to dilutions of the other constituents comparable to the experiments described above), and AscH and UA added to give 20 μM and 300 μM, respectively. Oxidation was monitored by LL-CL, of the complex alone or in the presence of 100 mU chondroitinase ABC, without addition of further Cu^{2+}. Fig. 3 shows that the gel-filtrated complex oxidized in the presence of the antioxidants (trace A), unless the integrity of the glycosaminoglycan was destroyed with chondroitinase (trace B).

3.3. Increased susceptibility to oxidation of LDL aggregates with C-4-S and SMase after seeding with 12/15-LOX

Incubation with 12/15-LOX has been shown to increase the content of LDL in hydroperoxides, in particular cholesteryl ester hydroperoxides [19]. Such 'seeding' has been proposed to be a potential mechanism for the atherogenicity of 12/15-LOX. When non-aggregated LDL was incubated with 12/15-LOX for 30 min at 37°C and then aggregated by addition of

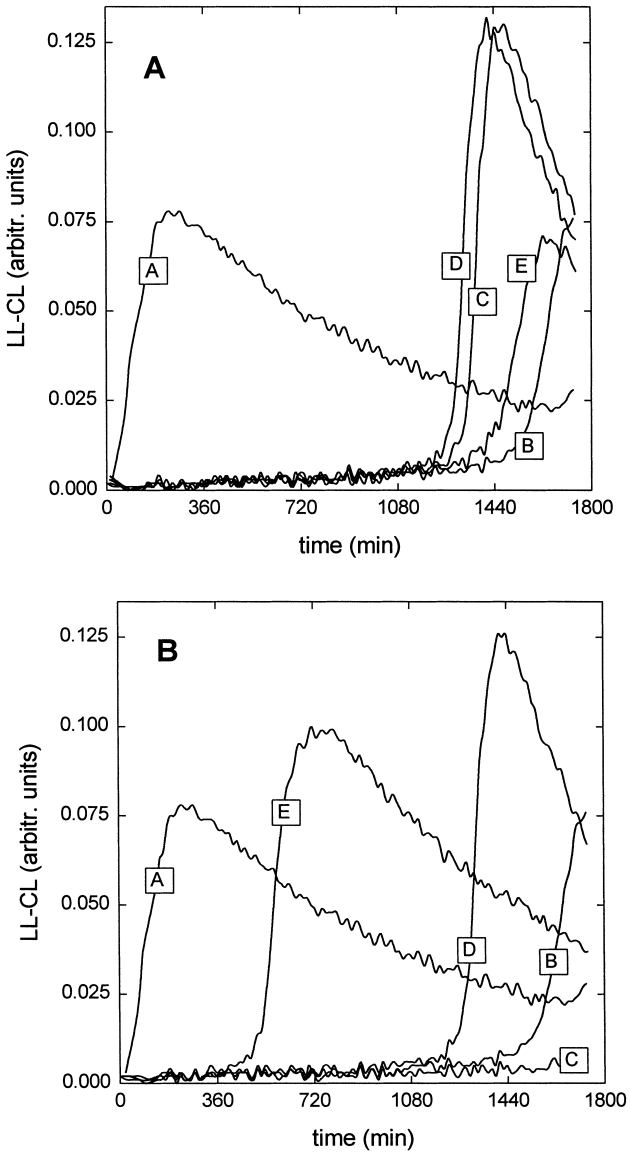

Fig. 2. LDL oxidation in the presence of C-4-S and C-6-S. LDL was incubated as in Fig. 1. A: (A) Control, (B) as (A) plus 20 μM AscH and 300 μM UA, (C) as (B) plus 0.25 mg/ml C-6-S, (D) as (B) plus 0.5 mg/ml C-6-S, (E) as (B) plus 1.0 mg/ml C-6-S. B: (A) Control, (B) as (A) plus 20 μM AscH and 300 μM UA, (C) as (B) plus 0.25 mg/ml C-4-S, (D) as (B) plus 0.5 mg/ml C-4-S, (E) as (B) plus 1.0 mg/ml C-4-S.

SMase, a strong prooxidant effect of 12/15-LOX was found (Fig. 4) after initiating lipid peroxidation by addition of Cu^{2+}. Practically identical effects were observed whenever LDL was aggregated first by C-4-S (or SMase – not shown for clarity) and then incubated with 12/15-LOX. It should be noted that incubation with 12/15-LOX does not increase oxidizability of non-aggregated LDL by Cu^{2+} in the presence of AscH and UA (not shown), hence under these conditions, 12/15-LOX-derived lipid hydroperoxides enhance the oxidation sensitivity of the complex only.

4. Discussion

The interaction of lipoproteins with glycosaminoglycans and proteoglycans of the endothelial extracellular matrix has been suggested to be one of the key factors in atherogenesis [23,24]. Increased retention of LDL in the subendothelial space of the arterial intima, and consequentially a prolonged exposure to oxidants was hypothesized to be involved in the 'response-to-retention' hypothesis. It has been demonstrated earlier that the interaction of LDL with glycosaminoglycans and proteoglycans (in particular those of C-6-S) increases its susceptibility towards oxidation, albeit this effect was observed only in the absence of water-soluble low-molecular

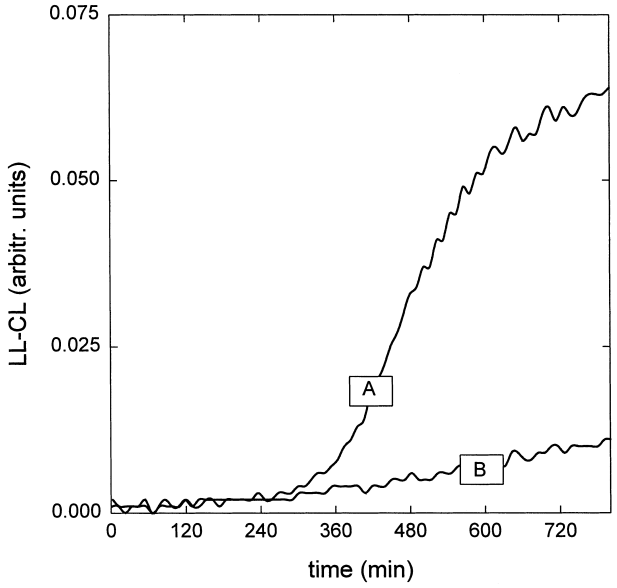

Fig. 3. LDL (3–4 mg/ml), C-4-S (1.3 mg/mg LDL) and Cu^{2+} (6.4 μM/mg LDL) were briefly vortexed, gel-filtrated and then diluted to 0.3 μM LDL. Oxidation was monitored in the presence of 20 μM AscH and 300 μM UA (without further addition of Cu^{2+}), in the absence (A) and presence of chondroitinase ABC (B).

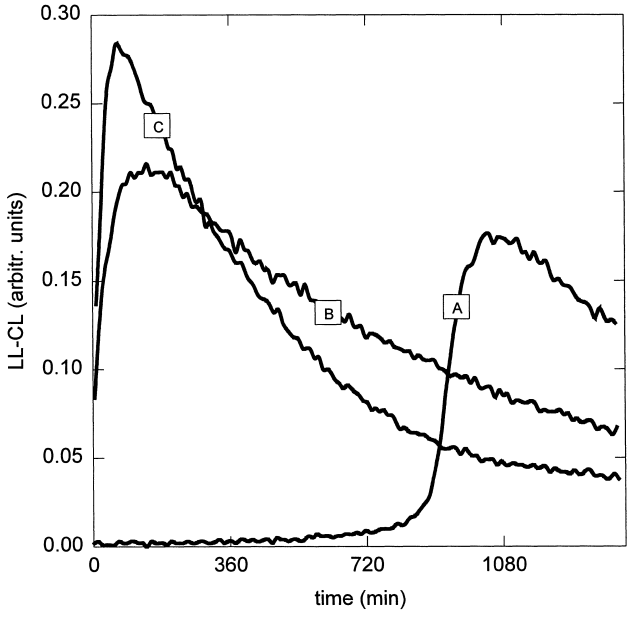

Fig. 4. LDL oxidation after aggregation and preincubation with 12/15-LOX. LDL was oxidized as in Fig. 1. (A) Control, (B) 30 min preincubation with 0.6 μM LOX in the presence of 0.5 mg/ml C-4-S, (C) 30 min incubation with 12/15-LOX after 30 min preincubation with SMase.

mass antioxidants [8,9]. However, the subendothelial compartment is rich in such antioxidants from the circulation, like UA and AscH [3], which can efficiently prevent LDL oxidation both by metal-catalyzed and by radical-induced mechanisms, probably even during prolonged retention.

It is quite striking that only the aggregation of C-4-S with LDL, as shown in this work, can circumvent the antioxidant protection by antioxidants in metal-catalyzed oxidation, while C-6-S and enzymatic aggregation proved ineffective. It has been described previously that C-4-S is able to bind Cu^{2+} ions which leads to inhibition of the oxidation of non-aggregated LDL, unlike C-6-S [25] which cannot bind Cu^{2+}. Hence, a possible explanation for the increased sensitivity of C-4-S/LDL aggregates towards Cu^{2+}-mediated oxidation might be entrapment of comparatively high concentrations of Cu^{2+} within these aggregates, corroborated by the fact that mixtures of LL, C-4-S and Cu^{2+} can oxidize after gel-filtration: if Cu^{2+} was not trapped within the complex, it would have been removed by gel-filtration. That C-4-S is responsible for the binding of Cu^{2+} in these complexes is also supported by the observation that low concentrations of C-4-S even increase resistance of LDL towards oxidation (Fig. 2B, curve C), while at higher concentrations (approaching concentrations in the arterial intima) the entrapment effect prevails, leading to decreased resistance towards oxidation.

These findings indicate that aggregation or interaction of glycosaminoglycans and proteoglycans alone with LDL may not be sufficient to increase sensitivity of LDL towards oxidation in the presence of water-soluble antioxidants, as was originally postulated [8,9], albeit for a system free of such antioxidants. Neither do other mechanisms of aggregation increase oxidizability under these conditions, such as shear forces and the action of enzymes, which do not lead to the additional incorporation of Cu^{2+} ions. Hence, for the efficient oxidation of LDL in the presence of AscH and UA, concomitant aggregation and entrapment of Cu^{2+} ions is probably a prerequisite.

Catalytic Cu^{2+} might thus catalyze extensive LDL oxidation protected from the interference by water-soluble antioxidants which would otherwise serve to regenerate the lipophilic antioxidants in LDL [26]. This is also consistent with our finding that preincubation of LDL with 12/15-LOX greatly increases the oxidizability of C-4-S/LDL aggregates, while there was no such effect of 12/15-LOX on pure LDL aggregates prepared by SMase, or on C-6-S/LDL aggregates, and neither on unaggregated LDL. Lipid hydroperoxides seeded into LDL by the action of 12/15-LOX become effective only when they are able to enhance redox-cycling of trapped Cu^{2+}, and when protected from water-soluble antioxidants. The additional antioxidant effect observed with SMase might be due to a general reduction in sensitivity of LDL towards oxidation by enzymatic cleavage of sphingomyelin [27].

In this work we have described a possible mechanism how the antioxidant load of plasma or endothelial lining fluid may be overcome to permit lipid peroxidation processes which eventually lead to the large amount of oxidized lipid found in atherosclerotic lesions. The role glycosaminoglycans and proteoglycans may play in atherogenesis is underlined by recent findings which indicate that minimally modified LDL can induce the secretion of such proteoglycans which increasingly retain native LDL in the arterial intima [28]. This process could trigger the increasing accumulation of lipoproteins which could then be extensively oxidized in the intima even though in this environment antioxidants are present in abundance.

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