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How Different Constituents of Low Density Lipoprotein Determine its Oxidizability by Copper: A Correlational Approach

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Although low density lipoprotein (LDL) susceptibility to oxidation is expected to be primarily related to its composition, the individual contributions of different constituents to its oxidizability remain unclear. The present study was undertaken to elucidate how different constituents of isolated LDL determine its susceptibility to oxidation induced by Cu^{2+} under conditions close to those of the well-known Cu^{2+}-oxidation assay (H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder (1989) *Free Radical Research Communications*, **6**, 67–75). We characterized antioxidant, fatty acid and total lipid composition of human LDL from healthy donors (n = 22) and compared each with LDL oxidizability by Cu^{2+}. LDL oxidizability was evaluated as oxidizability of antioxidant-containing LDL (rate of lipid peroxidation measured before total consumption of α-tocopherol, the major LDL antioxidant), oxidizability of antioxidant-depleted LDL (maximal rate of lipid peroxidation and maximal production of conjugated dienes within the propagation, antioxidant-depleted phase of oxidation) and overall LDL resistance to oxidation (duration of the lag-phase before the onset of the propagation phase). We found that the oxidizability of antioxidant-containing LDL correlated negatively with LDL content of ubiquinol-10 and free cholesterol, and positively with that of n-3 polyunsaturated fatty acids (PUFAs). LDL n-3 PUFAs, ubiquinol-10 and free cholesterol were the most important determinants of the oxidizability of antioxidant-containing LDL, contributing to about 35%, 25% and 25% of its total variability, respectively. Oxidizability of antioxidant-depleted LDL was largely determined by LDL PUFA content. The overall LDL resistance to oxidation correlated weakly with LDL chemical composition. α-Tocopherol was found to be only a minor contributor to the oxidizability of isolated LDL under oxidative conditions used (7.5 or 14 mol Cu^{2+}/mol LDL). It appears that the oxidizability of antioxidant-containing LDL represents a parameter highly sensitive to changing LDL composition, whereas the overall LDL resistance to oxidation combines contributions from different LDL constituents more uniformly, being weaker sensitive to individual factors. It is suggested that PUFAs, ubiquinol-10 and free cholesterol are the most important determinants of LDL oxidizability by Cu^{2+}.

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

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INTRODUCTION

There is strong evidence that low density lipoprotein (LDL) oxidation plays a key role in the early development of atherosclerosis.[1,2] Current knowledge implies that LDL oxidizability by physiological oxidants may be of a great importance for the development of this pathology. LDL oxidizability is likely determined by its content of components directly involved in the process of oxidation, namely oxidizable substrates and antioxidant compounds. Individual contributions of different LDL constituents to this process, however, remain unclear. LDL is known to contain a number of antioxidants, the most abundant being α-tocopherol.[3] α-Tocopherol is a classical lipophilic antioxidant that scavenges free radicals in a hydrophobic milieu. The rate of LDL oxidation is expected to be low in the presence and high in the absence of α-tocopherol (lag-phase and propagation phase of the oxidation, respectively).[3] However, LDL oxidizability by Cu^{2+} was only found to correlate negatively with LDL α-tocopherol when the lipoprotein was preliminary enriched with this antioxidant[4–7] and when relatively strong[8] oxidative conditions of the well-known Cu^{2+}-oxidation assay[9] were used. A number of studies have found no significant correlation between α-tocopherol content and susceptibility to oxidation in native LDL unsupplemented with antioxidants.[4,5,10–14] Moreover, this correlation has even become positive under mild oxidative conditions.[8] These findings suggest that, depending on oxidative conditions, α-tocopherol can develop both anti- and prooxidant activity in isolated LDL and therefore does not represent a main factor determining LDL oxidizability in the Cu^{2+}-oxidation assay.[9]

Among other endogeneous LDL antioxidants, ubiquinol-10 seems to be the only one that can efficiently protect the lipoprotein against oxidation. Ubiquinol-10 inhibits an early stage of LDL oxidation, being the antioxidant first consumed during this process.[15,16] Rate of LDL oxidation increases rapidly after ubiquinol-10 consumption, despite the presence of large amounts of α-tocopherol.[8,15,16] LDL oxidizability also depends on the amount of oxidizable substrate available within the LDL particle, i.e., on its polyunsaturated fatty acid (PUFA) content. LDL enrichment with PUFAs increases its susceptibility to oxidation,[17,18] while the enrichment with monounsaturated fatty acids (MUFAs) decreases it.[17,19] Lipid hydroperoxides preformed to the moment when oxidation begins, can also increase LDL oxidizability.[20] The susceptibility to oxidation and initial hydroperoxide content correlate positively in LDL unsupplemented with hydroperoxides.[21] Accordingly, LDL oxidizability has been shown to increase following supplementation with hydroperoxides of linoleic or arachidonic acid.[22,23] The rate of LDL oxidation likely also depends on factors affecting intrinsic structure and physical properties of the LDL particle. Of these factors, total lipid and fatty acid composition is probably the most important because of its greater influence on the fluidity of lipid parts of the particle.[24] This expectation corresponds well with the fact that LDL susceptibility to oxidation correlates negatively with free cholesterol content of LDL subfractions.[25,26]

The variety of factors influencing LDL oxidation makes it difficult to compare the relative importance of different LDL constituents for this process. The study of Frei and Gaziano[21] allows an explanation about 35% of the total variation in the lag-phase of LDL oxidation by variations in LDL levels of preformed lipid hydroperoxides and α-tocopherol. Kleinveld *et al.*[27] suggested that up to 50% of the lag-phase variation may be determined by LDL PUFA content. Tribble *et al.*[28] indicated that ubiquinol-10 might be responsible for more than 70% of the LDL oxidizability at an early oxidation stage. In a recent study, we showed that, at an early oxidation stage, LDL oxidizability by Cu^{2+} correlates negatively with its ubiquinol-10 and positively with its PUFA content.[29] The present study was undertaken to further characterize, how these and other LDL constituents contribute to LDL oxidizability by

Cu^{2+} measured under conditions close to those of the Cu^{2+}-oxidation assay.[9] LDL oxidizability was evaluated as oxidizability of antioxidant-containing LDL (rate of lipid peroxidation measured before total α-tocopherol consumption), oxidizability of antioxidant-depleted LDL (maximal rate of lipid peroxidation and maximal production of conjugated dienes within the propagation, antioxidant-depleted phase of oxidation) and overall LDL resistance to oxidation (duration of the lag-phase before the onset of the propagation phase).

MATERIALS AND METHODS

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-10 and ubiquinol-9 were prepared from ubiquinone-10 and ubiquinone-9 by reduction with sodium dithionite.[30] The concentration of the ubiquinols in the stock ethanol solutions obtained was determined spectrophotometrically by oxidation with 0.1 M KOH.[31]

Isolation of LDL

22 apparently healthy normolipidemic donors aged 22–51 years (mean 34.0) were recruited from the community and all gave their informed consent. None of the subjects were taking supplementary antioxidants or were on drug therapy. After an overnight fast, blood was taken in ethylenediaminetetraacetic acid (EDTA)-containing tubes (1.6 mg EDTA/ml blood). To obtain plasma, the blood was immediately centrifugated at 4°C for 10 min. LDL was isolated using density gradient ultracentrifugation of the plasma for 20 h at 4°C in the presence of 1.5 mM EDTA.[32]

Characterization of Chemical Composition of LDL

Ubiquinol-10, ubiquinone-10, α-tocopherol and γ-tocopherol content of LDL was quantified immediately after LDL isolation by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection as described elsewhere.[29] Total cholesterol, free cholesterol, triglyceride and phospholipid content of LDL were determined by commercially available enzymatic tests. To calculate LDL cholesterol esters, its total lipids were measured. Total protein was determined with 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.[33] Absolute amounts of fatty acids were calculated using the data on LDL lipid composition assuming that the lipoprotein does not contain any free fatty acids. Thiobarbituric acid-reactive substances (TBARS) were measured using 0.375% thiobarbituric acid and 15% trichloroacetic acid dissolved into 0.25 M HCl.[34] Lipid peroxides were evaluated with Merck CHOD-iodide cholesterol color reagent (E. Merck, Darmstadt, Germany).[35] To recalculate the values obtained into mol/mol LDL, an LDL molecular weight of $2.5 \cdot 10^6$ was used.[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 the final cholesterol concentration of 0.85 mg/ml (approximately 1.0 μM LDL). LDL oxidation was then started by adding a small volume of freshly prepared stock solution of $CuSO_4$ in PBS. The oxidation was performed in a water bath at 37°C at a Cu^{2+} concentration of 7.5 μM (corresponding to about 7.5 Cu^{2+} ions per 1 LDL particle). In an LDL concentration of

0.12 mg total cholesterol/ml (approximately 0.14 μM LDL) with Cu^{2+} concentration of 2.0 μM (corresponding to about 14 Cu^{2+} ions per LDL particle) oxidation was performed at 25°C to measure conjugated diene accumulation. LDL oxidation level measured under these different sets of experimental conditions, was also characterized using the same oxidizability index (accumulation of lipid peroxides after 1 h oxidation; see below). A highly significant correlation was observed ($r = 0.83$, $p = 0.005$, $n = 9$), justifying the comparison of results obtained under different conditions. All the oxidation experiments were completed within 30 hours of collecting the blood.

Estimation of the Level of LDL Oxidation

The level of LDL oxidation was characterized by an accumulation of lipid peroxides, TBARS and conjugated dienes in the sample. Lipid peroxides and TBARS were evaluated as described above. Conjugated dienes were measured according to Esterbauer *et al.*[9] by continuous registration of sample absorption at 234 nm. The duration of the lag-phase, the maximal oxidation rate (the rate of the propagation phase) and the maximal diene production (maximal amount of dienes formed) were calculated from the curve thus obtained.[12] The oxidizability of antioxidant-containing LDL was characterized as an accumulation of the lipid peroxidation products before total consumption of α-tocopherol, the major LDL antioxidant.[3] α-Tocopherol is known to be consumed directly before the onset of the propagation phase of conjugated diene accumulation.[3] Rate of conjugated diene accumulation was therefore calculated from the corresponding curve at different time-points within the lag-phase (after 0.25, 0.5, 1.0, 1.25 and 1.5 h oxidation). Lipid peroxides and TBARS were measured at a definite time-point before total α-tocopherol consumption (after 1 h oxidation). Presence of α-tocopherol in the sample at this time-point was confirmed by HPLC (see above). Oxidizability of antioxidant-depleted LDL was estimated as the maximal oxidation rate and maximal diene production. The lag-phase duration was chosen to characterize an overall LDL resistance to oxidation.

Statistical Analysis

All results are presented as means ± standard deviations ($n = 22$). Spearman's correlation coefficients were calculated to evaluate relationships between variables. Coefficient of determination (a square of the correlation coefficient) was used to characterize a proportion of a variation of one variable accounted for by a variation of the other.

RESULTS

Chemical Composition of LDL

LDL content of main lipid classes, antioxidants and fatty acids was found to correspond well with data published elsewhere (Table 1 and 2).[3,28] Of all the parameters measured, LDL content of

TABLE 1 Antioxidant, oxidation product and total lipid content of LDL

Compound	LDL content
	mol/mol LDL
Ubiquinol-10	0.282 ± 0.078
Ubiquinone-10	0.265 ± 0.118
Total ubiquinone-10 + ubiquinol-10	0.547 ± 0.144
α-Tocopherol	6.60 ± 3.29
γ-Tocopherol	0.56 ± 0.20
Lipid peroxides	4.93 ± 5.64
TBARS	0.16 ± 0.10
	Percent of total LDL weight
Cholesterol esters	40.5 ± 4.6
Free cholesterol	10.2 ± 2.6
Total cholesterol	33.0 ± 4.2
Phospholipids	18.3 ± 2.2
Triglycerides	5.0 ± 2.3
Total lipids	74.1 ± 5.7
Total protein	25.9 ± 5.7

Data are presented as means ± S.D.. LDL (n = 22) was isolated from freshly-collected plasma using density gradient ultracentrifugation for 20 h at 4°C. EDTA was present at all steps of isolation.

TABLE 2 Fatty acid content of LDL

Fatty acid	LDL content, mol/mol LDL
PUFAs	1154 ± 238
n-6 PUFAs	1061 ± 221
18:2	894 ± 190
20:3	37 ± 9
20:4	130 ± 44
n-3 PUFAs	93 ± 54
18:3	27 ± 10
20:5	28 ± 28
22:6	38 ± 20
MUFAs	541 ± 70
16:1	80 ± 39
18:1	461 ± 68
Saturated fatty acids	703 ± 94
16:0	510 ± 74
18:0	149 ± 27
20:0	9 ± 4
22:0	19 ± 5
24:0	17 ± 5
Total fatty acids	2842 ± 275

Data are presented as means ± S.D.. LDL (n = 22) was isolated from freshly-collected plasma using density gradient ultracentrifugation for 20 h at 4°C. EDTA was present at all steps of isolation.

antioxidants, oxidation products and minor fatty acids (n-3 PUFAs particularly) revealed a high between-subject variability, with a coefficient of variation higher than 25%. Lipid peroxidation products (lipid peroxides measured iodometrically and TBARS) were slightly decreased in our LDL as compared with others,[3] presumably due to our shorter procedure for isolating LDL. On the other hand, LDL content of lipid peroxides was several orders of magnitude higher than that measured by HPLC with chemiluminescence detection in rapidly (40 min) isolated LDL.[36] Ubiquinol-10/ubiquinone-10 ratio was concomitantly decreased in our LDL as compared with that isolated rapidly (1.1 vs. 4.3, respectively).[36] These differences could also reflect increasing LDL autooxidation on isolation. It is known, however, that the iodometric assay highly overestimates the peroxidation level in native, non-oxidized lipoproteins.[3,37] Therefore, the relatively high level of lipid peroxides found in our LDL may not be directly compared with that measured by specific chemilunimescence detection.

Oxidizability of LDL

Incubation of LDL with Cu^{2+} led to the oxidation of the lipoprotein as judged by accumulation of lipid peroxidation products and consumption of antioxidants (Table 3). After 1 h oxidation ubiquinol-10 was totally consumed, whereas α-tocopherol was consumed by 87%. These values of antioxidant consumption justified measuring the oxidizability of antioxidant-containing LDL at time-points between 0 and 1 h oxidation. Measurement of conjugated dienes showed that the oxidation was characterized by a distinct lag-phase followed by propagation and decomposition phases.[9,12,29] The accumulation of dienes paralleled that of lipid peroxides and TBARS. This corresponded well with intercorrelations found between these oxidizability indices after 1 h oxidation (r = 0.57–0.86, p = 0.000–0.005). After 1 h oxidation, LDL accumulated more lipid peroxides than conjugated dienes, indicating that the latter pathway represents only a part of the former.[3] LDL also accumulated more lipid peroxides than TBARS, suggesting that an early oxidation stage was implicated.[3] Of all the oxidizability indices studied, those related to the overall LDL resistance and to the oxidizability of antioxidant-depleted LDL varied moderately between subjects (coefficients of variation between 16 and 30%), whereas the indices related to the oxidizability of antioxidant-containing LDL exhibited a much higher between-subject variability (coefficients of variation between 28 and 99%).

Correlation between Oxidizability and Antioxidant Content of LDL

LDL antioxidant content revealed several significant correlations with the indices of LDL oxidizability by Cu^{2+} (Table 4). Initial LDL content of

TABLE 3 Indices of LDL oxidizability by Cu^{2+}

Lipid peroxide accumulation after 1 h oxidation[a], mol/mol LDL	53.6 ± 52.9
TBARS accumulation after 1 h oxidation[a], mol/mol LDL	7.06 ± 6.84
Conjugated diene accumulation[b], mol/mol LDL	
after 0.25 h oxidation	4.04 ± 2.35
after 0.5 h oxidation	8.45 ± 4.49
after 1 h oxidation	19.4 ± 10.7
after 1.5 h oxidation	38.2 ± 25.6
Lag-phase of diene accumulation[b], min	100.8 ± 24.7
Maximal oxidation rate[b], mol dienes/mol LDL min	2.44 ± 0.73
Maximal diene production[b], mol/mol LDL	259.1 ± 40.3
Ubiquinol-10 content after 1 h oxidation[a], mol/mol LDL	n.d.[c] (100)
α-Tocopherol content after 1 h oxidation[a], mol/mol LDL	0.83 ± 0.74 (87)
Ubiquinone-10 content after 1 h oxidation[a], mol/mol LDL	0.132 ± 0.139 (75[d])

Data are presented as means ± S.D.. LDL (n = 22) was isolated from freshly-collected plasma using density gradient ultracentrifugation for 20 h at 4°C. EDTA was present at all steps of isolation. Antioxidant consumption expressed as a percentage of initial antioxidant content are presented in parenthesis. [a]Oxidation by 7.5 μM Cu^{2+} (LDL concentration 0.85 mg total cholesterol/ml). [b]Oxidation by 2.0 μM Cu^{2+} (LDL concentration 0.12 mg total cholesterol/ml). [c]Not detected. [d]Total ubiquinone-10 + oxidized ubiquinol-10 consumed.

ubiquinol-10 correlated negatively with the oxidizability of antioxidant-containing LDL measured either as an accumulation of lipid peroxides (Figure 1, A), TBARS or conjugated dienes in the sample after 1 h oxidation. The correlation between ubiquinol-10 and conjugated dienes accumulated after 0.5 h oxidation was of a similar significance. These findings correspond well with recent data demonstrating a negative relationship between ubiquinol-10 content of LDL subfractions and their susceptibility to the oxidation at an early stage.[28] However, in another study[21] no correlation was found between the LDL ubiquinol-10 level and oxidizability (measured as a rate of lipid peroxidation during the lag-phase). This apparent discrepancy from our data could be explained, however, by the fact that in the other study[21] an average rate of the oxidation over the whole lag-phase was measured. As we have previously reported,[29] the rate of LDL oxidation correlates negatively with LDL ubiquinol-10 content only within the first part of the lag-phase (after 0.5 h oxidation under identical conditions). Accordingly, when the oxidation rate was averaged for the whole lag-phase, the correlation also became non-significant in our study (data not shown). This was accompanied by the absence of the correlation both at an early beginning of the lag-phase (after 0.25 h oxidation) and at its later stage (after 1.5 h oxidation; data not shown). Nor

TABLE 4 Correlation coefficients between oxidizability indices and initial antioxidant content of LDL

	Conjugated dienes[a]	Conjugated dienes[b]	Lipid peroxides[b]	TBARS[b]	Lag-phase[c]	Maximal rate[d]	Diene production[e]
Ubiquinol-10	−0.49*	−0.47*	−0.49*	−0.54**	0.22	−0.24	−0.29
Ubiquinone-10	−0.43	−0.42	−0.20	0.01	0.32	−0.31	−0.17
Total ubiquinone-10 + ubiquinol-10	−0.39	−0.38	−0.33	−0.07	0.30	−0.30	−0.17
α-Tocopherol	0.09	0.16	0.10	0.37	0.18	0.09	0.04

Data of 22 subjects as presented in Tables 1–3 were used. [a]Formed after 0.5 h oxidation; [b]formed after 1 h oxidation; [c]lag-phase of conjugated diene accumulation; [d]maximal oxidation rate; [e]maximal diene production. *$p<0.05$, **$p<0.01$.

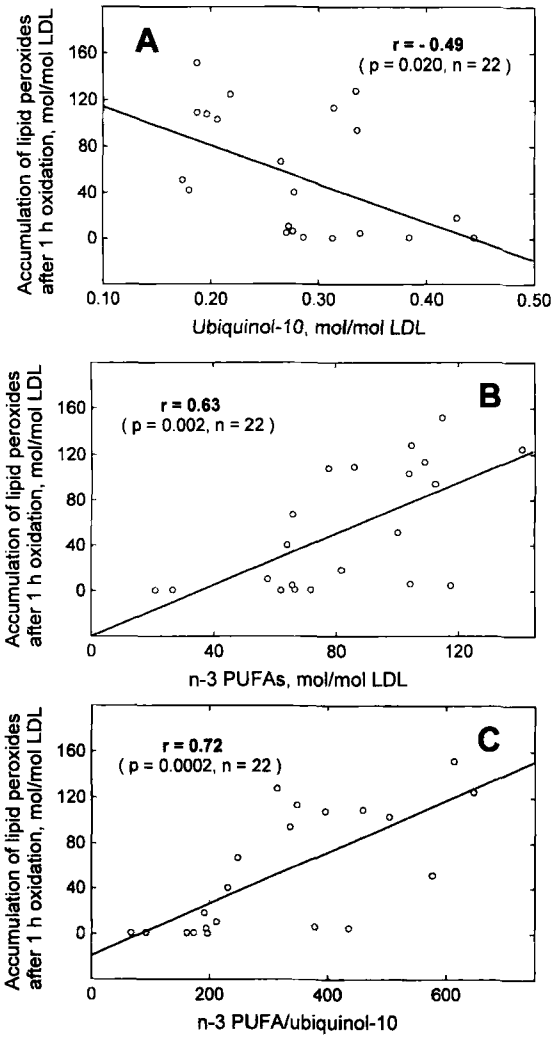

FIGURE 1 Correlation between LDL content of ubiquinol-10 (A), n-3 PUFAs (B), the n-3 PUFA/ubiquinol-10 ratio (C) and the rate of lipid hydroperoxide accumulation in the LDL sample measured after 1 h of LDL oxidation by Cu^{2+}. LDL concentration, 0.85 mg total cholesterol/ml; Cu^{2+} concentration, 7.5 μM; temperature, 37°C.

was a significant correlation found between the conjugated dienes measured after 0.25 h and any other parameter used to characterize LDL composition in our study (data not shown). The correlation between ubiquinol-10 and conjugated dienes was most pronounced when measured after 0.5 h oxidation.

In contrast to ubiquinol-10, initial LDL α-tocopherol content revealed no significant correlation with any of the oxidizability indices used. A weak positive correlations between α-tocopherol content and the oxidizability of antioxidant-containing LDL was found. The correlation between α-tocopherol and the lag-phase of diene accumulation was also found to be weakly positive. It has been reported[21] that this correlation is considerably improved and reaches significance if LDL α-tocopherol is expressed on a cholesterol, instead of a protein, base. In accordance with several other studies,[4,5,10–14] we found no significant correlation between the initial LDL α-tocopherol content and any of the oxidizability indices used regardless of the base on which the LDL α-tocopherol was calculated. No correlation between any oxidizability index studied and the initial LDL content of γ-tocopherol was also found in our study (data not shown).

Levels of preformed lipid peroxidation products (lipid peroxides measured iodometrically and TBARS) did not correlate with any of the indices of LDL oxidizability (data not shown). It has been recently reported that the initial LDL content of lipid hydroperoxides correlates positively with its oxidizability by Cu^{2+}.[21] In that study LDL hydroperoxides were measured by HPLC with a specific chemiluminescence detection. It appears, therefore, that our found lack of correlation could rather be due to the relatively low sensitivity and specificity of our used methods to measure lipid peroxidation products[3,37] than to an actual relationship between the parameters studied.

Correlation between Oxidizability and Fatty Acid Composition of LDL

In our study series of significant correlations were found between the oxidizability of antioxidant-containing LDL and its initial fatty acid content (Table 5). There was a positive correlation between the oxidizability of antioxidant-containing LDL and the initial LDL content of n-3 PUFAs (Figure 1, B). Of individual fatty acids of this type, LDL content of eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids revealed

TABLE 5 Correlation coefficients between oxidizability indices and fatty acid composition of LDL

	Conjugated dienes[a]	Conjugated dienes[b]	Lipid peroxides[b]	TBARS[b]	Lag-phase[c]	Maximal rate[d]	Diene production[e]
PUFAs	0.46*	0.43*	0.41	0.38	0.01	0.37	0.62**
n-6 PUFAs	0.54**	0.45*	0.37	0.30	0.03	0.27	0.61**
18:2	0.53*	0.39	0.30	0.25	0.07	0.20	0.57**
20:3	0.21	0.20	0.38	0.49*	−0.06	0.21	0.20
20:4	0.29	0.41	0.42	0.34	−0.06	0.26	0.54*
n-3 PUFAs	0.19	0.38	0.63**	0.56**	−0.04	0.43	0.54*
18:3	0.22	0.02	0.25	0.19	−0.13	0.46*	0.05
20:5	0.21	0.48*	0.63**	0.54**	−0.23	0.61**	0.57**
22:6	0.24	0.34*	0.53*	0.54*	0.04	0.27	0.45*
n-3 PUFA/UQH_2	0.47*	0.57**	0.72***	0.68***	−0.20	0.53*	0.55*
n-3 PUFA/α-Toc	0.10	0.24	0.49*	0.25	−0.34	0.40	0.35
PUFA/UQH_2	0.49*	0.48*	0.56**	0.57**	−0.17	0.42	0.48*
PUFA/α-Toc	0.17	0.10	0.16	−0.11	−0.25	0.04	0.19

Data of 22 subjects as presented in Tables 1–3 were used. For explanation of the abbreviations used, see Table 4. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

correlations of comparably high significance. Adjustment for the total LDL content of PUFAs did not substantially reduce the significance of the correlations (data not shown). LDL n-6 PUFAs correlated only with the rate of conjugated diene accumulation. The rate of diene accumulation also correlated positively with total LDL PUFAs. When the PUFA/ubiquinol-10 ratios were examined, the correlations reached even greater significance compared with those calculated solely for LDL PUFAs. The n-3 PUFA/ubiquinol-10 and PUFA/ubiquinol-10 ratios correlated with all three indices used to characterize the oxidizability of antioxidant-containing LDL (Table 5; Figure 1, C). The improvement of the correlations observed was accompanied by a negative correlation between LDL PUFAs and ubiquinol-10 ($r = -0.59$, $p = 0.004$). As a noteworthy comparison, the n-3 PUFA/α-tocopherol and PUFA/α-tocopherol ratios revealed no correlation with the oxidizability of antioxidant-containing LDL, partly due to the existence of a weak positive relationship between LDL PUFAs and α-tocopherol ($r = 0.17$, $p = 0.459$).

Indices of the oxidizability of antioxidant-depleted LDL also revealed a pronounced correlation with LDL fatty acid composition. The maximal diene production correlated positively with LDL content of total, n-6 and n-3 PUFAs. The maximal oxidation rate correlated positively with LDL content of linolenic and eicosapentaenoic acid. Using the PUFA/antioxidant ratio did not considerably improve the correlations in this case. The duration of the lag-phase of diene accumulation revealed no correlation with fatty acid composition. The n-3 PUFA/α-tocopherol ratio provided the highest correlation coefficient with the lag-phase ($r = -0.34$, $p = 0.148$). No correlations between LDL content of monounsaturated or saturated fatty acids and any of the indices of LDL oxidizability used were found (data not shown).

Correlation between Oxidizability and Total Lipid Composition of LDL

The oxidizability of antioxidant-containing LDL was found to correlate positively with LDL content of cholesterol esters (Table 6). LDL cholesterol esters also correlated positively with its PUFAs ($r = 0.55$, $p = 0.01$) and n-3 PUFAs ($r = 0.66$, $p = 0.001$) pointing out that the PUFAs are the main fatty acids in the LDL cholesterol ester fraction. This conclusion corresponds well with data published elsewhere.[38] When the correlations calculated for one of these variables were adjusted for the other,

TABLE 6 Correlation coefficients between oxidizability indices and total lipid composition of LDL

	Conjugated dienes[a]	Conjugated dienes[b]	Lipid peroxides[b]	TBARS[b]	Lag-phase[c]	Maximal rate[d]	Diene production[e]
Cholesterol esters	0.37	0.46*	0.50*	0.54**	− 0.06	0.29	0.46*
Free cholesterol	− 0.05	− 0.11	− 0.49*	− 0.54*	0.08	− 0.33	0.08
Phospholipids	0.52*	0.36	0.04	0.19	− 0.22	0.42	0.28
Triglycerides	− 0.23	− 0.14	0.04	0.05	− 0.12	− 0.28	− 0.25
Total lipids[f]	0.18	0.11	− 0.02	− 0.05	0.06	− 0.03	0.45*

Data of 22 subjects as presented in Tables 1–3 were used. For explanation of the abbreviations, see Table 4. *$p<0.05$, **$p<0.01$.

only the correlations between LDL oxidizability and its n-3 PUFAs remained significant (data not shown). These results imply that the contributions of LDL cholesterol esters and PUFAs into the total variability of LDL oxidizability should not be considered as independent.

LDL free cholesterol revealed a negative correlation with the oxidizability of antioxidant-containing LDL. This was not accompanied by a negative correlation between LDL free cholesterol and cholesterol esters ($r = -0.20$, $p = 0.373$). LDL phospholipids correlated positively with conjugated dienes measured after 0.5 h oxidation. No correlation between the LDL oxidizability indices used and its triglyceride content was found. It is interesting to note that the LDL triglycerides correlated with its MUFAs ($r = 0.70$, $p = 0.0003$) and saturated fatty acids ($r = 0.64$, $p = 0.001$), indicating that these fatty acids predominated in the LDL triglyceride fraction.

Of the indices of oxidizability of antioxidant-depleted LDL, the maximal diene production correlated positively with the LDL content of cholesterol esters and total lipids. Maximal oxidation rate was found to correlate positively only with the LDL cholesterol ester/free cholesterol and phospholipid/free cholesterol ratios (data not shown). The lag-phase of diene accumulation revealed no correlation with the LDL total lipid composition.

DISCUSSION

The oxidizability of antioxidant-containing LDL, measured as an accumulation of lipid peroxidation products before total α-tocopherol consumption (after 0.5 or 1 h oxidation) under conditions close to those of the Cu^{2+}-oxidation assay,[9] correlated significantly with the LDL content of ubiquinol-10, n-3 PUFAs, PUFAs, cholesterol esters and free cholesterol. We have previously reported[29] that LDL oxidizability by Cu^{2+} correlates with its content of ubiquinol-10 and PUFAs at an early oxidation stage. The present study confirms an important role of these constituents in the LDL oxidation. The negative correlation between the oxidizability of antioxidant-containing LDL and its ubiquinol-10 content points out that quinol inhibits oxidation at an early stage. This is hardly surprising, taking the fact into account that ubiquinol-10 is known to function as a powerful lipid-soluble antioxidant towards protection of unsaturated lipids in cell membranes[39] and LDL.[15] In human LDL ubiquinol-10 has been found to provide efficient protection of LDL lipids only at an early stage of LDL oxidation.[8,15,16] The mechanisms by which ubiquinol-10 can protect LDL against oxidation involve direct scavenging of free radicals within the LDL particle[40,41] and continual recycling of LDL α-tocopherol.[42,43] Both free radical scavenging[44] and α-tocopherol recycling[45] appear to occur fast enough to inhibit LDL oxidation until all the quinol is consumed. The low LDL content of ubiquinol-10 (< 1 mol/mol LDL), however, puts some doubt on its potential importance for LDL oxidation. It has been suggested by Tribble *et al.*[28] that the ubiquinol-10 content may represent a surrogate measure of some other LDL property affecting its oxidizability, namely its

initial lipid hydroperoxide content. Our recent results (A. Kontush and C. Hübner, unpublished data) show that ubiquinol-10 can significantly decrease LDL oxidizability by Cu^{2+} at an early oxidation stage, when present in the lipoprotein in sufficient amounts (about 0.5 mol/mol LDL and higher). The close proximity of the latter value to physiological levels of ubiquinol-10 in human LDL[15,28,29,36] suggests that the quinol can serve as an independent factor determining the LDL oxidative susceptibility.

The positive correlations between the oxidizability of antioxidant-containing LDL and its content of n-3 PUFAs, PUFAs and cholesterol esters suggest the role of all of these LDL constituents in enhancing the LDL oxidation. However, all of these factors should not be considered as independent because of an existence of the intercorrelations between them. This presumably reflects the fact that LDL content of either PUFAs, n-3 PUFAs or cholesterol esters represent three different measures of an amount of substrate available for oxidation within the LDL particle. Chemically, this substrate includes PUFA moieties of LDL lipids (predominantly those of LDL cholesterol esters[38]), with n-3 PUFA moieties being the most vulnerable to oxidation due to their higher level of unsaturation. This means that, of all the three LDL constituents, n-3 PUFAs may be considered as contributing most importantly to the oxidizability of antioxidant-containing LDL.

The lack of a significant correlation between any index of LDL oxidizability studied and its α-tocopherol content indicates a complex effect of this compound on LDL oxidation. We have previously suggested[29] that this well-documented finding[4,5,10–14] might be due to the fact that α-tocopherol develops both anti- and prooxidant activity in a course of LDL oxidation. The prooxidant activity has been suggested to prevail at early stages and the antioxidant activity at later stages of the oxidation. In the present study, LDL α-tocopherol content revealed weak positive correlations with both the oxidizability of antioxidant-containing LDL and the lag-phase duration, corresponding well with this hypothesis. Recent data[46–48] suggest that, at an early stage of LDL oxidation by Cu^{2+}, the prooxidant activity of α-tocopherol is related to the reduction of Cu^{2+} by the vitamin. Both Cu^{+} ions and α-tocopheroxyl radicals formed in this reaction possess a prooxidant activity. Cu^{+} can decompose lipid peroxides present in the sample to oxyl radicals.[46–48] The α-tocopheroxyl radical, if present in the LDL particle for a sufficient time, i.e., at low free radical fluxes, can directly initiate the oxidation of LDL lipids.[8,49] A comparison between the rates of α-tocopherol consumption and lipid peroxide accumulation (Table 3) indicated that peroxidation in the lag-phase proceeded via a chain reaction. This mechanism of α-tocopherol-mediated peroxidation[8] might substantially contribute to its prooxidant activity at an early oxidation stage. On the other hand, the classical chain-breaking activity of α-tocopherol might prevail over its chain-propagating or Cu^{2+}-reducing activity at high free radical fluxes, i.e., at later stages of LDL oxidation.

We could not also find any significant correlation between the initial LDL content of lipid peroxidation products and LDL oxidative susceptibility. This does not mean, however, that preformed lipid peroxides have no influence on LDL oxidizability by Cu^{2+}, rather demonstrates additionally[3,37] the inadequateness of applying the iodometric and TBARS-based assays to measure lipid peroxidation products in native, non-oxidized lipoproteins. Of different physical factors which can potentially influence the LDL oxidizability, only LDL free cholesterol content revealed a significant negative correlation with the oxidizability of antioxidant-containing LDL. It is well-known that free cholesterol can efficiently decrease fluidity of the LDL particle.[24] Hence, our results might imply that free cholesterol can delay LDL lipid peroxidation, decreasing the LDL fluidity and thus restricting the diffusion of free radicals within the LDL particle. A direct chain-breaking action of free cholesterol on LDL lipid peroxidation might also be of importance.[50]

Altogether, our results make it possible to estimate individual contributions of different constituents of LDL towards its oxidizability by Cu^{2+} under our experimental conditions. The correlation coefficients calculated in our study showed that the variations in the initial level of n-3 PUFAs could explain about 35% of the total variation in the oxidizability of antioxidant-containing LDL. LDL ubiquinol-10 and free cholesterol content presumably represented the factors of almost equal importance for the oxidizability of antioxidant-containing LDL, each accounting for approximately 25% of its total variation. The contribution of ubiquinol-10 is likely to represent a sum of contributions of ubiquinol-10 itself and preformed lipid peroxides,[28] as discussed above. According to Frei and Gaziano,[21] variations in the initial LDL hydroperoxide content may explain about 10–15% of the variation in the oxidizability. The contribution of ubiquinol-10 itself might be therefore estimated as 10–15% as well. It is worth mentioning, however, that in vivo LDL is ab initio expected to be virtually free of peroxides[36,47] Therefore, LDL contents of n-3 PUFAs, ubiquinol-10 and free cholesterol might be the most important determinants of LDL oxidizability at an early oxidation stage in vivo. The overall contribution of α-tocopherol to the oxidizability of antioxidant-containing LDL is obviously determined by a subtle balance of the pro- and antioxidant activities of this compound, was rather pro- than antioxidant and did not exceed 5–10% in our case. The fact that our analysis leaves only about 15% of the variability unexplained suggests that no other factor contributes much to LDL oxidation at an early stage. The unexplained variability might be related to some minor factors influencing LDL oxidation, for example to its intrinsic phospholipase activity[14] or vitamin A content.[51]

High variability of LDL antioxidant and PUFA content, on the one hand, and the oxidizability of antioxidant-containing LDL on the other, indicates that the latter parameter is highly sensitive to changing LDL composition. The oxidizability of antioxidant-depleted LDL did not vary greatly and was primarily determined by the total amount of oxidizable substrate in the LDL particle. This was judged by positive correlations between the maximal oxidation rate and maximal diene production, on the one hand, and LDL content of various PUFAs, cholesterol esters and total lipids on the other. Alternatively, the lack of any correlation between LDL chemical composition and its overall resistance to oxidation, measured as the lag-phase of LDL oxidation, suggests that this parameter combines contributions from different LDL constituents more uniformly, being weaker sensitive to individual factors. PUFAs,[27] α-tocopherol[21] and preformed lipid hydroperoxides[21] might be the most important determinants of the lag-phase duration. We also found no correlation between the rate of LDL oxidation measured at an early beginning of the lag-phase (after 0.25 h oxidation) and any parameter used to characterize LDL composition. This indicated that the activities of all LDL constituents influencing its oxidation (including those acting at an early oxidation stage, such as ubiquinol-10) were not developed immediately after adding Cu^{2+} but after some delay.

In the present study we used LDL oxidation by Cu^{2+} to model in vivo LDL oxidation. It is currently unclear to what extent the results obtained from such a model are applicable to biological systems since our knowledge about actual mechanisms of LDL oxidation in vivo is limited.[2] We also do not know, which stage of LDL oxidation contributes most to the early development of atherosclerotic lesions. It has been suggested that mild rather than extensive oxidation of LDL might be responsible for the atherogenic properties of this lipoprotein.[52] Potential suppression of an early stage of LDL oxidation by ubiquinol-10 and later stages by α-tocopherol might considerably delay the development of such lesions.

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