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Low density lipoprotein oxidizability by copper correlates to its initial ubiquinol-10 and polyunsaturated fatty acid content

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Abstract

At an early stage of oxidation induced by Cu^{2+}, the rate of oxidative modification of human low density lipoprotein (LDL) from healthy donors correlated negatively to its ubiquinol-10 ($r = -0.58$, $P < 0.01$) and positively to its polyunsaturated fatty acid (PUFA) ($r = 0.53$, $P < 0.05$) content. The PUFA/ubiquinol-10 ratio was the best predictor of LDL susceptibility to oxidation ($r = 0.68$, $P < 0.01$). No significant correlation between LDL oxidizability and its α-tocopherol content was found at any oxidation stage. It is suggested that ubiquinol-10 plays a central role in the early protection of LDL PUFAs against Cu^{2+}-induced oxidation whereas α-tocopherol posesses both pro- and antioxidant activity.

Key words: Low density lipoprotein; Lipid peroxidation; Ubiquinol-10; Polyunsaturated fatty acids; α-Tocopherol; Atherosclerosis

1. Introduction

Much evidence exists to implicate the oxidation of low density lipoprotein (LDL) in the early development of atherosclerosis [1,2]. Owing to this implication, a possible conjunction between oxidizability and chemical composition of human LDL has recently been extensively studied [3,4]. The relative importance of different LDL constituents in the prevention of LDL oxidation, however, remains unclear.

It can be assumed that LDL antioxidant content should be one of the most important factors determining LDL susceptibility to oxidation. It is well established that α-tocopherol is, on a molar base, the major antioxidant in LDL [3,4]. All other antioxidants (γ-tocopherol, carotenoids, ubiquinol-10) are present in LDL in much smaller amounts. However, a number of researchers have found no correlation between α-tocopherol content and susceptibility to oxidation in native LDL not supplemented with antioxidants [5–12]. It has been shown that ubiquinol-10 can protect human LDL more efficiently against lipid peroxidation than α-tocopherol [13–15], despite the much higher LDL content of the latter.

LDL oxidizability may also depend on an amount of oxidizable substrate available within the LDL particle, i.e. on LDL polyunsaturated fatty acid (PUFA) content: it was recently reported that the linoleic-to-oleic acid ratio correlates positively to the oxidizability of native LDL not enriched in fatty acid [16]. Some other factors, such as initial LDL hydroperoxide [17,18] or protein [14,16] content, may also play an important role in the process of LDL oxidation.

In order to better understand how different LDL constituents might influence its susceptibility to oxidation, we characterized antioxidant as well as total composition of human LDL and compared each with LDL oxidizability by copper, measured as accumulation of conjugated dienes in the LDL sample. The results suggest that, at an early stage of the oxidation, protection of LDL PUFAs is provided mainly by ubiquinol-10 while α-tocopherol functions as a pro- rather than an antioxidant. Consequently, LDL ubiquinol-10 and PUFA contents presumably represent the most important factors determining the susceptibility of LDL to the copper-induced oxidation.

2. Materials and methods

2.1. Chemicals

Ubiquinone-10, ubiquinone-9, α-tocopherol and δ-tocopherol were obtained from Fluka (Neu-Ulm, Germany). All other chemicals and solvents were from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Ubiquinol-9 was prepared from ubiquinone-9 by reduction with sodium dithionite [19].

2.2. Isolation and characterization of LDL

LDL was isolated using density gradient ultracentrifugation of human plasma for 20 h at 4°C in the presence of 1.5 mM EDTA [20].

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; MUFA, monounsaturated fatty acid; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances.

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Plasma was obtained by low-speed centrifugation of blood from 20 apparently healthy donors aged 22–51 years (mean 34.3) who were on neither an antioxidant nor a fatty acid diet. Blood taken in EDTA-containing tubes was immediately centrifuged at 4°C for 10 min.

Ubiquinol-10, ubiquinone-10, α-tocopherol and γ-tocopherol content of LDL was quantified immediately after LDL isolation by reversed-phase HPLC with electrochemical detection (Finckh, B., unpublished data). Briefly, 100 μl LDL was mixed with 100 μl ethanol containing necessary amounts of ubiquinol-9, ubiquinone-7 and δ-tocopherol as internal standards. After adding 100 μl water and 500 μl hexane, the mixture was vortexed, centrifuged and the hexane extract was collected and evaporated under argon. The residue was dissolved in 125 μl reagent alcohol/methanol (1:1 v/v) and subjected to HPLC with a Coulochem 5100A electrochemical detector (Environmental Sciences Assoc., Bedford, MA, USA) equipped with a Model 5011 analytical cell and a Model 5020 conditioning cell. The system was set as follows: conditioning cell −0.60 V, detector 1 −0.15 V, detector 2 +0.60 V. A methanol/ethanol/2-propanol (1,025:451:24 v/v) mixture containing 13 mM lithium perchlorate was used as a mobile phase at a flow rate of 1.2 ml/min.

Total cholesterol, free cholesterol, triglyceride, phospholipid and total lipid content of LDL were determined with commercially available enzymatic kits. LDL cholesterol ester content was calculated from the values obtained. Total protein was measured with the Pierce reagent (Pierce, Oud-Beijerland, The Netherlands) using human serum albumin as a standard. Fatty acid composition of LDL was characterized by capillary gas chromatography as described elsewhere [21]. Absolute amounts of fatty acids in LDL were calculated using the data on LDL lipid composition assuming that the lipoprotein does not contain any free fatty acids.

LDL TBARS were measured using 0.375% thiobarbituric acid and 15% trichloroacetic acid dissolved into 0.25 M HCl [22]. Lipid peroxides were estimated with Merck CHOD-iodide cholesterol color reagent (Merck, Darmstadt, Germany) [23].

To recalculate the values obtained into mol/mol LDL, an LDL molecular weight of $2.5 \cdot 10^6$ was used [4].

2.3. Oxidation of LDL

Immediately after LDL isolation, EDTA and potassium bromide were removed from the LDL suspension by gel filtration on Sephadex PD-10 columns (Sephadex G-25M; Pharmacia Fine Chemicals, Uppsala, Sweden). The EDTA-free LDL suspension was diluted with phosphate-buffered saline (PBS) to a final cholesterol concentration of 0.12 mg/ml (approximately 0.14 μM LDL) and the oxidation was started by addition of freshly prepared copper sulphate to a final concentration of 2.0 μM. All the oxidation experiments were performed at 25°C and completed within 30 h from the moment of blood collection.

The level of LDL oxidation was estimated by accumulation of conjugated dienes in the LDL sample. Conjugated dienes were measured according to Esterbauer et al. [24] as an increase in the absorption of the sample at 234 nm. Duration of the lag-phase of diene accumulation was calculated from the time courses obtained (see Fig. 1).

3. Results

LDL used in the experiments revealed no abnormalities in chemical composition (Table 1). LDL content of the main lipid classes corresponded well with data published elsewhere [3,4]. The LDL concentration of ubiquinol-10 was almost 3-fold higher than that reported by Esterbauer et al. [4] but about 2-fold lower than that reported by Bowry et al. [25]. It is well known that ubiquinol-10 is a highly unstable compound and easily oxidized into the corresponding quinone, even under careful handling [26]. Hence, the apparent discrepancy found might simply be due to the fact that our LDL isolating procedure lasted longer than that of Bowry et al. [25] but was shorter than that of Esterbauer et al. [4].

LDL content of ubiquinone-10 ranged within previously reported values [25,27]. LDL content of α-tocopherol and γ-tocopherol, as well as of major classes of fatty acids, corresponded well with reference values [4]. Lipid peroxidation products (lipid peroxides measured iodometrically and TBARS) were slightly decreased in our LDL as compared with others [4], also presumably due to our relatively short procedure for isolating LDL.

Incubation of LDL with Cu^{2+} (about 17 Cu^{2+} ions per 1 LDL particle) led to the oxidation of the lipoprotein as judged by accumulation of conjugated dienes in the LDL sample (Fig. 1). This was confirmed by parallel measurements of TBARS and lipid peroxides (data not shown). The time–course of diene accumulation was similar to that originally reported by Esterbauer et al. [24]. The oxidation was characterized by a distinct lag-phase (100.2 ± 25.4 min) followed by propagation and decomposition phases.

In accordance with previous findings [4,7,8], no significant correlation was found between the duration of the lag-phase of diene accumulation and the initial LDL content of α-tocopherol ($r = 0.295$, $P = 0.207$) or PUFAs ($r = 0.097$, $P = 0.684$). Nor was a significant correlation found between the duration of the lag-phase and initial LDL ubiquinol-10 concentration ($r = 0.160$, $P = 0.501$). Nevertheless, when the data were examined as rates of diene accumulation measured between definite moments within the lag-phase (see Fig. 1), a series of significant correlations were found (Fig. 2). The correlations were only significant at an early stage of LDL

Table 1
Chemical composition of LDL preparations

Compound	LDL content (mol/mol LDL)
Ubiquinol-10	0.280 ± 0.071
Ubiquinone-10	0.263 ± 0.121
Total ubiquinone-10 + ubiquinol-10	0.543 ± 0.153
α-Tocopherol	6.11 ± 2.63
γ-Tocopherol	0.56 ± 0.20
PUFAs	1,194 ± 224
MUFAs	536 ± 66
Saturated fatty acids	676 ± 56
Double bond index	1.05 ± 0.17
Lipid peroxides	3.69 ± 2.76
TBARS	0.16 ± 0.10
	Percent of total LDL weight
Cholesterol esters	41.0 ± 4.3
Free cholesterol	10.5 ± 2.5
Total cholesterol	33.8 ± 3.3
Phospholipids	18.5 ± 2.1
Triglycerides	4.7 ± 1.7
Total lipids	74.7 ± 4.6
Total protein	25.3 ± 4.6

Data are presented as means ± S.D. LDL ($n = 20$) was isolated from freshly collected plasma using density gradient ultracentrifugation for 20 h at 4°C. EDTA was present at all steps of isolation.

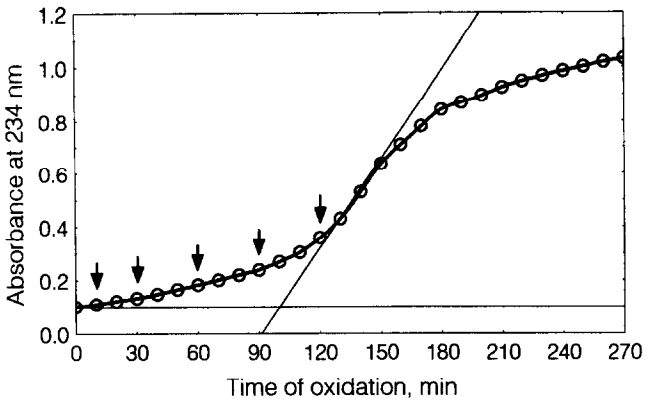

Fig. 1. Accumulation of conjugated dienes in LDL samples measured as an increase of the absorbance of the sample at 234 nm after addition of Cu^{2+}. The curve was averaged for 20 samples studied. The straight lines illustrate the lag-phase calculation method [4]. The arrows show the moments used for calculation of correlations. LDL concentration, 0.12 mg total cholesterol/ml; Cu^{2+} concentration, 2.0 μM; temperature 25°C.

oxidation, i.e. within the first part of the lag-phase (making up approximately 30–50% of the total duration of the lag-phase). The rate of LDL oxidation measured between 10 and 30 min of oxidation and the initial LDL ubiquinol-10 content correlated negatively ($P < 0.01$) (Fig. 2A). The rate of LDL oxidation measured within the same period correlated positively ($P < 0.05$) with the initial LDL content of PUFAs (Fig. 2C). Upon analysis of the PUFAs/ubiquinol-10 ratio, the correlations yielded an even greater significance (Fig. 2D). The early (10–30 min) LDL oxidizability also correlated positively ($P < 0.05$) with the ratio of LDL content of PUFAs over that of monounsaturated fatty acids (MUFAs) (Fig. 2F). All correlations were most pronounced when measured between 10 and 30 min of incubation with Cu^{2+} and became less or non-significant at later stages of oxidation. No significant correlation was found between the variables shown in Fig. 2 and an initial rate of LDL oxidation measured after 10 min of incubation.

In contrast to ubiquinol-10 and PUFAs, no significant correlation was found between LDL content of α-tocopherol and the rate of diene accumulation within any period studied (Fig. 2B). However, it should be noted that the correlation tended to be positive at early stages and negative at later stages of oxidation induced by Cu^{2+}. Similarly, no significant correlation was found between PUFAs/α-tocopherol ratio and the rate of LDL oxidation despite a tendency towards a positive correlation at the later stages of the oxidation (Fig. 2E). The values of the rate of diene accumulation measured between 10 and

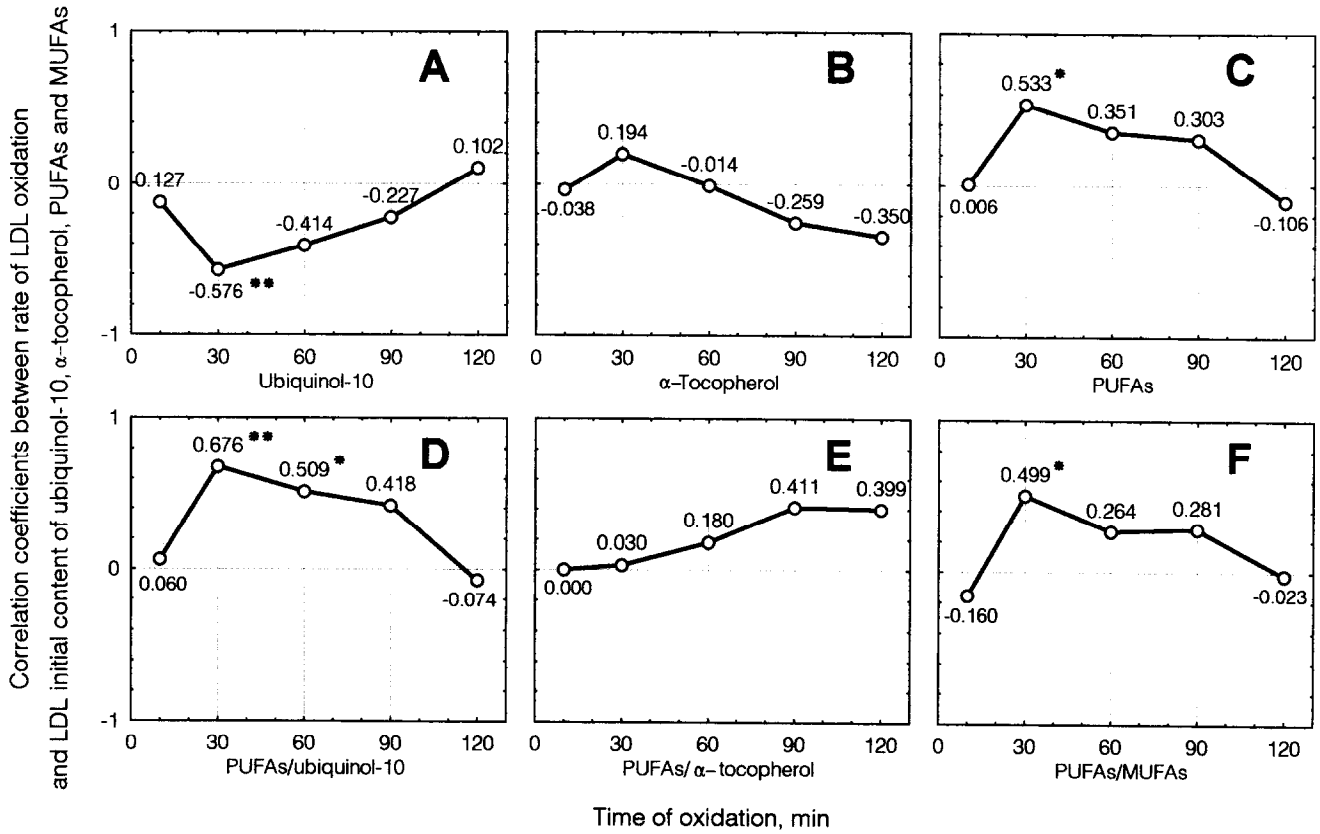

Fig. 2. Correlation coefficients between rate of diene accumulation in LDL samples measured within different periods (between 0 and 10, 10 and 30, 30 and 60, 60 and 90, 90 and 120 min of oxidation) and initial LDL content of ubiquinol-10 (A), α-tocopherol (B) and PUFAs (C). Analogous correlation coefficients for PUFAs/ubiquinol-10 (D), PUFAs/α-tocopherol (E) and PUFAs/MUFAs (F) are also shown. Numbers on the curves denote correlation coefficients calculated within definite periods. LDL concentration, 0.12 mg total cholesterol/ml; Cu^{2+} concentration, 2.0 μM; temperature 25°C. * $P < 0.05$; ** $P < 0.01$.

30 min of oxidation and LDL contents of ubiquinol-10, α-tocopherol and PUFAs for all the samples studied are shown in Table 2.

4. Discussion

At an early stage of oxidation induced by Cu^{2+}, i.e. within the first part of the lag-phase of diene accumulation, the rate of oxidative modification of human LDL correlated significantly with its ubiquinol-10 and PUFA content. On the other hand, no significant correlation between LDL oxidizability and its α-tocopherol content was found.

The correlation between the rate of LDL oxidation and its ubiquinol-10 content was found to be negative, suggesting that quinol delays the oxidation in the early phase. Ubiquinol-10 is known to be a powerful lipid-soluble antioxidant towards protection of unsaturated lipids in various model and biological systems [28]. Stocker et al. [13] found a high antioxidative activity of ubiquinol-10 in human LDL, and that, as long as ubiquinol-10 is present, no significant amounts of hydroperoxides are accumulated in LDL. Hence, our correlation may be explained by the inhibitory action of the quinol on LDL lipid peroxidation. Stocker et al. [13] also demonstrated that ubiquinol-10 is consumed long before α-tocopherol during in vitro LDL oxidation. Our discovered existence of a correlation only at an early stage of oxidation is in accordance with this finding.

How ubiquinol-10 functions as an LDL antioxidant is unclear at present. It is generally assumed that the quinol can eliminate free radicals formed directly by their scavenging [29–31] and/or indirectly through the recycling of LDL α-tocopherol [32,33]. Whatever the exact mechanism, however, the antioxidative activity of ubiquinol-10 appears to be high enough to inhibit LDL oxidation until all the quinol is consumed. It has been shown that ubiquinol-10 scavenges peroxyl radicals and regenerates α-tocopherol radicals in an hydrophobic medium at a rate constant of about 10^4–$10^5 \cdot M^{-1} \cdot s^{-1}$ [31,34]. On the other hand, an initial step of LDL oxidation by Cu^{2+} presumably occurs at a rate which is at least several orders lower [15]. The suggestion of a central role of ubiquinol-10 in the early protection of LDL against Cu^{2+}-induced oxidation corresponds well with these observations.

The correlation between the LDL oxidizability and its PUFA content in the present study was found to be positive, suggesting an increased rate of LDL oxidation in samples rich in unsaturated lipids. It appears quite obvious that the rate of lipid peroxidation depends on the amount of substrate available for the process. Recent findings have demonstrated an increased LDL susceptibility to oxidation in subjects on a PUFA-enriched diet [35]. Our discovered positive correlation between LDL oxidizability and its PUFAs/MUFAs ratio also corresponds well to the positive correlation between linoleic-to-oleic acid ratio and oxidizability of LDL not enriched in fatty acid [16]. Altogether, these data underline the role of PUFAs in enhancing the process of LDL oxidation.

The lack of a significant correlation between the rate of diene accumulation and LDL α-tocopherol content points to a complex effect of this compound on LDL oxidation. The correlation tended to be positive at early and negative at later stages of Cu^{2+}-induced oxidation. This time–course might be explained by the prevalence of a prooxidant activity of the vitamin at early stages and an antioxidant activity at later stages of oxidation. The fact that the correlation between LDL PUFAs/α-tocopherol ratio and the rate of diene accumulation tended to be positive at the later stages of oxidation corresponds well with this assumption. Prooxidant action of α-tocopherol on the process of LDL oxidation has been found by Bowry et al. [36]. According to their proposed scheme, a free radical of α-tocopherol, if present in the LDL particle for a sufficient time (at low free radical fluxes), can initiate peroxidation of LDL PUFAs. Obviously, this mechanism can not be valid in our case because of the high copper concentration used (17 Cu^{2+} ions per 1 LDL particle), which corresponds to significantly higher free radical fluxes [15].

The second possibility involves a participation of α-

Table 2
Values of LDL content of ubiquinol-10, α-tocopherol and PUFAs and rate of LDL oxidation measured between 10 and 30 min of incubation with copper in all the LDL samples studied

Ubiquinol-10 (mol/mol LDL)	α-Tocopherol (mol/mol LDL)	PUFAs (mol/mol LDL)	Rate of LDL oxidation (units of absorbance)
0.174	5.39	1,560	0.063
0.277	8.34	1,401	0.045
0.276	3.90	1,217	0.018
0.206	3.10	1,315	0.033
0.272	3.35	1,111	0.008
0.265	6.88	1,034	0.035
0.286	5.95	717	0.014
0.313	5.80	911	0.022
0.315	4.46	1,233	0.025
0.384	5.11	1,180	0.022
0.444	4.53	1,006	0.008
0.314	2.64	1,161	0.030
0.335	5.72	1,043	0.036
0.196	12.99	1,260	0.030
0.334	4.58	1,368	0.011
0.270	11.44	1,572	0.026
0.218	8.47	1,437	0.035
0.187	5.87	1,209	0.041
0.187	6.43	1,298	0.020
0.338	7.31	852	0.010

Rate of LDL oxidation (0.12 mg total LDL cholesterol/ml, 2 μM Cu^{2+}, 25°C) is expressed as an increase of absorbance of LDL sample at 234 nm between 10 and 30 min of oxidation.

tocopherol in the initiation of lipid peroxidation by Cu^{2+} [4]. Presumably, this process begins with one-electron reduction of Cu^{2+} to Cu^{2+} by an unknown reductant. Recently, it has been shown that in deoxycholate micelles α-tocopherol can be easily oxidized by Cu^{2+} [18]. Under similar conditions, α-tocopherol incorporated into phospholipid liposomes rapidly reduced Fe^{3+} to Fe^{2+} [37]. This suggests that α-tocopherol can also function as a reductant for Cu^{2+} in a course of LDL oxidation, providing an initial step of the process [4]. This scheme might explain the weak positive correlation between the rate of diene accumulation and LDL α-tocopherol content that we observed within the first 30 min of oxidation. On the other hand, the weak negative correlation observed later might correspond to the classical scavenging of free radicals by α-tocopherol at high free radical fluxes. The weakness of both correlations might be due to the existence of the two concurrent processes at all the stages of oxidation.

The prevalence of the prooxidant activity of α-tocopherol at early stages of LDL oxidation supports the suggestion of a crucial role of ubiquinol-10 in the early protection of LDL PUFAs. It has been reported that under conditions of a constant flux of water-soluble peroxyl radicals, α-tocopherol provides almost 90% of the total radical-trapping capacity of human LDL, whereas ubiquinol-10 contributes insignificantly to the overall trapping [12]. However, at early stages of oxidation induced by Cu^{2+}, when α-tocopherol acts as a pro- rather than antioxidant, ubiquinol-10 might represent the only actual antioxidant in LDL.

Finally, it should be mentioned that the mechanism of early LDL oxidation may be of great importance in vivo where the oxidation of LDL in the arterial wall can give rise to the formation of early atherosclerotic lesions [1]. It has been suggested that mild rather than extensive oxidation of LDL might be responsible for the atherogenic properties of this lipoprotein [38]. In our study, the PUFAs/ubiquinol-10 ratio was the best predictor of the early LDL oxidation. Hence, the ubiquinol-10 and PUFA content of LDL might represent an important factor influencing the early development of such lesions.

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