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Copper-induced and photosensitive oxidation of serum low-density lipoprotein. The relation to cholesterol level and inter-species differences

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Abstract

Hypercholesterolemia is associated with a higher risk for developing atherosclerotic coronary heart disease. During the past few years, evidence has been increasing that modification of lipoproteins, particularly low-density lipoprotein (LDL) oxidation, might be involved in the pathogenesis of atherosclerosis. To compare these factors metal-dependent and -independent photodynamic methods were used for the screening of several indexes of LDL oxidation. Lipid oxidation has been continuously monitored by the increase of conjugated dienes and verified by iodometric and thiobarbituric reaction assay. A close association between LDL cholesterol concentration (and/or serum cholesterol concentration) and LDL maximum diene formation was found using both methods and different sources of sera. With copper-induced oxidation, highly significant correlation coefficient $r = 0.86$, and with photo-sensitive oxidation $r = 0.84$ were noted. The data standardized to protein unit showed a reduced but still significant correlation. The extent of LDL oxidation was also closely related to preformed dienes, i.e., to the data obtained before the start of oxidation ($r = 0.91$). The rate of LDL oxidation was positively linked to LDL cholesterol using both oxidation methods but with photo-sensitive oxidation the rate was much higher. The lag time was inversely related to LDL cholesterol (standardized data) with Cu^{2+} induced oxidation but it was absent in the photosensitive oxidation. In animals known to be resistant to spontaneous atherosclerosis (rats, guinea pigs) a prolonged lag time, markedly reduced diene formation and lower LDL cholesterol in LDL in parallel was demonstrated. The fact that, using various methods (epidemiology, arteriography, autopsy), the cholesterol level in men was found positively linked to atherosclerosis development on the one hand, and positively associated to oxidation of human LDL on the other, strongly supports the concept on the important role of LDL oxidative modification in this pathological process.

Keywords: Lipid peroxidation; Copper-induced oxidation; Photosensitive oxidation; Low-density lipoprotein; Serum; Cholesterol; (Human); (Rat); (Guinea-pig)

1. Introduction

Hypercholesterolemia is one of the principal risk factors of coronary heart disease, and treatment aimed at reducing the level of circulating cholesterol is essential in preventing lipid deposition in the arterial wall [1–3,5].

An increasing body of evidence supports the hypothesis that plasma lipoproteins, primarily low-density lipoproteins (LDL), must be modified before they can be taken up and degraded by macrophages in the arterial wall intima to such an extent that these cells accumulate lipids [4].

Oxidation is apparently an important mechanism in this modification. Oxidized low-density lipoproteins (ox-LDL) may be atherogenic in several ways. They are recognizable by the non down-regulating macrophage scavenger receptor resulting in the formation of foam cells characteristic of early-stage atherosclerosis. The site and mode of endogenous (in vivo) ox-LDL formation have not been identified as yet, and little is known about ox-LDL turnover rate.

That was why the main goal of this study was investigate LDL susceptibility to oxidative stress in vitro and to compare the results with cholesterol levels as a widely recognized risk factor of atherosclerosis.

2. Materials and methods

All the reagents, specific inhibitors, photosensitizers, vitamins, lipid substrates (free cholesterol, cholesterol esters, phospholipids, polyunsaturated fatty acids) used were

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of reagent grade and were purchased from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany), Bayer (Leverkusen), Boehringer-Mannheim (Mannheim, Germany) and Lachema (Brno, Czech Republic).

Commercial kits for the enzymatic determination of cholesterol and triacylglycerols in serum were from Lachema-Diagnostica (Brno, CR).

The color reagent for iodometric assay (cat. No. 14106) and precipitation reagent for LDL isolation (cat. No. 14992) were from Merck (Darmstad, Germany).

Physiological salt solution was Krebs-Henseleit buffer (KH) containing (in mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 2.5 $MgSO_4$, 25 $NaHCO_3$, 11 glucose, 1 μmol/l CaEDTA and saturated with 95% O_2 plus 5% CO_2. Phosphate-buffered saline was prepared by mixing 90 parts of 0.145 mol/l NaCl and 10 parts of 0.1 mol/l Na_2HPO_4, pH 7.4.

Human sera obtained from 67 male and female volunteers, aged 40 to 70 years and animal sera from 24 rats (Wistar strain) and 6 guinea pigs were used fresh or stored at +2°C not longer than 7 days. The results gained with the same fresh or stored sera did not differ appreciably.

2.1. Preparation of low-density lipoprotein

Because of the well-known unstability of LDL and time consuming sequential ultracentrifugation during its isolation [6] and subsequent purification we preferred for serial assays a more rapid and convenient precipitation technique based on the formation of a reversible complex of heparin with LDL in an isoelectric point. An almost perfect correlation with ultracentrifugation or quantitative electrophoresis method was found [7].

LDL isolation was carried out according to the manufacturer instructions (Merck) by mixing in centrifugation tube with a conical bottom, 0.5 ml of serum with 5 ml of precipitation solution at laboratory temperature, followed by 10 min standing and centrifugation at $2000 \times g$ for 10 min. The supernatant was discarded, the tube was rinsed with precipitation solution and then the inner wall of a tilted tube was thoroughly washed with redistilled water with the aid of a micropipette to remove the residua of serum and precipitation solution. The precipitate of LDL was dissolved in 10 ml of KH solution at ambient temperature under slow and gentle stirring of the precipitate with a tiny glass rod. Thus, the final concentration of clear solution of LDL was 20-times lower as compared to its value in serum. Reproducibility of the method was found to be satisfactory with a variation coefficient of about 7%. Problems with clear-cut separation of LDL precipitate arose only with sera which were extremely hypertriglyceridemic.

Instead of KH solution, 10 mmol/l phosphate saline buffer with 1 μmol/l EDTA could be alternatively used for dissolution of LDL precipitate as well.

Cholesterol and triacylglycerol in serum were measured spectrophotometrically using the enzymatic kit mentioned above. Cholesterol in isolated LDL was measured using a proportionately greater volume than that in the measurement of serum cholesterol and treating the standard cholesterol solution in the same way.

Protein content was measured as described by Bradford [8].

Electrophoresis of LDL was performed on 1.5% agarose in barbital buffer (pH 8.6) and stained with Fat Red 7B (Sigma-Deisenhofen).

Iodometric assay for lipid peroxides of serum lipoproteins was carried out basically according to El-Saadami [9] and Wieland [10].

The breakdown products of lipid peroxides, so-called thiobarbituric acid reactive substances (TBARS), were measured according to Yagi [11] as modified by Simon [12].

The increase in conjugated dienes in LDL exposed to oxidative stress was monitored according to Esterbauer [13]. Both wavelength scanning between 220–300 nm and multiwavelength photometry close to absorbance peak were used. Peak differential absorbance was close to 234 nm in LDL and 246 nm in 5% serum.

The absorbance units were recalculated to conjugated dienes using molar extinction coefficient $\epsilon_{234\ nm} = 2.95 \cdot 10^4\ M^{-1}\ cm^{-1}$ [13].

2.2. Oxidative modification of low density lipoproteins

Of the several available methods the most frequently used copper-induced (method A) and metal-ion independent photosensitive oxidation (method B) with a much faster reaction rate were chosen.

Using method A 2.5 or 5 ml of LDL samples and copper sulfate at a final concentration of 10 μmol/l (routine assay) were mixed directly in photometry quartz cuvettes (1×1 cm), closed and incubated for 24 h at 37°C in water bath. The formation of conjugated dienes was monitored every 15–30 min.

Using method B 10 ml of freshly prepared LDL solution and Rose Bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein, the stock solution 4 mmol/l in destilled water) as photo-sensitizer [14,15] at a final concentration of 16 μmol/l was mixed. One half of this volume served as a photometry control sample. The other half was placed in recirculating water bath at 37°C and irradiated sidewise from a fixed distance (about 1 cm) by 150 W white light or a narrow band light source 560 ± 4 nm. For routine assays, rotation of cuvettes was omitted because it did not change the reaction rate. Electron absorbance spectra of conjugated dienes were monitored routinely at min 0, 1, 2, 3, 4, 5 and 10 whereas the control sample remained in the dark. After 10 min, lipid peroxides and TBAR were determined.

The reaction was oxygen dependent, inhibited by sodium azide (1–10 mmol/l), histidine (1–10 mmol/l) and ascorbic acid (0.01–0.1 mmol/l) suggesting that an oxidation

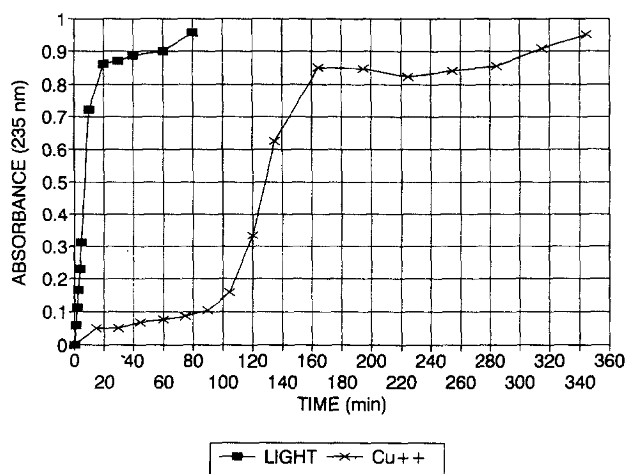

Fig. 1. Time course comparison of conjugated diene formation in isolated human low-density lipoproteins induced by irradiation or by $CuSO_4$ (5 μM). The LDL protein content 0.15 mg/ml. See Materials and methods.

process of type II and singlet oxygen as active oxygen species were involved. The relative values for quantum yields and stability of the light source were determined chemically by irradiation of iodide color solution (supplied by Merck) in the presence of Rose Bengal under the same conditions as those used for LDL samples. Irradiation of iodide solution resulted in I_3-formation with an absorbance increase of 0.93 units within 1 min. (Peak absorbance at 356 nm and molar extinction coefficient $2.47 \cdot 10^{-4}$ M^{-1} cm^{-1}).

2.3. Data statistical analysis

Data are expressed as means ± S.E. and *n* refers to the number of analyzed serum samples of men or animals. ANOVA and *t*-tests were used to evaluate the statistical significance of differences. A two-tailed probability value $P < 0.05$ was considered to be statistically significant. Parametric, non-parametric tests, regression parameters, correlation coefficient and cluster analysis were calculated with the aid of computer programs (Statistica, Statsoft and Excel, Microsoft).

3. Results

3.1. Lag period of human LDL oxidation

(a) In several previous papers [16–18] it has been demonstrated that oxidation of LDL (ox-LDL) catalyzed by Cu^{2+} was preceded by a lag phase. This could be estimated by the intercept of lag time and the following propagation phase data plot. According to the experimental protocol and the amount of anti-oxidants and their effectiveness, the length of the lag period varied between

Table 1
Levels of cholesterol in serum and LDL, protein in LDL and LDL oxidability indexes, lag-time, half-time and oxidation rate

Group A (men, Cu^{2+}-induced oxidation)	Mean ± S.E.	Range	*n*
Total serum cholesterol (Tc; mmol/l)	5.86 ± 0.23	3.9–8.5	27
LDL total cholesterol (LDL-tc; mmol/l)	3.56 ± 0.2	2.2–6	27
LDL cholesteryl esters (LDL-ce; mmol/l)	2.32 ± 0.16	1.3–4.1	25
LDL protein (p; mg/ml)	0.15 ± 0.006	0.1–0.24	25
Lag-time (min)	63.3 ± 2.7	30–90	27
Half-time (min)	101 ± 3.6	69.6 ± 142.8	21
Max. diene concentration (diene conc.; nmol diene/ml per 24 h)	44.3 ± 2.5	23.4–70.9	27
Rate (nmol diene/mg p per min)	2.9 ± 0.16	1.52–5.2	25
Group B (photosensitive oxidation)	**mean ± S.E.**	**range**	***n***
Men			
Total serum cholesterol (Tc; mmol/l)	5.7 ± 0.34	1.98–7.84	21
LDL total cholesterol (LDL-tc; mmol/l)	2.5 ± 0.27	0.1–4.7	22
LDL protein (p; mg/ml)	0.12 ± 0.006	0.1–0.15	21
Max. diene concentration (diene conc.; nmol diene/ml per 10 min)	16.5 ± 0.95	7.7–30.9	40
Rate (nmol diene/mg p per min)	15.5 ± 1.14	6.69–24.1	21
Guinea pig			
Total serum cholesterol (Tc; mmol/l)	1.08 ± 0.1	0.88–1.52	6
LDL total cholesterol (LDL-tc; mmol/l)	0.46 ± 0.08	0.31–0.79	6
LDL protein (p; mg/ml)	0.11 ± 0.008	0.09–0.12	6
Max. diene concentration (diene conc.; nmol diene/ml per 10 min)	4.8 ± 0.6	3–6.9	6
Rate (nmol diene/mg p per min)	4.36 ± 0.55	2.7–4.9	6
Rat			
Total serum cholesterol (Tc; mmol/l)	2.46 ± 0.13	1.96–3.1	8
LDL total cholesterol (LDL-tc; mmol/l)	0.3 ± 0.019	0.26–0.4	8
LDL protein (p; mg/ml)	0.15 ± 0.007	0.14–0.17	8
Max. diene concentration (diene conc.; nmol diene/ml per 10 min)	4.5 ± 0.3	2.7–6.9	8
Rate (nmol diene/mg p per min)	3.0 ± 0.2	1.8–4.6	8

Cu^{2+}-induced and photosensitive oxidation was studied in serum LDL from two group of men and animals. *n*, number of LDL samples; p, protein.

30–120 min during which time the antioxidants were oxidatively inactivated to get lipid oxidation started afterwards. In the illustrating Fig. 1, we can see that our LDL preparation oxidized in the presence of a 5 μmol/l concentration of copper ions exhibited slow and monotonous increase of absorbance (lag time) lasting about 90 min followed by an abrupt absorbance change afterwards. The lag time shortened by 20–30 min in the presence of 10 mmol/l of Cu^{2+} and negligibly changed by increasing the concentration of Cu^{2+} from 10 to 40 μmol/l. The average value was 63.3 ± 2.7 min (10 μmol/l Cu^{2+}, 37°C, see Table 1).

(b) With light-induced oxidation and under our routine assay conditions, the latent period was virtually abolished (Fig. 1). The separate experiments indicated that endogenous antioxidants were most likely destroyed already at the very beginning of measurement. One of the most effective natural antioxidants, ascorbic acid, if added to isolated LDL at a concentration of 9–90 μmol/l significantly delayed the start of LDL oxidation from 0.5 to 5 min. Although latent time could be substantially extended simply by reducing the light energy or photosensitizer concentration we selected these conditions for routine assays because our main concern was a rapid monitoring of lipid substrate oxidation.

3.2. Time course of oxidation of human LDL and diluted serum

(a) Following the lag period, LDL oxidation induced by Cu^{2+} proceeded relatively quickly (propagation phase) towards a maximum 1 and, except for a short-term decline, it asymptotically reached a maximum 2 in about 24 h (Fig. 1). From the maximum 2 and molar extinction coefficient (see Materials and methods) the maximum diene concentration and/or diene production has been calculated. The oxidation rate has been proposed to be expressed by means of plotting of the data in propagation phase. Alternately, half-time of the whole oxidative process time can be calculated according to the equation:

$$\text{Half-time} = (((T_u - T_l)/(A_u - A_l)) \times (A_{hf} - A_l)) + T_l$$

where A_{max} = absorbance at maximum 1, A_{min} = absorbance minimum, $A_{hf} = (A_{max} - A_{min})/2$, T_u, A_u = upper time data nearest A_{hf}, T_l, A_l = lower time data nearest A_{hf}. Processing of a greater number of data can be advantageously automated using, e.g., a spreadsheet calculator. The accuracy of calculated half-time depends on the length of measurement interval but it showed not to influence the results in practice appreciably. The mean values of all these LDL oxidizability indexes are given in Table 1, group A (10 μmol/l Cu^{2+}, 37°C).

Cu^{2+}-induced oxidation (using a 10 μmol/l concentration of Cu^{2+}) of serum diluted with KH solution to 5% – which corresponded to the dilution of isolated LDL – was not observed.

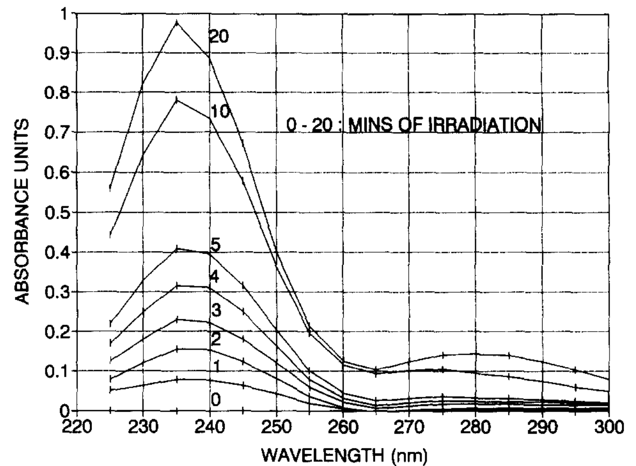

Fig. 2. Photosensitive oxidation of human LDL irradiated from 0 to 20 min. Data of control sample left in the dark were subtracted. Note scan peaks at 235 nm (conjugated dienes) and at 275 nm (conjugated trienes). See Methods and materials.

(b) The photosensitive oxidation rate of LDL allowed to see the rise in conjugated dienes (at 235 nm) and/or conjugated trienes (at 275 nm) already within one minute of irradiation. Typical wavelength scanning can be seen in Fig. 2. The observed increase of absorbance was essentially linear with time up to minute 10 and reaching a plateau afterwards (Figs. 1 and 3). The reaction rate was about 5-times greater than with Cu^{2+} induced LDL oxidation (Table 1). No change in the reaction rate was observed due to the absence or presence of 10 μmol/l EDTA.

In serum diluted with KH solution to 5%, the absorbance changes were faster and reached the maximum within a shorter time (Fig. 3). Wavelength scanning showed a red shift of maximum absorbance of about 12 nm. This peak shift has been demonstrated to diminish proportionately to greater dilution of serum.

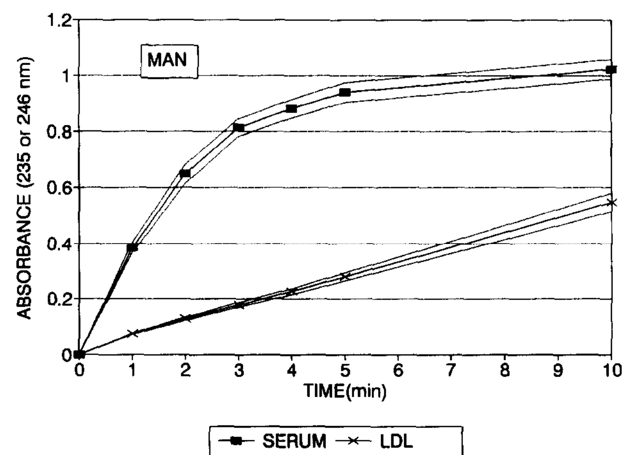

Fig. 3. Absorbance changes after irradiation of 5% human serum (peak absorbance 246 nm) and human low-density lipoproteins (LDL, peak absorbance at 235 nm). Dotted lines indicate S.E. $n = 22$.

3.3. Further characterization of oxidized LDL

The electrophoretic mobility of oxidized LDL induced by the 10 mmol/l concentration of Cu^{2+} was found to be about twice as fast as that of native LDL.

The correlation between the amount of conjugated dienes after 10 min irradiation and lipid peroxides was very close with a correlation coefficient of $r = 0.83$ ($P < 0.001$).

In several samples the results were compared with TBAR showing a similar association.

3.4. Association between donors sex and age, LDL composition and LDL oxidation

To simplify statistical processing, the data related to maximum increase of dienes were selected as observed after 24 h of exposure to 10 μmol/l copper sulfate and/or after 10 min irradiation of LDL (Table 2).

Although there was a positive trend of greater diene formation seen in men as compared to women as well as in increasing age, statistical tests and regression parameters were of marginal significance.

Several dyslipidemic sera exhibited the highest values above average of LDL oxidation. By comparing all data of maximum dienes formation with LDL cholesterol we found these data closely related.

As can be seen from Table 2, in Cu^{2+}-induced oxidation, correlation coefficient between LDL-tc and maximum dienes concentration was found to be $r = 0.86$.

A multiple regression analysis of these data resulted in a function

$$\text{Maximum diene} = 0.71 \times \text{LDL-tc} + 0.2 \times \text{LDL protein}$$

(for LDL-tc coefficient, $P < 0.001$ and for LDL protein coefficient, $P > 0.1$) and partial correlation coefficient between maximum diene and LDL-tc as $r = 0.7$ (constant protein, $P < 0.001$) and non-significant correlation between maximum diene and LDL protein (constant LDL-tc). This indicate that the major predictor of diene formation was LDL-tc. This is most likely due to the fact that LDL-tc is related to the amount of polyunsaturated fatty acids concentration and thus to the amount of oxidizable substrate.

Standardization of data, i.e., their recalculation to LDL-tc per mg of LDL protein should theoretically minimize the correlation. In fact, however, it resulted in the decrease of correlation coefficient $r = 0.67$ only. This could mean that with the same LDL concentration the oxidation increased with increasing cholesterol content. The correlation coefficient and the conclusion is similar to the previous papers [18,19] in which the LDL samples were adjusted to the same LDL protein concentration before oxidation.

Table 2
Coefficients of correlation (r-values) for cholesterol level and/or initial diene and oxidation indexes in LDL exposed to Cu^{2+}

	Tc	Diene conc.	Lag time	Rate
LDL-tc	0.82 **	0.86 **	0.33	0.52 **
LDL-tc/mg p		0.67 **	−0.39 **	0.34 *
Initial diene	0.80 **	0.91 **	0.04	0.46
Initial diene/mg p		0.71 **	−0.57 **	0.66 **

For abbreviations, see Table 1. Statistical significance levels are: * $P < 0.05$; ** $P < 0.01$.

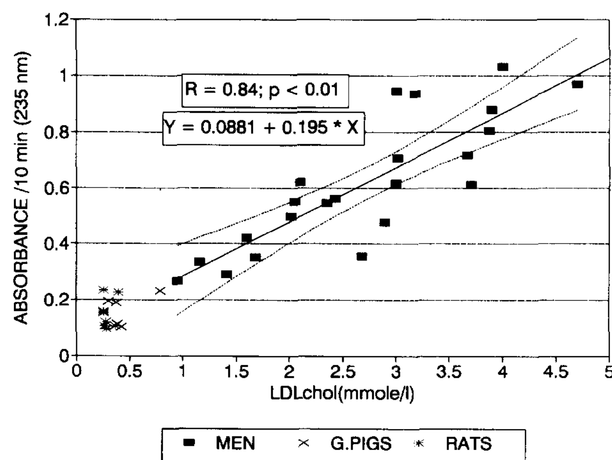

Fig. 4. Correlation between cholesterol level and conj. dienes formed (peak absorbance at 235 nm) after 10 min irradiation of human low-density lipoproteins. Similar data from guinea-pigs and rats are included. n(men) = 22, n(guinea pigs) = 6, n(rats) = 8.

The inverse association between lag time (reflecting the resistance of LDL towards the oxidation) and positive association between the rate of oxidation was found on one hand and LDL-tc/mg LDL protein on the other hand.

A positive association was also demonstrated between LDL-tc and initial dienes, i.e., LDL absorbance measured before the beginning of oxidation. Interestingly, the latter data were also closely related to LDL oxidation indexes and in fact they exhibited remarkably high correlation coefficients.

Also light-induced LDL oxidation rate and LDL cholesterol were closely associated ($r = 0.84$, $P < 0.001$), although the assay and the set of sera were entirely different (Fig. 4). With standardized data (recalculated to LDL-tc/mg protein) the correlation decreased to $r = 0.8$ only, obviously because the differences between protein content in each LDL sample were small.

In addition, we found that oxidation indexes were also proportionate to total serum cholesterol. This could be readily explained by the fact that, in our cohorts, LDL cholesterol and total serum cholesterol were very closely related as well ($r = 0.82$, Table 2).

3.5. Oxidation of animal low-density lipoproteins

Fresh sera of rats and guinea pigs were used and isolation of LDL and both above oxidation procedures were carried out exactly in the same way as in human sera.

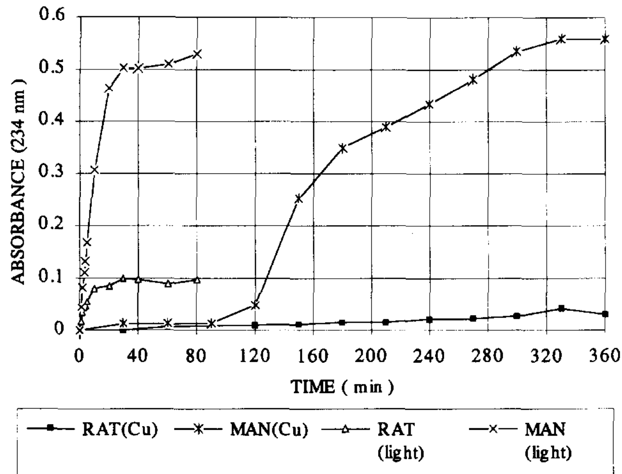

Fig. 5. Time course of Cu^{2+}-induced and photosensitive oxidation of man and rat LDL under standardized conditions, i.e., both LDL were adjusted to the same protein concentration 0.07 mg/ml before experiment.

(a) In contrast to human LDL, Cu^{2+}-induced oxidation of animal LDL advanced extremely slowly. This is demonstrated in Fig. 5 where both human and rat LDL samples were adjusted to the same protein concentration. In fact, the lag period was shorter than 6 h in 4 of 44 LDL samples only and in average would reach maximum dienes concentration of about 25% of that found in human LDL after 24 h incubation. Thus, a more accurate evaluation of oxidative indexes was difficult if not impossible.

(b) The formation of conjugated dienes at 0, 1, 2, 3, 4, 5 and 10 min of irradiation was linear with time, similarly to those in human LDL but again the average reaction rate was considerably slower in both animal species reaching about 27% of corresponding value in men (Fig. 6).

The absorbance changes of 5% sera of animals were also proportionately smaller than in the sera of human beings.

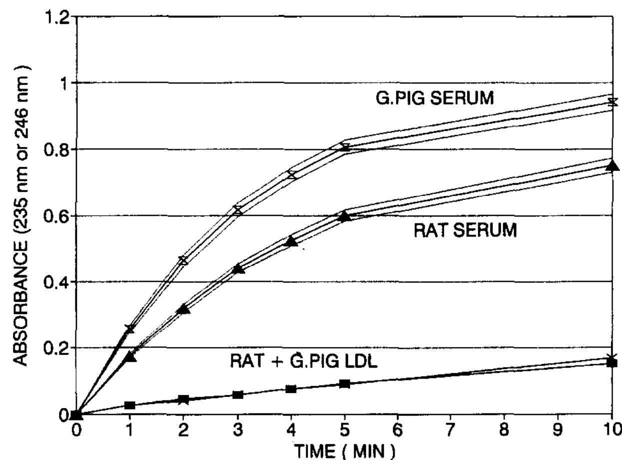

Fig. 6. Absorbance changes after irradiation of 5% serum (peak absorbance at 246 nm) and low-density lipoproteins (LDL, peak absorbance at 235 nm) of rats ($n = 8$) and guinea pigs ($n = 8$). Dotted lines indicate S.E.

Species differences, similar to those in the photooxidation rate of LDL, were observed in average LDL cholesterol level. The latter was substantially lower in animals than in man, i.e., 0.3 ± 0.019 in rats and 0.46 ± 0.08 mmol/l in guinea pigs. Data of man and animals, if taken together, raised the correlation coefficient between LDL cholesterol and oxidation rate of LDL to $r = 0.94$, $P < 0.001$. However, the interindividual variation among animals was clearly smaller than that found in man and created a distinct cluster of data (see Fig. 4). Squared Euclidean distances between man/guinea pig/rat were 0:14.6:17.7 in ox-LDL and 0:0.93:1.15 in LDL-cholesterol. Thus, the merging of data for correlation calculation appears theoretically not quite justified.

3.6. Photooxidation of model lipids

Since it showed that total cholesterol levels and the rate of conjugated dienes formation in LDL during irradiation were closely interrelated, we sought to identify the lipid fraction of LDL that might be a potential source of the optical signal. The lipids were dissolved in ethanol at a concentration roughly corresponding to the concentration in LDL. The ethanolic extract of LDL was carried out by the addition of 10 ml of absolute ethanol to the LDL precipitate prepared from 0.5 ml of human serum followed by mixing, 20 min incubation and centrifugation (10 min at $2000 \times g$). The irradiation was performed in a similar way as before with isolated LDL.

We found that free cholesterol, cholesterol oleate, trioleate glycerol gave negligible optical signal of conjugate dienes during 10 min of irradiation whereas cholesterol esters, phospholipids and triglycerides containing polyunsaturated fatty acids as well as the ethanolic extract of LDL produced a positive signal of conjugated dienes.

4. Discussion

Oxidative modification of LDL can be carried out in a biological, enzymatic or purely chemical way [19,20]. A feature characteristic of all these techniques is that the central role is played by the active oxygen species which attacks organic compounds including antioxidants and polyenic fatty acids.

In Cu^{2+}-induced oxidation, the appropriate oxygen species was not yet clearly defined but it has been proposed to be initiated by metal-catalyzed breakdown of preformed lipid hydroperoxid [21–23].

In the photodynamic technique, the active oxygen species is represented by short-living singlet oxygen, possibly generated in a variety of biological systems [24,25], the oxidative reaction chain is metal-independent and can take place very quickly. Similarly to metal-dependent oxidation, the target of photosensitive oxidation are obviously

unsaturated fatty acids as shown in this as well in earlier papers [26].

A marked difference between these two oxidation methods is the virtual absence of lag time in photosensitive oxidation unless the exogenous antioxidant has been added in surplus.

The fundamental mechanism of lag time in metal-induced oxidation remains unclear. It has been firmly established, however, that during this time, the level of a number of antioxidants – mainly tocopherol and carotenoids – slowly decreases and lipid oxidation get started afterwards [17]. But, at the same time, it was shown that there is a poor association of the lag period with vitamin E content in LDL unless the plasma were enriched previously with tocopherol [17,18].

Interestingly, it has been shown earlier that lag phase could be virtually eliminated by treating ultracentrifugally isolated LDL with chondroitin sulfate proteoglycan, chondroitin-6-sulfate and heparin [27]. Further analysis led the authors to suggest that oxidative lag time in native LDL could be connected with intact apoB protein structure not allowing free and rapid access of Cu^{2+} to the hydrophobic core of LDL. It should be noted that we found, in our LDL prepared by heparin precipitation, no such anomaly in lag phase. This is likely due to protective effect of serum proteins during precipitation, or very short precipitation time, or both.

Recently, a clear association between the lag period and linoleic to oleic acid ratio has been found, indicating that the lipid composition of LDL might play a significant role [28]. This fact, together with the formerly mentioned hypothesis on the metal-dependent breakdown of preformed lipid hydroperoxids and consequently oxygen chain radical formation, could explain not only why metal-independent photosensitive oxidation is lacking an induction period but also, why in Cu^{2+}-induced oxidation the presence of preformed ox-LDL might predetermine further oxidation of native LDL.

Our finding that the formation of conjugated dienes of LDL lipid was statistically proportionate to the cholesterol level is consonant with the study of Miyazawa and Fujimoto [29] who showed, using high performance chromatographic system and chemiluminiscence detection, an even closer correlation ($r = 0.94$) between LDL-phosphatidylcholine hydroperoxide and total cholesterol concentration in human plasma. Recently, in experiments on rabbits fed with cholesterol-rich diet, Kanazawa et al. [30] found a fast increase of lipid peroxides parallel with cholesterol level in plasma. This appears to indicate that LDL lipid oxidation, as measured in vitro, is also reflected in processes occurring under in vivo conditions.

One cannot of course completely exclude the possibility that some amount of preformed ox-LDL originates during manipulation of blood in vitro. If so, then this effect would be worthwhile to be studied in detail because it could occur fairly quickly by unknown mechanism (e.g., linked to coagulation or similar processes), again in dependence on the cholesterol and/or lipid level and under pathological conditions in vivo.

These considerations raise the question unresolved to date, that is, whether efficient in vivo oxidative stress occurs in the plasma, in the elements of plasma or in the area of vascular intima only. We found in our experiment that a diluted serum (human or animal) was readily modified by photooxidation. However, the chemical complexity of serum and the absorbance peak anomaly (shift of the peak absorbency) appearing after irradiation of serum could indicate that the modification may not be strictly limited to lipids only. This seems to be supported by the fact that the thiobarbituric reaction was about 25% of expected values. However, because a decomposition of lipid oxidation products in serum cannot be excluded more detailed analyses are needed.

The controversial fact that the susceptibility of LDL towards photo-oxidation is related to cholesterol levels even though irradiation of cholesterol alone does not result in a rise in the photometric optical signal of conjugated dienes could be explained either by the relatively fixed proportion of cholesterol to the unsaturated fatty acids in cholesterol esters or by assuming that the presence of cholesterol in LDL facilitates the peroxidation of unsaturated fatty acids as found in the experiments with platelets [31]. In the experiment of Wieland [10] it has been clearly shown that, in human LDL oxidized in the presence of Cu^{2+}, lipid hydroperoxides were predominantly in the cholesterol ester fraction. This together with previously mentioned facts indicate that the oxidation of LDL as related to cholesterol content is indirect through a common link to the poly-unsaturated fatty acids.

Because administration of both mono- and polyenic fatty acids tends to decrease the cholesterol level in vivo [32,33], these variables are certainly under complicated feedback control.

The relatively stable proportion of unsaturated fatty acids to cholesterol level does not rule out the possibility that it could be affected by an intensive dietary intervention. It has been shown that the administration of olive oil rich in oleic acid makes LDL resistant to peroxidation [34]. This is consonant with our lipid model experiments showing that cholesterol oleate, unlike cholesterol linoleate or cholesterol linolenate, is very little oxidatively modified.

The very slow oxidation of LDL and lengthy lag time in rats and guinea pigs could be most readily explained by the low cholesterol level in their LDL and/or by the lipid content relative to protein in LDL that is substantially lower in animals than in man.

The other explanation might be based on the idea that animal but not human LDL contain some potent protective factor. However, any attempt to transfer the LDL oxidative resistance of rats to that of man by mixing their LDL or sera almost completely failed. Only in 2 from 11 experiments, the LDL prepared from a mixture of the rat and

human serum (ratio 1:1) were oxidized in a manner similar to the rat LDL. Otherwise the expected mean of oxidation of these two kind of LDL were found. Specific interspecies differences will nevertheless require further attention in the future.

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