papers and notes on methodology

A simple test for predisposition to LDL oxidation based on the fluorescence development during copper-catalyzed oxidative modification

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Abstract When human low density lipoprotein (LDL) obtained from 10 volunteers was incubated in air at 37°C in the presence of various concentrations of copper, an increase in fluorescence was observed with emission maximum at 430 nm when excitation was performed at 360 nm. The fluorescence increase was inhibited by ethylenediamine tetraacetic acid and by 4-methyl-2,6-di-tert-butylphenol. The fluorescence was found to be tightly bound to the protein moiety. Furthermore, Cu^{2+} modification of LDL was associated with a decrease in the reactive amino groups of apolipoprotein B and in the uptake of the lipoprotein by rabbit fibroblasts. Under our conditions, the fluorescence increase showed two consecutive periods: an inhibition period during which the fluorescence increased only weakly, and a propagation period with a rapid increase in fluorescence that was linear for at least 5 h. Both periods were influenced by copper concentration. The study also shows that the extent of fluorescence generated upon LDL oxidation varied greatly in the volunteers. Thus, while the results demonstrate that the fluorescence increase may likely monitor the extent of the apoB derivatization, the calculation of the fluorescence development rate of the propagation period together with the duration of the inhibition period may constitute a quantitative measurement of the susceptibility of apoB to be derivatized. — **Cominacini, L., U. Garbin, A. Davoli, R. Micciolo, O. Bosello, G. Gaviraghi, L. A. Scuro, and A. M. Pastorino.** A simple test for predisposition to LDL oxidation based on the fluorescence development during copper-catalyzed oxidative modification. *J. Lipid Res.* 1991. **32:** 349–358.

Supplementary key words atherosclerosis • oxidation • conjugated dienes • malondialdehyde • free amino groups

It has been suggested that the oxidative modification of low density lipoprotein (LDL) could be of importance in the development of atheromatous lesions (1–5). Endothelial cells (6), smooth muscle cells (7), and macrophages (8) have all been shown to be capable of oxidizing LDL in vitro. LDL can also be oxidized in a cell-free medium containing a sufficiently high concentration of copper or iron (9).

Oxidation of LDL is a lipid peroxidation process in which the polyunsaturated fatty acids contained in the LDL lipids are rapidly converted to lipid hydroperoxides and then to some unsaturated aldehydes, which can alter the property of apolipoprotein (apo) B (10, 11). In particular, these aldehydes seem to react with the free amino groups of the lysines, thus neutralizing a certain cluster of positive charges that are recognized by ionic interaction by the classical LDL receptor (9, 12). The loss of positive charges due to blockage of lysyl residues gives to the particle a higher anionic electrophoretic mobility and reduced recognition by the LDL receptor on fibroblasts (9, 12, 13) while it enhances its recognition by macrophages (8).

The same process that alters the properties of apoB also generates a fluorescent product with a strong emission maximum at 430 nm when excitation is performed at 360 nm (14).

Therefore, the aim of this work was to set up a method which, by means of the fluorescence increase during the first phases of the oxidative process, could represent a useful monitor of the LDL apoB modification and thereby evaluate the susceptibility of LDL to the same oxidative stress.

Abbreviations: ANOVA, analysis of variance; apoB, apolipoprotein B; BHT, 4-methil-2,6-di-tert-butylphenol; CD, conjugated dienes; CV, coefficient of variation; EDTA, ethylenediamine tetraacetic acid; F, fluorescence; FDR, fluorescence development rate; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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MATERIALS AND METHODS

LDL isolation

Whole blood, obtained by venipuncture from 10 healthy volunteers after 12 h of fasting, was collected into Vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) (1 mg/ml) and immediately centrifuged at 2000 rpm for 20 min at 4°C. Plasma was stored at 4°C and processed for LDL separation within 1 day. LDL was isolated by sequential ultracentrifugation in NaBr solutions (15) containing 1 mg/ml EDTA (d 1.019–1.063 g/ml), stored at 4°C and used within 4 days.

Lipoprotein oxidation

Immediately before the oxidation incubations, LDL was separated from EDTA and from diffusible low molecular weight species by gel filtration (deprotection period) on AcA22 gel (LKB, Bromma, Sweden), a gel that does not retain particles with a molecular weight greater than 1.2×10^6. The column (1.6 × 20 cm) was previously equilibrated overnight in 0.01 M phosphate, pH 7.4, buffer (buffer A) and continuously bubbled with argon to wash out the dissolved oxygen. A sample of LDL (2 ml) was layered on the top of the column and the elution was carried out with the buffer A at 0.13 ml/min. The fractions eluting at the void volume were pooled, adjusted to a protein concentration of 0.5 mg/ml, and used immediately. For the oxidation experiments, aliquots (1.5 ml) of deprotected LDL, alone or in presence of copper (usually 50 μM), were placed in 3-ml conical screw-capped vials with a perforatable septum (Supelco Inc., Bellefonte, PA) and kept in a water bath at 37°C under mild agitation. By means of a needle inserted in the cap, the samples were maintained under gentle gas flow (ultrapure air or argon: gas chromatographic grade). Both air and argon were filtered on line with a 0.45-μm cellulose filter unit (MSI, Westborough, MA) and saturated with water by bubbling them in the buffer A at the same temperature. At prefixed intervals of time (every 20 min over the first 140 min and every 40 min over the second 160 min for a total of 300 min) a 250-μl sample was withdrawn and collected into a test tube containing EDTA (final concentration: 1.5 mg/ml) to quench the reaction.

A number of incubations were also carried out in the presence of 4-methyl-2,6-di-tert-butylphenol (BHT) and ascorbic acid. BHT was added at the start of the experiment in concentrated ethanol solution (20 μl in 1.5 ml LDL) and ethanol-only controls were done with the same amount of ethanol.

Fluorescence measurements

For fluorescence measurements the quenched samples were diluted 20 times with buffer A and the emission was measured at 430 nm with excitation at 360 nm (Spectrophotofluorimeter Shimadzu RF-5000). The excitation and emission slit width were both maintained at 5 nm. During the measurements the instrumental drift of sensitivity was checked by measuring the intensity of the Raman band of cyclohexane by excitation at 360 nm (16). The fluorescence was corrected daily for intensity of the Raman band of cyclohexane and for protein concentration into the measurement cell.

Binding of the fluorophore

In order to ascertain whether the fluorophore developed during the reaction was really attached to LDL and in particular to apoB, fluorophore binding was determined as follows. Native and oxidized LDL (after 4 h oxidation) were first gel filtered by AcA22 gel (LKB, Bromma, Sweden). The column (60 × 0.9 cm) was equilibrated in 20 mM Tris, 0.3 mM EDTA, and 0.15 M NaCl buffer, pH 7.4, and the elution was carried out with the same buffer at 0.10 ml/min. The eluted samples where checked for fluorescence, absorbance at 280 nm, and protein and cholesterol concentration. Then the fluorescence bound to LDL particles, i.e., to the protein or lipid domain, was better localized through the delipidation of the lipoprotein with isopropanol; after redesolving the protein component by means of a detergent (Nonidet-P40: Sigma, St. Louis, MO), the fluorescence of the aqueous and organic phase was measured. Furthermore, native and oxidized LDL were delipidated with Nonidet-P40 and ultracentrifuged in a sucrose gradient as described by Melnick and Melnick (17). The gradient consisted of our 2.25-ml steps of 6, 13, 24, 46% sucrose, respectively, and a final step of 1 ml of 54% sucrose. All density solutions contained 15 mM CHAPS (Sigma), 0.15% Nonidet-P40, and 10 mM Tris-HCl (w/v), pH 8.6. The samples were centrifuged in a Beckman T-41 swinging bucket rotor at 40000 rpm for 20 h. Fractions were collected from the bottom and analyzed for protein and cholesterol content and for fluorescence.

Measurement of lipoprotein uptake

In order to ascertain whether, under our conditions, the oxidative chain reaction reduced the affinity of the modified LDL to the LDL receptors, a series of displacement curves was performed. The fibroblasts used in this study were established from explants of rabbit skin as previously described (18). Human LDL (prepared from blood collected in 0.1% EDTA from healthy subjects (d 1.019–1.063 g/ml) was labeled with ^{125}I (Sorin Biomedica, Saluggia) by using the method of McFarlane (19) modified by Bilheimer, Eisenberg, and Levy (20). The specific activity of the preparation ranged between 130 and 180 cpm per ng of protein. Human lipoprotein-deficient serum (LPDS) was prepared as described previously (21) utilizing blood from healthy donors.

Cells were grown according to the method of Shimada et al. (18). Briefly, 8.0×10^4 cells were seeded into dishes

(35 × 10 mm) containing 1.5 ml of the culture medium. After 3 days, cells were preincubated for 16 h at 37°C in the culture medium in which fetal calf serum had been replaced with 4 mg protein/mg of LPDS. The medium was then removed and replaced with 1 ml of fresh LPDS containing 10 μg/ml of ^{125}I-labeled LDL plus increasing amounts of cold LDL (native and oxidized) at 37°C for 4 h. Fibroblasts were washed as described (18) and dissolved in 0.1 M NaOH for assay of protein and radioactivity. Trichloroacetic acid-soluble iodide-free radioactivity was determined as described elsewhere (21). Data were expressed as cell-associated ^{125}I-labeled LDL (% of uninhibited control).

Other methods

Cholesterol was determined with an enzymatic method (Boehringer Biochemia Robin CHOD-PAP high performance). Protein was measured by the method of Lowry et al. (22).

Thiobarbituric acid (TBA) reaction was determined according to Asakawa and Matsushita (23). Reactive free amino groups were measured with 2,4,6-trinitrobenzenesulfonic acid (TNBS reactivity) as described by Habeeb (24). The values, at various oxidation times, were expressed as percent variations with respect to the initial values.

Conjugated dienes (CD) were measured as described by Pryor and Castle (25). The native and oxidized LDL was analyzed for electrophoretic mobility by using agarose gel (26).

Statistical analysis

Data were analyzed by the analysis of variance (ANOVA) for random effects and by one-way ANOVA followed by a multiple comparison test (27).

RESULTS

The results of this study show that LDL presented a weak native fluorescence before the oxidation process. The fluorescence of LDL increased considerably during the incubation at 37°C in air in the presence of copper. The fluorescence intensity developed by LDL after 4 h of oxidation was linear with the concentration of LDL protein in cuvette (r = 0.998, $P<0.001$). Since the reaction rate was slightly affected by the LDL protein concentration, for routine measurements, all the samples to be oxidized were diluted to 0.5 mg LDL protein/ml.

Fig. 1 shows that the fluorescence increase was sensitive to the presence of oxygen and that the substitution of oxygen with argon considerably prevented its development. The fluorescence increase showed two consecutive periods: an inhibition period during which the fluorescence increased only weakly, and a propagation period with a rapid increase of fluorescence. The same figure also demonstrates that EDTA completely suppressed the fluorescence increase.

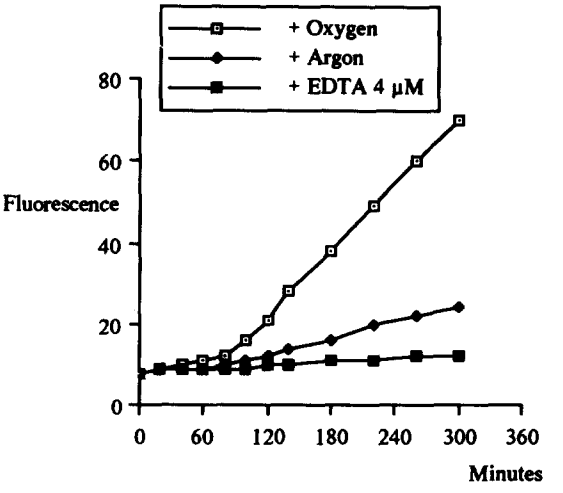

Fig. 1. Increase of LDL fluorescence during the oxidative process. EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air or argon flow in the presence of 50 μM Cu^{2+}. The same lot of LDL was incubated under the same conditions in air after the addition of EDTA (4 μM). After appropriate dilution, the fluorescence of the samples was measured every 20 min from time 0 to 140 and every 40 min from time 140 to 300 at 430 nm with excitation at 360 nm. Fluorescence was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into measurement cell.

Fig. 2 shows the effect of different concentrations of BHT on the fluorescence increase. BHT at 30 μg/ml completely quenched the reaction. Ascorbic acid (data not shown) blocked the reaction at a concentration of 20 μg/ml. The addition of BHT or ascorbic acid, at 30 and

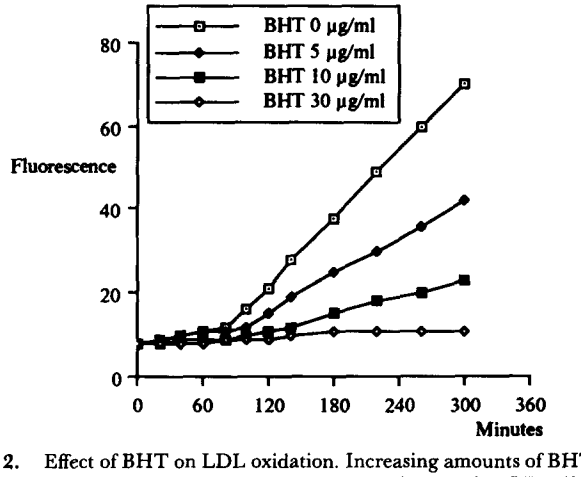

Fig. 2. Effect of BHT on LDL oxidation. Increasing amounts of BHT were added to different samples of the same lot of EDTA-free LDL (0.5 mg protein/ml). The incubations were performed at 37°C under air flow in presence of 50 μM Cu^{2+}. After appropriate dilution the fluorescence of the samples was measured every 20 min from time 0 to 140 and every 40 min from time 140 to 300 at 430 nm with excitation at 360 nm. Fluorescence was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into measurement cell. BHT was added at the start of the experiment in concentrated ethanol solution (20 μl in 1.5 ml LDL).

20 μg/ml, respectively, at the end of the incubation had no effect on the fluorescence measurement, thus excluding the possibility that the decrease of fluorescence was due to a quenching effect of the two substances.

Gel-filtration studies showed that the main peaks of fluorescence, protein, and cholesterol of both native and oxidized LDL coeluted at the void volume. **Fig. 3** shows that the specific fluorescence of LDL peak greatly increased after oxidation. Moreover, after delipidation with isopropanol, the major part of the fluorescence bound to oxidized LDL was found in the protein extract (>95%) both in the native and oxidized LDL, while only a negligible fraction was found in the organic solvent. The results obtained by using delipidation with isopropanol was confirmed by gradient ultracentrifugation in detergent. The major fluorescence peak, in fact, was superimposable on the main protein fraction, while the lipids floated on the top of the gradient (**Fig. 4**). Also in this case an increase in the fluorescence of protein LDL peak was observed. It must also be pointed out that the protein fraction was slightly fluorescent before oxidation.

Fig. 5 shows the electrophoresis on agarose-gel of LDL during the oxidative process. During the reaction the electrophoretic mobility increased progressively. In all oxidized samples a band corresponding to the migration of the native LDL was never detected. However, when the oxidized samples were mixed with native LDL, two different bands separated, the faster corresponding to the oxidized LDL at any given time, and the slower to the native LDL. The presence of BHT during exposure to Cu^{2+}/air abolished electrophoretic changes in the LDL.

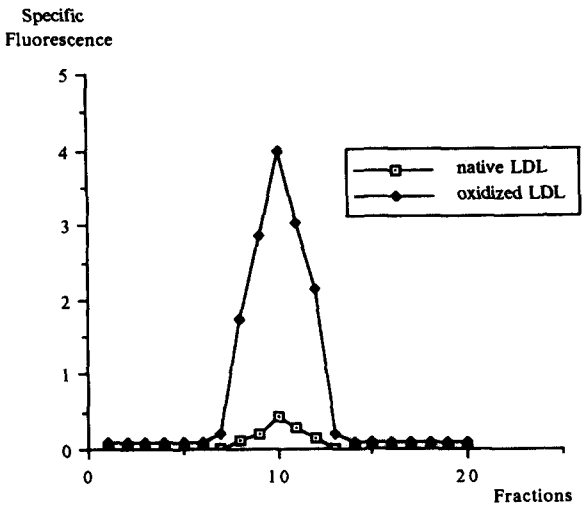

Fig. 3. Specific fluorescence of LDL of native and oxidized LDL after gel filtration. Native and oxidized LDL were gel-filtered on AcA22 (a gel that does not retain particles with a molecular weight greater than 1.2×10^6) in 20 mM Tris, 0.3 mM EDTA, and 0.15 M NaCl buffer, pH 7.4. The eluted fractions were checked for protein concentration and fluorescence. Specific fluorescence was expressed as fluorescence/mg protein.

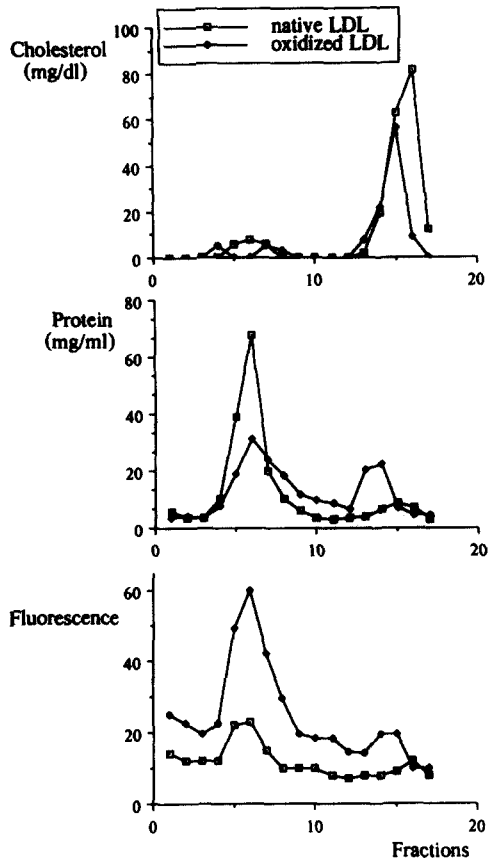

Fig. 4. Gradient ultracentrifugation of native and oxidized LDL. Native and autoxidized LDL were ultracentrifugated (Beckman T-41 swinging bucket rotor, 40.000 rpm for 20 h) in a sucrose gradient containing 15 mM CHAPS and 0.15% Nonidet P-40 buffered with 10 mM Tris-HCL, pH 8.6. In the 20 fractions collected from the bottom, protein, cholesterol, and fluorescence were determined.

Our results also show that during the oxidation of LDL there was a progressive decrease in the free amino groups (TNBS reactivity) of LDL apoB. **Fig. 6** shows the behavior of free amino groups and fluorescence during the oxidation of LDL. It must be pointed out that the decrease in free amino groups behaved inversely to the increase in fluorescence and that both profiles showed a superimposable inhibition period.

Fig. 7 shows the displacement of the uptake of ^{125}I-labeled native and autoxidized LDL in normal rabbit fibroblasts.

The reproducibility of the fluorescence increase was verified with the same pool of LDL. In **Table 1** are indicated the values of 10 determinations of the same LDL pool or of 10 different control subjects performed on different days. Each determination was done in triplicate. The coefficient of variation (CV) calculated on the triplicate values of fluorescence at any time of the reaction both in the LDL pool and in control subjects was never greater than 2%; the CV calculated on the values

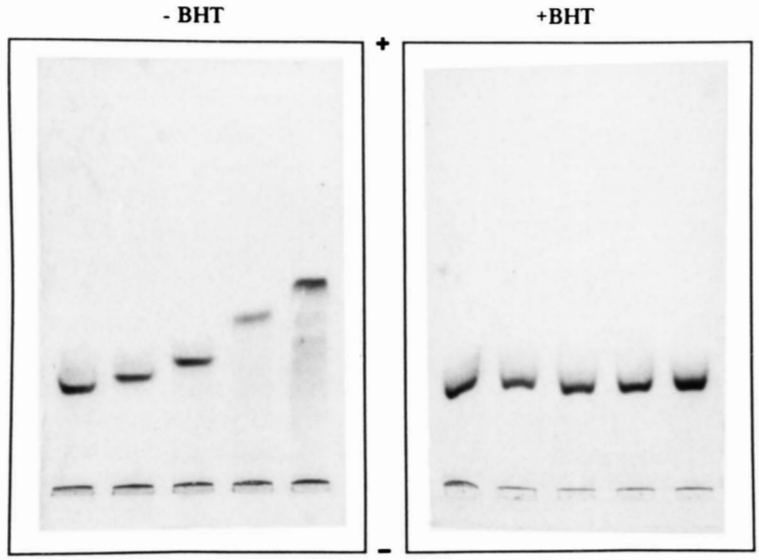

Fig. 5. Electrophoretic mobility of LDL during the oxidative process. LDL (0.5 mg protein/ml) containing 50 μM Cu^{2+} was incubated at 37°C under air flow without (on the left) or with (on the right) BHT (20 μg/ml) for 4 h. Aliquots were taken every 60 min and the reaction was quenched in EDTA (see Material and Methods). Agarose gel electrophoresis was carried out in Tris buffer, pH 8.6, at 4°C. The sample was pre-stained with Sudan Black. The 0-time is on the left of each gel.

of fluorescence in the LDL pool at any time of the reaction in 10 different days was never greater than 3%; the CV calculated in the 10 control subjects at any time of the reaction ranged between 15 and 25%. The analysis of variance for random effects showed that the between-subjects variability was significantly greater than the experimental variability (triplicates) at any time of the reaction (F test ranging from 500 to 2000; $P<0.001$).

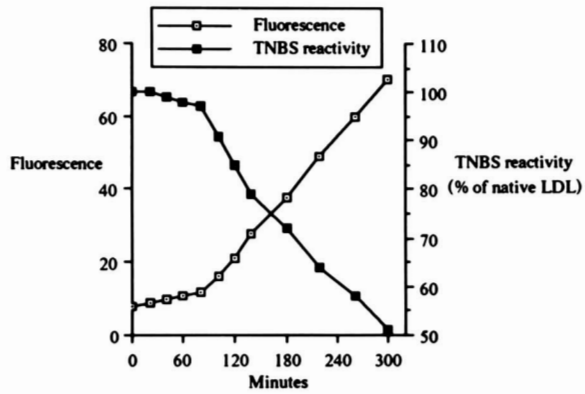

Fig. 6. Decrease of free amino groups (as TNBS reactivity) and fluorescence development during LDL oxidation. EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air flow in the presence of 50 μM Cu^{2+}. The samples collected every 20 min from time 0 to 140 min and every 40 min from 140 to 300 min were checked for free amino groups with 2,4,6-trinitrobenzenesulfonic acid and for fluorescence at 430 nm with excitation at 360 nm. Fluorescence was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into measurement cell. Free amino groups were expressed as a percent of initial value (native LDL).

Since the plot of the fluorescence intensity against the time was a straight line both in the inhibition period and in the propagation period (a plateau or post-propagation period was rarely seen and, in any case, never before 240 min), the calculation of the fluorescence development rate (FDR) of the two periods and also of the duration of the inhibition period (intersection of the two lines) was allowed. In practical terms the process of fluorescence development from time 0 to 240 min was approximated by using the following model:

$$\begin{cases} y = a_1 + b_1 t, & t < t_i \\ y = a_2 + b_2 t, & t \geq t_i \end{cases}$$

where t_i is the reaction time corresponding to the intersection of the two lines (inhibition period), and b_1 and b_2 represent the slopes (rates) of the inhibition period and of the propagation period. The values of t_i, b_1, and b_2 were estimated by least squares analysis.

In **Table 2** are indicated the mean values of the inhibition period and the slopes of the inhibition and propagation period in LDL pool and control subjects. Each determination was done in triplicate. The CV calculated on the triplicates of each parameter both in the LDL pool and in controls subjects was never greater than 2%; the CV of each parameter calculated for the LDL pool on 10 different days was never greater than 3%; the CV for each parameter calculated in the 10 control subjects ranged between 20 and 25%.

The analysis of variance for random effects showed that the between-subjects variability for each parameter was

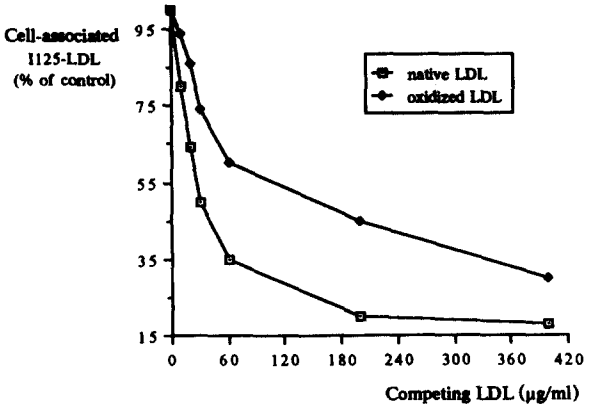

Fig. 7. Displacement of the uptake of ^{125}I-labeled LDL in normal rabbit fibroblasts by native and oxidized LDL. Confluent monolayers of fibroblasts were incubated with lipoprotein-deficient medium containing ^{125}I-labeled LDL (15 μg/ml; 160 dpm/ng) plus the indicated concentrations of native and oxidized LDL at 37°C for 4 h. Cell-associated radioactivity was determined as described in Materials and Methods and expressed as a percent of uninhibited control.

significantly greater than the experimental variability (triplicates). (F test ranging from 300 to 1800; $P<0.001$).

In **Table 3** are indicated the mean values of the FDR of the two periods and the duration of the inhibition period with different copper concentrations. Increasing copper concentrations were associated with an increase in the FDR of the two periods and with a decrease in the duration of the inhibition period. However, they did not change proportionally and, as shown in Table 3, a tenfold increase in copper concentration changed the duration of the inhibition period and the FDR of the propagation period by about 40%.

Fig. 8 shows the relationship between the copper concentration and the intensity of fluorescence developed after 5 h of LDL oxidation. After a definite value the increase in copper concentration did not produce any variation in the fluorescence which, on the contrary, remained constant.

In **Table 4** the values of fluorescence generated upon oxidation of LDL are compared with the values of conjugated dienes and thiobarbituric acid reactive substances (TBARS). Under conditions only conjugated dienes and TBARS seemed to reach an obvious third stationary phase.

DISCUSSION

The results of this study show that the fluorescence of LDL increased considerably during incubation at 37°C in air in presence of copper. The increase in fluorescence required the presence of oxygen and substitution of oxygen with argon considerably prevented the fluorescence development. Furthermore, the fact that EDTA completely suppressed the fluorescence increase confirms that the process required the presence of copper, but apparently not as a chemically reactive chelate.

As already reported, the fluorescence increase during the oxidative modification is due mainly to a derivatization reaction of the free amino-groups of apoB, possibly induced by some reactive aldehydes and, in particular, by 4-hydroxynonenal originating from the oxidation of unsaturated fatty acids of the LDL phospholipids (14). The increase in fluorescence during the LDL oxidation is said to be mainly due to the protein-bound fluorophore with emission at 430 nm when the excitation is at 360 nm (28). The chemical structure of this fluorophore is still unknown even though it has been hypothesized that it may be related, as recently reviewed (29, 30), to a conjugated Shiff base or to some dihydropyridinaldehydes or, as more recently suggested, to beta-addition products (31).

According to previous results (28), the fluorophore generated under our conditions during the oxidative process, with emission at 430 nm when the excitation is at 360 nm, seems mainly bound to the protein and possibly reflects apoB derivatization. The weak protein-bound fluorescence found in freshly prepared LDL, and its protein fraction in particular, is most likely indicative, as

TABLE 1. Mean values (± SD) of fluorescence generated upon oxidation of the same LDL pool on different days compared with the results obtained in 10 control subjects

	Minutes											
Sample	0	20	40	60	80	100	120	140	180	220	260	300
LDL pool	6.3 ± 0.25	7.7 ± 0.28	8.7 ± 0.36	9.6 ± 0.41	10.9 ± 0.31	12.2 ± 0.42	14.8 ± 0.42	18.8 ± 0.79	30.5 ± 0.50	40.2 ± 0.70	50.2 ± 0.75	57.1 ± 0.65
Controls	7.3 ± 1.82	8.3 ± 1.68	9.0 ± 2.17	11.7 ± 2.87	13.9 ± 4.24	18.3 ± 4.75	24.9 ± 5.25	30.9 ± 5.85	40.2 ± 6.16	50.3 ± 9.33	58.6 ± 11.13	68.2 ± 16.05

EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air flow in the presence of 50 μM Cu^{2+}. The fluorescence of the samples was measured every 20 min until the 140th minute and every 40 min until the 5th hour at 430 nm with excitation of 360 nm. It was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into the measurement cell. Values (± SD) are means of 10 determinations of the same LDL pool or of 10 different control subjects performed on different days. Each determination was done in triplicate. See text for details of coefficient of variation.

TABLE 2. Mean values (± SD) of the inhibition period and of the fluorescence development rate (FDR) of the inhibition period (IP) and of propagation period (PP) utilizing the same LDL pool on different days and 10 control subjects

Samples	Inhibition Period	FDR-IP	FDR-PP
	min	*10^2F/min*	*10^2F/min*
LDL pool	110.96 ± 2.12	5.8 ± 0.16	25.5 ± 0.37
Control subjects	75.47 ± 19.28	8.3 ± 1.70	29.6 ± 5.80

EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air flow in presence of Cu^{2+} (50 μM). The fluorescence (F) of the samples was measured every 20 min until the 140th minute and every 40 min until the 5th hour at 430 nm with excitation at 360 nm. F was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into the measurement cell. FDR-IP represents the slope of the line obtained by plotting F against the time during the inhibition period. FDR-PP represents the slope of the line obtained by plotting F against the time during the propagation period. The inhibition period was extrapolated on the time axis at the intersection of the two regression lines. Values (± SD) are mean of 10 determinations of the same LDL pool or of 10 different control subjects performed on different days. Each determination was done in triplicate. See text for details of coefficient of variation.

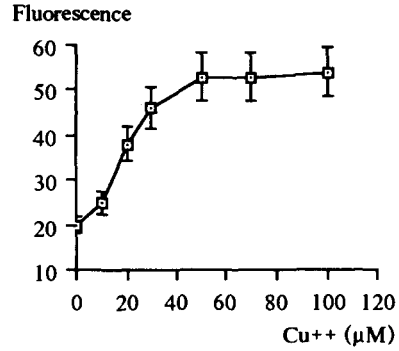

Fig. 8. Relationship between copper concentration and intensity of fluorescence developed after 5 h of LDL oxidation. Increasing amounts of Cu^{2+} (from 10 to 100 μM) were added to different samples of the same lot of EDTA-free LDL (0.5 mg protein/ml). The incubations were performed at 37°C under air flow. After appropriate dilution the fluorescence of the samples was measured after 300 min at 430 nm with excitation at 360 nm. Fluorescence was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into measurement cell. Values are means ± SD of triplicate determinations.

already stated (28), of the presence of remnants from lipid peroxidation that had occurred in vivo before the isolation of LDL from plasma.

In agreement with previously reported data (12, 32–34), our study also demonstrates that copper modification of LDL was associated with an increased anodic electrophoretic mobility of the lipoprotein on agarose gel and with a decrease in the reactive amino groups of apoB. The loss of reactive amino groups is said to be accompanied by a decreased degradation of LDL in vitro and in vivo via LDL receptor (12), and increased degradation via acetyl LDL receptor (12). The result showing that oxidized LDL displaced ^{125}I-labeled at a lower extent than native LDL from rabbit fibroblast receptors is consistent with several other reports (35, 36) in which it was shown that blocking of lysine residues lowered the affinity of apoB for its receptor. Therefore, the fact that the fluorescence behaved inversely to the decrease of the TNBS reactivity of LDL and that both profiles showed a superimposable inhibition period supports the idea that the fluorescence increase may likely monitor the extent of the apoB derivatization.

TABLE 3. Effect of different copper concentrations on the inhibition period and on the fluorescence development rate (FDR) of inhibition period (IP) and propagation period (PP)

Copper Concentration	Inhibition Period	FDR-IP	FDR-PP
μM	*min*	*10^2F/min*	*10^2F/min*
5	129.33 ± 24.97	4.5 ± 1.00	16.6 ± 3.20
20	94.02 ± 18.55	5.8 ± 1.20	22.9 ± 4.50
50	75.47 ± 19.28	8.3 ± 1.70	29.6 ± 5.80

EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air flow in the presence of different copper concentrations (5–20–50 μM). The fluorescence (F) of the samples was measured every 20 min until the 140th minute and every 40 min until the 5th hour at 430 nm with excitation at 360 nm. F was expressed as fluorescence intensity corrected for cyclohexane Raman band and protein concentration into the measurement cell. FDR-IP represents the slope of the line obtained by plotting F against the time during the inhibition period. FDR-PP represents the slope of the line obtained by plotting F against the time during the propagation period. The inhibition period was extrapolated on the time axis at the intersection of the regression lines. Values of the inhibition period, FDR-IP, and FDR-PP represent the mean ± SD of the values obtained (in triplicate) under different copper concentrations in 10 control subjects. Analysis of variance indicated a significant difference of the three parameters under different copper concentrations ($P < 0.01$). Multiple comparison test revealed that at 5 μM Cu^{2+} the mean of the parameters was significantly different than at 20 and 50 μM Cu^{2+} ($P < 0.01$); at 20 μM Cu^{2+} the mean of the parameters was significantly different than at 50 μM Cu^{2+} ($P < 0.01$).

Under our conditions the fluorescence increase was biphasic and consisted of an initial inhibition period and a second propagation period during which the fluorescence rose quickly. During the inhibition period the reaction rate was likely to be reduced by the presence of some antioxidants, the more representative being the vitamin E (14, 37). When the antioxidants are completely destroyed, the kinetics reaches the rate characteristic of the uninhibited oxidative process (37, 38). Contrary to the model established by Esterbauer et al. (14), under our experimental conditions the generation of new fluorophores did not cease during the time of incubation, suggesting that the functional groups with a potential to be converted to fluorophores were not completely depleted after 5 h of LDL oxidation.

Increasing copper concentrations were associated with a decrease in the duration of the inhibition period and with an increase in the FDR of the two periods. However,

TABLE 4. Kinetics of the generation of fluorescence (F), conjugated dienes (CD), and thiobarbituric acid reactive substances (TBARS) during oxidation of LDL

	Minutes											
Sample	0	20	40	60	80	100	120	140	180	220	260	300
F	6.1 ± 0.0	7.9 ± 0.0	8.6 ± 0.0	10.0 ± 0.3	11.1 ± 0.4	12.1 ± 0.4	14.5 ± 0.4	17.6 ± 0.5	29.8 ± 0.8	39.6 ± 0.6	50.2 ± 1.1	57.2 ± 1.0
CD	176 ± 14	194 ± 22	202 ± 17	227 ± 15	236 ± 10	283 ± 24	391 ± 34	557 ± 47	612 ± 58	664 ± 57	671 ± 61	665 ± 59
TBARS	2.7 ± 0.4	2.8 ± 0.4	2.5 ± 0.4	6.0 ± 0.6	9.9 ± 1.6	22.8 ± 2.8	59.4 ± 6.7	100.1 ± 12.1	129.2 ± 15.2	132.4 ± 17.2	132.6 ± 16.1	135.9 ± 14.3

EDTA-free LDL (0.5 mg protein/ml) was incubated at 37°C under air flow in the presence of Cu^{2+} (50 μM). The fluorescence (F) of the samples was measured every 20 min until the 140th minute and every 40 min until the 5th hour at 430 nm with excitation of 360 nm. F was expressed as fluorescence intensity corrected for Raman band (cyclohexane) and protein concentration into the measurement cell. CD and TBARS were expressed in nmol/mg protein. Values are mean (± SD) of triplicate determinations.

they did not change proportionally and a tenfold increase in copper concentration changed the duration of the inhibition period and of the FDR of the propagation period by about 40%. Interestingly, in the case of the propagation period, after a definite value the increase in copper concentration did not produce any variation in the reaction rate which, on the contrary, remained constant. While on the basis of our present results we cannot exclude that this phenomenon may have been caused by a limited number of peroxides, even if at this time of reaction all the endogenous antioxidants are supposed to have been destroyed, it is possible that the saturation-like behavior we found may depend on the presence of any enzymatic step between the oxidation period and the derivatization of the apoB, an that at higher initiation rates the enzyme becomes substrate-saturated.

That an enzymatic activity, and in particular a phospholipase A_2 activity, may play an essential role in the oxidative modification of LDL has been previously reported by Parthasarathy et al. (39). These authors found that not only endothelial cell-modification, but also copper-modification of LDL was accompanied by hydrolysis of phosphatidylcholine at the *sn*-2 position (39), suggesting the presence of a phospholipase A_2 activity associated with LDL as it is isolated.

This study also shows that the extent of fluorescence generated upon LDL oxidation varied greatly in the control subjects, contrary to results with the LDL pool. Similar data under different experimental conditions were previously obtained Esterbauer et al. (14) who found that the maximum increase in fluorescence after oxidative modification of LDL ranged from 135 to 280% in different subjects as compared to the native LDL. The difference in the extent of fluorescence generated at any time of LDL oxidation process gave rise to a great variability in the length of the inhibition period and in the FDR of the two periods among the control subjects. According to the classical kinetics proposed by Niki (40) for lipid peroxidation, the length of the inhibition period is directly correlated with the amount of antioxidants and inversely with the initiation rate. Furthermore, at a fixed rate of radical chain initiation, the slope of the inhibition period is correlated with the potency of radical scavengers, while the slope of the propagation period is determined by the amount and kind of polyunsaturates as well as by the number of hydroperoxides in the unprotected system (40). Extrapolating to LDL, the variability in the duration of inhibition period and in the FDRs may express compositional differences in the lipoproteins that may give rise to different susceptibilities of LDL to be modified in the same oxidative process. Since the derivatization of apoB is likely to start only after the end of the induction period and the slope of the propagation period is likely to assess the rate of apoB derivatization, the possibility of measuring these parameters could be useful in a clinical laboratory if we accept the idea that LDL can penetrate macrophages only after its modification (11). Further studies are needed to clarify the mechanisms that regulate the different parameters extrapolated from the oxidative curve and their distribution in normal populations and those at risk for atherosclerosis.

As expected and already demonstrated (38), conjugated dienes and TBARS showed about the same behavior as far as the first two periods were concerned but, contrary to the fluorescence, they reached a steady-state concentration. The fact that under our conditions only conjugated dienes and TBARS reached an evident third stationary phase confirms that these compounds represent intermediate products of the oxidative chain reaction (38, 41). Fluorescence, on the contrary, accumulated progressively and may represent at least one of the products of the final step, thus possibly matching the overall rate of apoB modification. So far there are several different methods available to measure the modification of LDL apoB: electrophoresis on agarose gel (32–34), macrophage degradation of modified LDL (6, 7), and apoB free amino

groups measurement (12). Electrophoresis is simple and easy to perform, unfortunately, it is unsuitable for quantitative measurements and therefore cannot evaluate the parameters we propose. Macrophage degradation, even if very predictive, is time-consuming and not suitable for kinetic measurement. Measurement of apoB free amino groups, when expressed as TNBS reactivity of LDL, could evaluate the same parameters we propose to measure with fluorescence; however, it does not seem to match entirely the measurements of intact lysine residues by amino acid analysis and therefore the derivatization of apoB (12, 42).

Fluorescence measurement is easy to perform and kinetic measurements can properly assess parameters (40) related to the total protection (inhibition period duration), radical scavenger potency (FDR during inhibition period), and susceptibility of unprotected LDL (FDR during propagation period).

The first main drawback of fluorescence measurement and comparison of data over a long period of time may be connected with the decrease in the emission power of the excitation light. To avoid this possibility a day-to-day sensitivity calibration is required. In our case the use of the ratio between the sample values and the intensity of cyclohexane Raman band seems to overcome the problem. Furthermore, fluorescent compounds with the same excitation/emission characteristics and, in particular, drugs carried by the LDL particles may generate high fluorescent background and therefore make the reading impossible.

It must also be pointed out that the fluorescence measurement cannot at present be expressed in absolute values since we lack a proper standard. Therefore, future investigative efforts on the nature of the fluorophore(s) are needed. Finally, it must be recognized that the method only recognizes how susceptible LDL is to the Cu^{2+}-dependent oxidation. As we do not know how oxidation is catalyzed in vivo, this may have nothing whatsoever to do with the in vivo situation. Further work will be needed to assess whether the LDL susceptibility to oxidation by Cu^{2+} in vitro is an indicator of the in vivo situation. Data from patients with severe atherosclerosis could therefore be most interesting.

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