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DIPHENYLHEXATRIENE AS A FLUORESCENT PROBE FOR MONITORING LOW DENSITY LIPOPROTEIN PEROXIDATION

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The use of the fluorescent probe diphenylhexatriene (DPH) for monitoring low density lipoprotein (LDL) peroxidation has been investigated. The DPH incorporation into LDL results in a high fluorescence signal which decreases with time after addition of cupric ions. A strong correlation was found between the decay of the DPH fluorescence signal and the appearance of the thiobarbituric reactive substances (TBARS). HPLC and spectrofluorometric analyses demonstrated that DPH is destroyed during the time course of the copper-induced LDL peroxidation. The decrease in DPH fluorescent signal is prevented by addition of EDTA, vitamin E and drugs which protect LDL against peroxidation such as probucol or calcium antagonists. The high fluorescence of DPH allows the use of very small quantities of LDL (less than 5 μg/ml LDL protein). We thus suggest that DPH could be of use for continuous monitoring of LDL autooxidation, especially for the *in vitro* testing of the protective effect of antioxidant compounds.

KEY WORDS: Diphenylhexatriene, low density lipoprotein peroxidation.

Abbreviations: DPH, diphenylhexatriene; EDTA, ethylene diamine tetraacetic acid; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; PBC, probucol; PBS, Phosphate Buffered Saline; TBARS, thiobarbituric acid reactive substances.

INTRODUCTION

It is admitted that low density lipoprotein (LDL) peroxidation plays a crucial role in the formation and in the progress of atherosclerotic lesions (1–3). The LDL is modified *in vitro* by incubation in the presence of endothelial (4) or smooth muscle cells (5), or monocytes (6). This process involves lipid peroxidation, as demonstrated by the appearance of thiobarbituric acid reactive substances (TBARS), and results in an increased negative net charge of the particle (4,7,8). As a consequence, the LDL is no longer recognized by its specific (apo B/E) receptor, but catabolized *via* the scavenger receptor pathway of macrophages (1–3). Stimulation of cholesteryl ester formation by such a modified LDL is currently believed to play a role in the formation of the foam cells derived from the monocyte-macrophage (1–3). The demonstration of the existence

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of oxidatively modified LDL *in vivo* (9,10) also supports the hypothesis for a role of the oxidative process in atherogenesis.

LDL oxidative modification can also be achieved *in vitro* by incubation of the particle with copper ions (8,11,12). This method provides an interesting model for the study of the mechanisms of LDL lipid peroxidation and for the evaluation of the antioxidant properties of drugs. In particular, it appears of interest to continuously follow the time course of the oxidative modification of the particle, and various methods have already been proposed for this purpose. The TBARS formation is most usually checked by the fluorometric assay of Yagi (13), but this method is not suitable for a continuous monitoring of lipid peroxidation during the time course of LDL modification. As a consequence, several authors tried to set up non destructive assays in order to more precisely study the mechanisms involved in LDL peroxidation and in the protective action of endogenous antioxidants such as vitamin E. Thus, Esterbauer *et al.* developed a method based on the measurement of conjugated diene bonds by spectrophotometry at 234 nm (14). More recently Cominacini *et al.* proposed to follow the increase of the LDL fluorescence at 430 nm upon excitation at 360 nm (15), based on previous observations of the group of Esterbauer (11). However, it must be observed that all these methods require relatively high LDL amounts. We therefore tried to develop a more sensitive and non destructive assay for the *in vitro* monitoring of LDL peroxidation. This method is based on our observation that the fluorescence signal of diphenylhexatriene (DPH) exhibited a progressive decay during the time course of LDL lipid peroxidation.

MATERIALS AND METHODS.

Chemicals.

The DPH was purchased from Interchim (Montluçon, France). Probucol was a generous gift from Merrell Dow France (Levallois-Perret, France). Tris buffer, EDTA, NaCl, $CuS0_4$, vitamin E and flunarizine were obtained from Sigma (St. Louis, MO, USA). Solvents for high pressure liquid chromatography (HPLC) were provided by Merck (Darmstadt, Germany).

LDL preparation.

The LDL, taken as the 1.024–1.050 fraction, was prepared from plasma of healthy donors by sequential ultracentrifugation according to Havel *et al.* (16). After extensive dialysis against a pH 7.4 0.005M Tris/0.15M NaCl/0.04% EDTA buffer, the LDL was filtered through a 0.45μM Millipore filter, and used within 48 h.

Fluorometric assay of LDL peroxidation.

Before experiments, an aliquot of the LDL preparation was dialysed against 0.005M Tris buffer/0.15M NaCl pH 7.4 devoid of EDTA. The labeling of LDL was performed using a 5×10^{-4}M dispersion of DPH in phosphate buffered saline (PBS). The final concentration of the LDL was 20 μg/ml, expressed on a protein basis (10 μg LDL protein in a 0.5 ml cuvette). The final concentration of DPH was 5×10^{-7}M. The dispersion of the probe into the lipidic core of the particle resulted in a progressive increase in the fluorescence signal (excitation and emission wavelengths were fixed at

354 and 432 nm, respectively). Fluorescence measurements were done using a Perkin Elmer LS5 spectrofluorometer equipped with a 4 position thermostatically controlled cuvette holder, with automatic recording using a Perkin Elmer R 100 A instrument. The kinetic of DPH incorporation into the LDL reachd a plateau after 90 min. of incubation of the rticle with the probe. However, about 80–90% of the signal is obtained within 60 min. of incubation. Thus, for experiments, except otherwise indicated, LDL labeling with DPH was performed by a 60 min. incubation of the particle with the probe, at 37°C. At the end of the labeling period, Cu^{2+} ions (5×10^{-6} to 5×10^{-5}M, final concentration) were added and the mixture was gently shaken. Fluorescence was then measured at various times after copper addition. In some experiments, the fluorescence spectra of DPH in LDL was checked before and after peroxidation of the particle induced by overnight incubation in the presence of 5×10^{-6}M Cu^{2+} ions.

HPLC determination of DPH in LDL extract.

In order to investigate whether the decay of the DPH fluorescent signal was due to a progressive release of the probe into the aqueous medium as a consequence of LDL peroxidation or to an actual destruction, we also performed HPLC analysis of the DPH-labeled LDL at various times following the addition of cupric ions. Aliquots (0.5 ml) of the DPH-labeled LDL solution were extracted with an ethanol/hexane (1:2, v/v) mixture by vigorous shaking during 5 minutes. After centrifugation, the upper phase was removed and an aliquot applied to a Waters ODS C18 column coupled to a Shimatzu RF 535 fluorometric detector (excitation at 354 nm, emission at 432 nm). The elution mixture was ethanol/methanol/water (80:10:4, v/v) and the flow rate was fixed to 1.5 ml/minute. Under these conditions, DPH appeared as a sharp peak, eluting 2 min. after the solvent. No other peak was observed.

TBARS measurements.

The appearance of TBARS was measured at intervals after addition of copper ions by the fluorometric assay of Yagi (13). In most experiments, TBARS were assayed on 50 μg (on a protein basis) of unlabeled LDL. In some cases, in order to study the possible influence of DPH on the lipid peroxidation process, TBARS were comparatively measured on samples of unlabeled or DPH-labeled LDL (see Figure 6). Results were expressed as nmol equivalent MDA/mg LDL protein. Protein measurement was performed according to Peterson (17).

All experiments were carried out at least in triplicate. Statistical analysis was performed by Student's t test.

RESULTS

Figure 1 displays a typical plot of the kinetics of DPH incorporation into native LDL. It can be noted that the curve tends to reach a plateau after 90 min. of incubation of the probe with the LDL solution at 37°C. Further incubation results in a very slow increase in the fluorescence signal, and the overall kinetics fits to a first order process with a regression coefficient close to 1. This is in agreement with a passive diffusion mechanism for the probe incorporation, as it would be expected from its lipophilic nature. The main point which has to be stressed in these experiments is that in the absence of any other additive in the LDL solution, the fluorescence of DPH is stable.

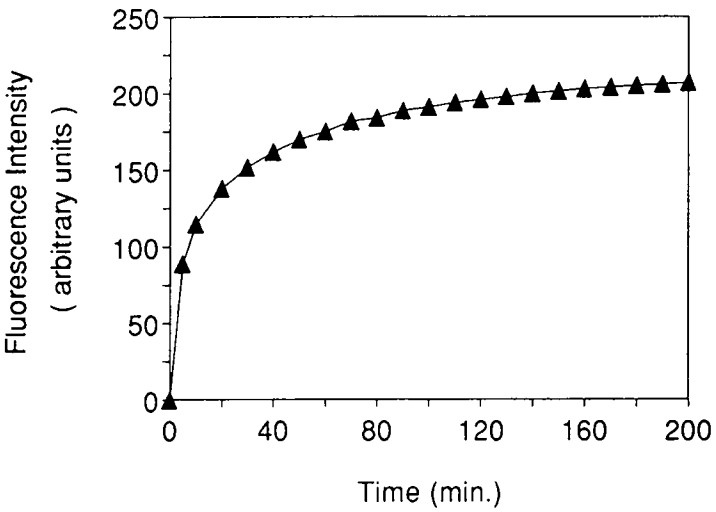

FIGURE 1 Typical kinetics of DPH incorporation into low density lipoprotein. The labeling of LDL was performed using a 5×10^{-4}M dispersion of DPH in phosphate buffered saline (PBS). The final concentration of the LDL was 20 μg/ml, expressed on a protein basis. The final concentration of DPH was 5×10^{-7}M. The excitation of the probe was performed at 354 nm, and fluorescence was recorded at 432 nm.

In other words, although DPH has been shown to be bleached by ultraviolet light (18), this phenomenon does not take place under our experimental conditions probably because the intensity of the excitation beam is too low to induce photochemical alteration of the probe.

A typical plot demonstrating the decrease in the DPH fluorescence upon addition of cupric ions to the LDL solution is shown in Figure 2a. The LDL solution has been labeled for 90 min at 37°C with 5×10^{-7}M DPH, then 5×10^{-6}M or 5×10^{-5}M cupric ions were introduced in the solution. First, a lag period is observed, the extent of which is inversely correlated with the copper ion concentrations (about 30–40 min. for 5×10^{-6}M Cu^{2+}, and only 10 min. for 5×10^{-5}M Cu^{2+}). This lag period probably corresponds to the consumption of the LDL endogenous antioxidants, as it has been previously shown (11). It must be observed that the duration of the lag period can vary with the LDL solution used for experiments (*i.e.* with the donor, and also with the storage time, although the LDL solution is always stored at 4°C in the presence of 0.04% EDTA, data not shown). This lag time is followed by a decrease in the DPH fluorescence, which occurs at increasing rate, suggesting an auto-propagated radical process. A third phase is then observed, with a slowered rate of decrease in the DPH fluorescence, which tends to zero. The disappearance of the DPH fluorescence is achieved with a delay which depends, for the same LDL preparation, upon the copper concentration. It must also be observed in Figure 2a that in the control LDL solution (no copper ions added) the DPH fluorescence does not exhibit any tendency to a decrease all along the experiment. Figure 2b shows that, concomitant to the decrease in the DPH fluorescence, an increase in the amount of TBARS was observed during the copper- induced LDL autooxidation. This increase in the TBARS content of the particle was inversely related to the fluorescence signal with a regression coefficient of –0.95 using a linear regression analysis (Figure 2c).

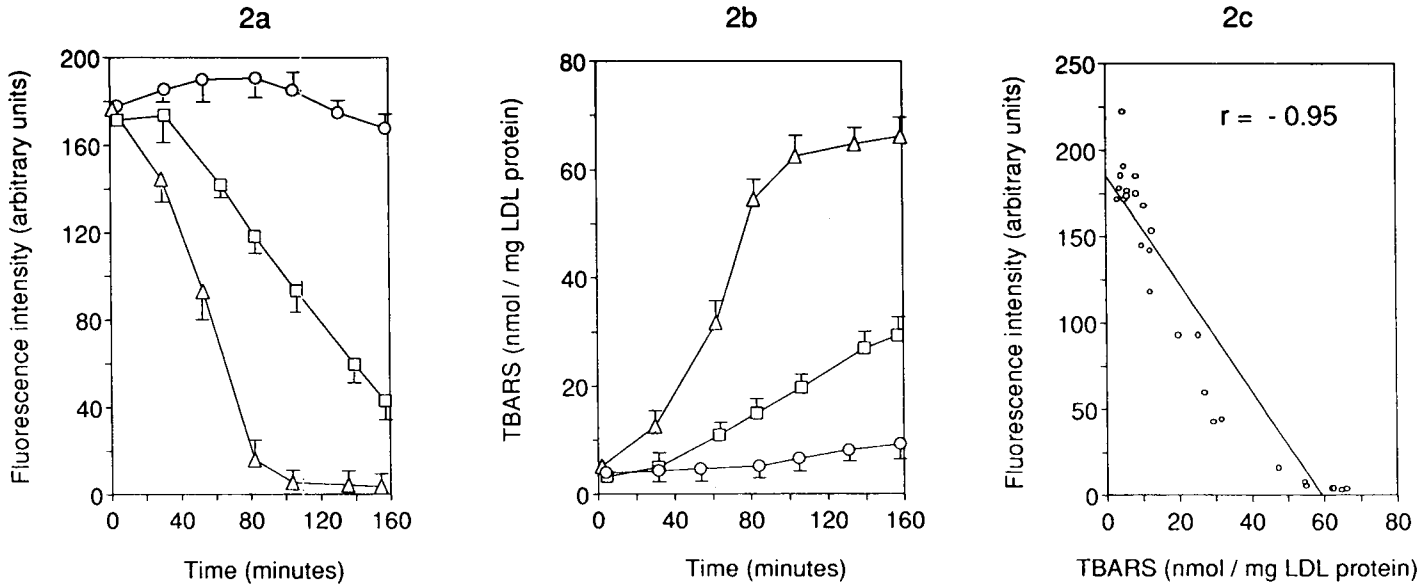

FIGURE 2 Effect of copper ions on the fluorescence of DPH-labeled LDL and TBARS formation as a function of incubation time with copper ions. Cu^{2+} ions were added to the LDL (20 μg/ml) solution, and after gently mixing, the fluorescence of the probe was recorded as described in Materials and Methods. TBARS formation was measured by the fluorometric method of Yagi on samples incubated in the same conditions. Figure 2a: fluorescence intensity at 430 nm; figure 2b: TBARS formation, expressed as nmol/mg LDL protein (○: no addition; ❑: 5×10^{-6}M Cu^{2+}; Δ: 5×10^{-5}M Cu^{2+}). Figure 2c: linear regression analysis of fluorescence intensity *v.s.* TBARS content of LDL during the time course of LDL oxidation. Each point corresponds to the mean of 6 experimental values±S.D.

Since previous studies demonstrated that addition of vitamin E results in an increase in the lag phase of LDL oxidation induced by copper ions (19), we tested the effect of various concentrations of alpha-tocopherol on the kinetics of the decrease in the DPH fluorescence during copper-induced LDL oxidation. In these experiments, the LDL solution was preincubated for 1 hour with vitamin E at concentrations ranging from 2×10^{-6} to 5×10^{-5}M. It can be observed in Figure 3a that the duration of the lag phase increases and that the kinetics of the oxidation process is notably slowered but not totally prevented in the presence of vitamin E. This is in accordance with previous results from the group of Esterbauer (19), using other experimental procedures to monitor the kinetics of the copper-induced LDL peroxidation. Figures 3b and 3c show that in these experiments a good correlation was also observed between the decrease in the DPH signal and the TBARS production studied in the presence of different concentrations of vitamin E ($r = -0.98$, using all experimental values).

In order to further establish the correlation between the decrease in the DPH fluorescence and the copper-induced oxidative process, we also tested the influence of EDTA, a divalent cation chelator which might be expected to impede the decrease in the DPH fluorescence if the latter was related to the presence of copper ions. Table I shows that the presence of 10^{-4}M EDTA completely prevents the decrease in the DPH fluorescence, while at the same time no significant amounts of TBARS were produced. Thus, the results presented in Figure 2, 3 and Table I strongly support the hypothesis that the reduction in the DPH fluorescence is actually related to the oxidative modification of the LDL induced by the addition of copper ions.

At this stage, the main question which had to be answered was to specify the mechanism(s) by which the DPH fluorescent signal was decreased during LDL peroxidation. Two hypothesis may be envisaged:

i Due to changes in the LDL structure, the probe is expelled from the lipidic core of the particle, resulting in its aggregation in the aqueous medium and in fluorescence quenching.

ii The probe is actually destroyed, as a "co-substrate" of the oxidative process.

In order to check these possibilities, the probe was extracted from the LDL by an hexane/ethanol mixture at intervals after addition of copper ions, and the amount of intact DPH recovered in the solution was measured by HPLC. Results in Figure 4 show that there is a good correlation between the decrease in the DPH fluorescence measured

TABLE I
Effect of EDTA on DPH fluorescence and TBARS formation in LDL incubated after overnight incubation at 37°C in the absence or presence of copper ions.

	Fluorescence intensity (arbitrary units)	TBARS formed (nmol/mg LDL protein)
Without Cu^{2+}:		
– EDTA:	203.5 ± 18	0.3 ± 0.1
+ EDTA	234.0 ± 16	0
Cu^{2+} 5×10^{-6}M:		
– EDTA	1.5 ± 0.9	58 ± 8
+ EDTA	198.5 ± 15	0.5 ± 0.2

The DPH-labeled LDL (20 μg/ml) was incubated for 18 hours in the absence or in the presence of 5×10^{-6}M Cu^{2+} or/and 10^{-4}M EDTA. The fluorescence was then measured at 432 nm upon excitation at 354 nm. The TBARS measurement was performed by the fluorometric method of Yagi (see Materials and Methods). Means of 6 values ± S.D., for 2 experiments carried out with the same LDL preparation.

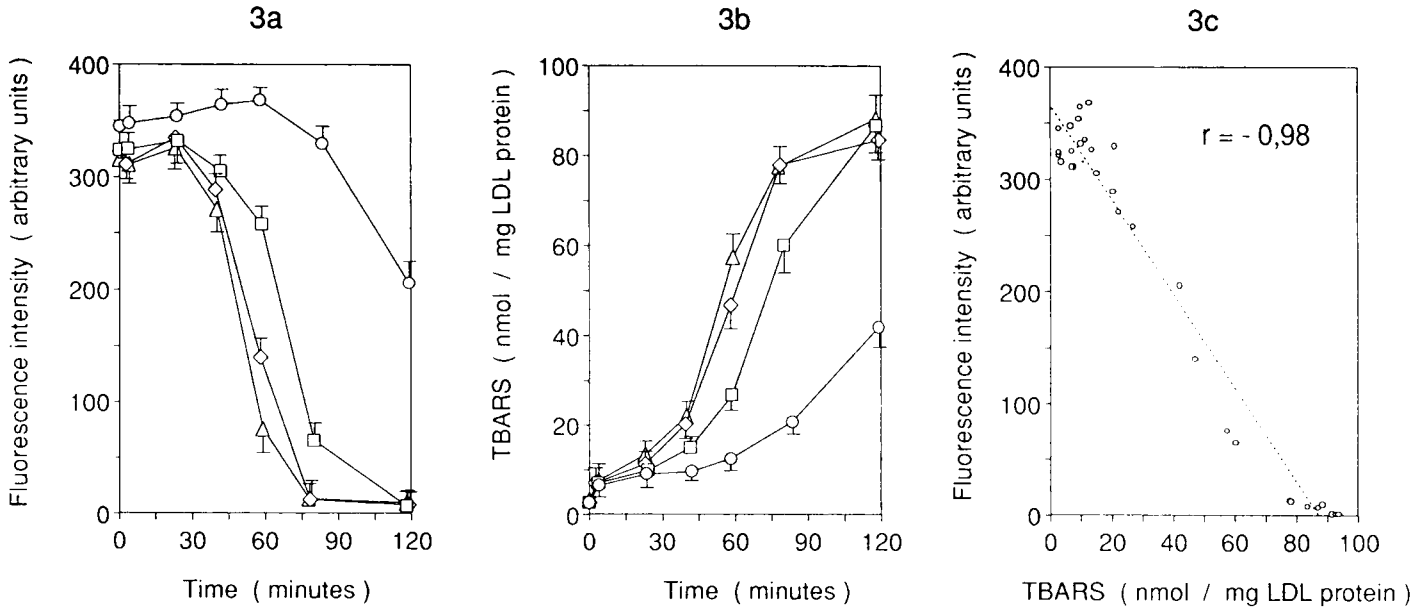

FIGURE 3 Effect of various concentrations of vitamin E on the time course of the DPH fluorescence and on the TBARS formation in LDL incubated with 5×10^{-6}M copper ions. The LDL solution was first incubated for 1 hour at 37°C with a concentrated solution of vitamin E in ethanol (final concentration of vitamin E: 2×10^{-6} to 5×10^{-5}M; final concentration of ethanol: 0.5‰). Figure 3a: fluorescence intensity at 432 nm; figure 3b: TBARS formation, expressed as nmol/mg LDL protein (Δ: no addition; ◊: 2×10^{-6}M vitamin E; ❑: 10^{-5}M vitamin E; ○: 5×10^{-5}M vitamin E). Figure 3c: linear regression analysis of fluorescence intensity *v.s.* TBARS content of LDL during the time course of LDL oxidation. Each point corresponds to the mean of 6 experimental values ± S.D.

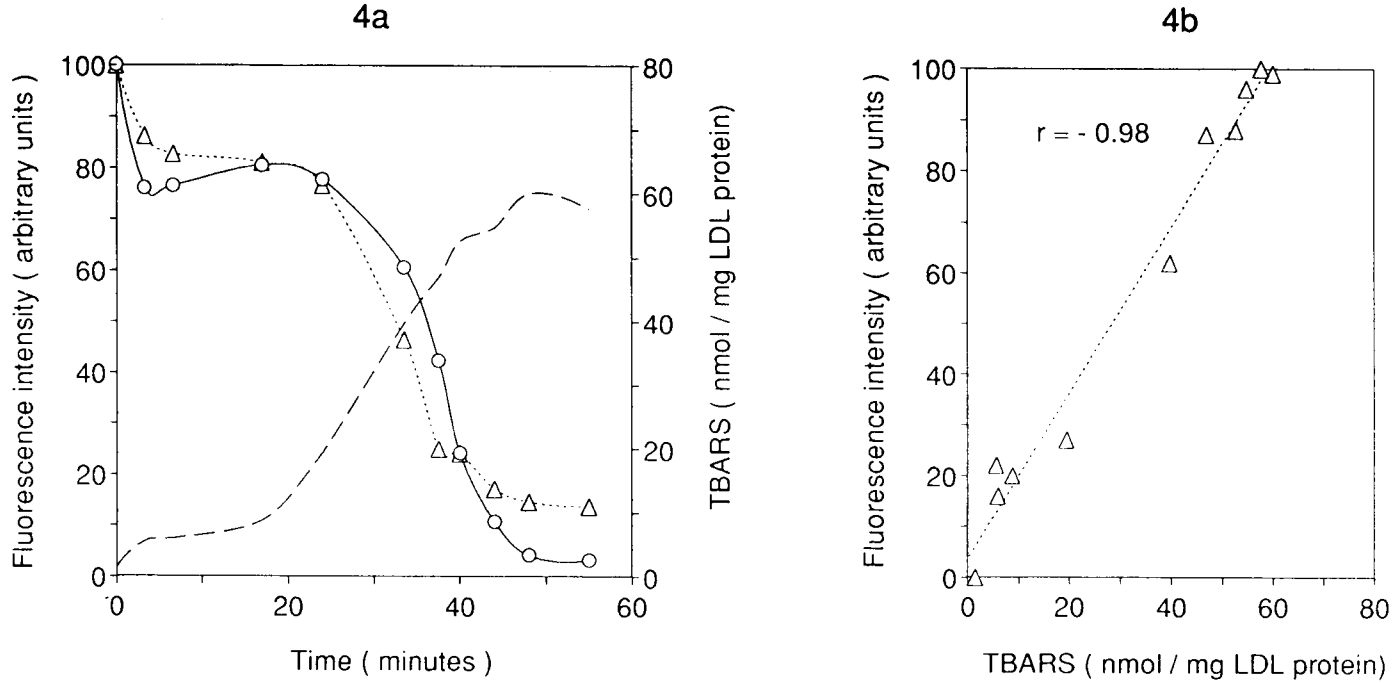

FIGURE 4 Correlation between the fluorescence of DPH-labeled LDL and the amount of intact DPH extracted from LDL as a function of the incubation time of the particle with 5×10^{-6}M Cu^{2+} ions, in relation to TBARS formation. The solvent-extracted DPH was measured by HPLC as specified in Materials and Methods. Figure 4a: ○: fluorescence intensity of intact DPH-labeled LDL; Δ: fluorescence intensity of DPH measured by HPLC after extraction with ethanol/hexane (see Materials and Methods);— — —: TBARS formation (nmol/mg LDL protein). Figure 4b: linear regression analysis of fluorescence intensity *v.s.* TBARS content of LDL. The data presented correspond to a typical experiment.

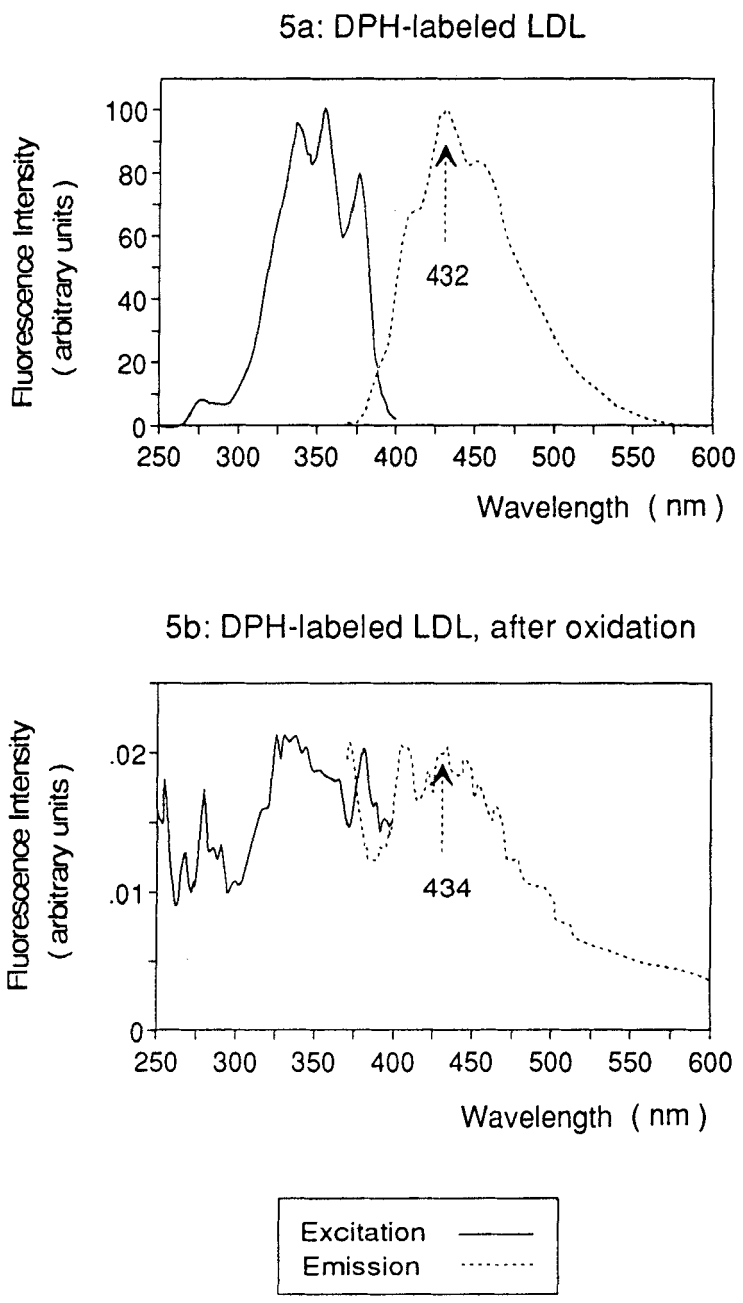

FIGURE 5 Typical results showing the fluorescence spectra of DPH-labeled LDL before (Figure 5a) and after (Figure 5b) 18 hour incubation in the presence of 5×10^{-5}M cupric ions. Experimental conditions are specified in Materials and Methods. The fluorescence intensities are expressed as percentages of the maximum values measured in LDL before oxidation (at 354 nm for excitation and at 432 nm for emission, respectively).

on the intact LDL solution and the decrease in the amount of DPH recovered by HPLC. The correlation coefficient between the DPH content of LDL measured by HPLC and the appearance of TBARS was r = –0.98 using linear fitting (not shown). However, it must be noted that the DPH concentrations measured after extraction and HPLC determination was never zero, even in experiments conducted during 18 hours (complete oxidation of the LDL particle). The percentage of intact DPH was usually about 10% of the amount of probe initially present in the labeled LDL. This suggests that if the major part of the probe is actually destroyed during the course of LDL

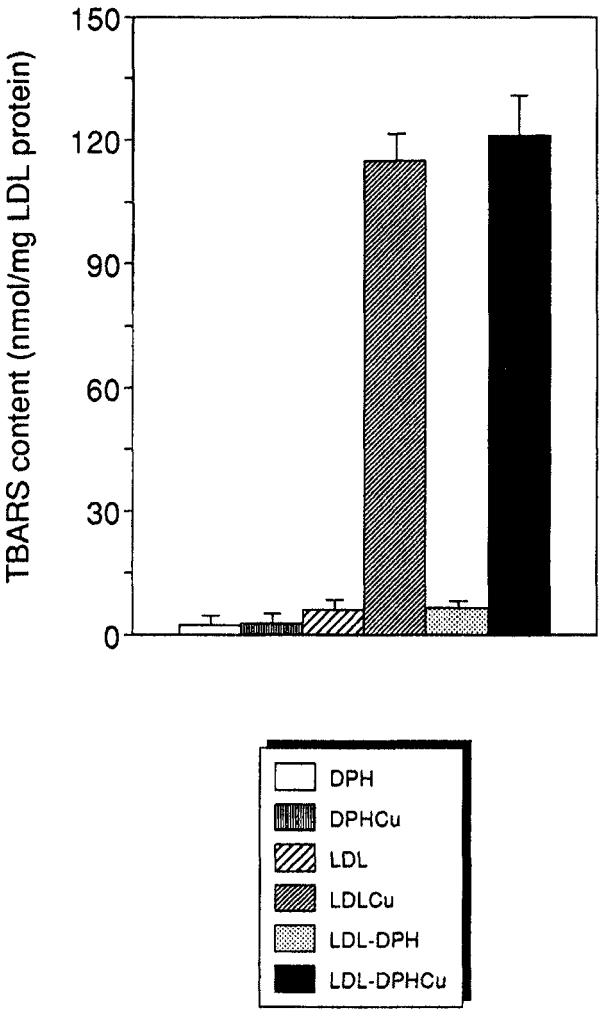

FIGURE 6 Influence of DPH on the TBARS formation during the time-course of LDL oxidation. The lipoprotein oxidation was carried out in PBS during 24 hours at 37°C using 20 μg/ml LDL protein and 5×10^{-6}M Cu^{2+}. The final concentration of DPH in the LDL solution, or alone in PBS, was 5×10^{-7}M. DPH: DPH alone (without LDL) in the absence of copper ions; DPH-Cu: DPH alone in the presence of Cu^{2+}; LDL: LDL without DPH, in the absence of copper: LDL-Cu: LDL without DPH in the presence of Cu^{2+}; LDL/DPH: LDL labeled with DPH, in the absence of Cu^{2+}; LDL/DPH-Cu: LDL labeled with DPH, in the presence of Cu^{2+}. Means of 4 experimental values ± S.D.

peroxidation, a small amount remains unsensitive to the oxidative process. This may be due to another localization in the lipoprotein particle, or to microaggregates of the probe not incorporated into the LDL. Thus a slight discrepancy exists between the signal directly measured on the LDL solution and the actual amount of probe destroyed during the oxidative process. It is also possible that during the course of LDL peroxidation, some structural changes could occur which may affect the quantum yield of the probe. However, the strong correlation existing between the reduction of the signal either directly measured or measured after extraction and the amount of TBARS formed suggests that the observed phenomenon fairly reflects the kinetics of the oxidative modification of the LDL by cupric ions. Another argument supporting the hypothesis of an oxidative destruction of the probe is given in Figure 5, which shows the fluorescence spectrum of the probe in the LDL solution before (Figure 5a) or after (Figure 5b) a 18 hour incubation in the presence of copper ions. It clearly appears that a broad spectrum is observed after oxidation, suggesting the appearance of DPH degradation products, whereas the fluorescence intensity at the peak is at least 50 times reduced.

Another important point for the validation of the method is to demonstrate that the presence of the probe, at the used concentration (5×10^{-7}M), does not influence the oxidative process. To this end, we compared the TBARS formation in unlabeled or DPH-labeled LDL after a 24 hour incubation in the presence of 5×10^{6}M Cu^{2+}. Controls with DPH alone in the presence or absence of copper were also done. Results in Figure 6 clearly show that

i DPH incubated in the presence of copper did not give rise to significant measurable amounts of TBARS and
ii DPH did not significantly modify the TBARS content of oxidized LDL (see columns 4 and 6).

Thus under our experimental conditions, DPH neither compete with polyunsaturated fatty acid (PUFA) peroxidation nor accelerate it.

We finally investigated the usefulness of this method for the screening of pharmacological agents which could protect LDL against oxidative modification. For this purpose, we studied the effects of probucol and flunarizine under our experimental system. Probucol is well known to prevent LDL from oxidation by cupric ions (20). We also reported that several calcium antagonists, including flunarizine, are also able to protect LDL against oxidative modification (21). Figure 7 clearly demonstrates that a 1 hour preincubation of LDL either with 5×10^{-5}M probucol or flunarizine almost completely inhibited the decrease in the DPH fluorescence induced by cupric ions while in untreated LDL the fluorescence of the probe was reduced to zero over 2 hours (Figure 7a). Concomitantly, no significant formation of TBARS was noted in probucol or flunarizine-treated LDL (Figure 7b).

DISCUSSION

In the present work, we demonstrate that the fluorescence of DPH incorporated into LDL readily decreased under oxidative conditions. The dependence of the DPH fluorescence decrease upon the cupric ion concentration, the excellent reciprocal correlation between the reduction of the fluorescence and the appearance of TBARS in the LDL particle, and the inhibition of the phenomenon by EDTA strongly suggest that it is actually related to the copper-induced LDL peroxidation. The HPLC

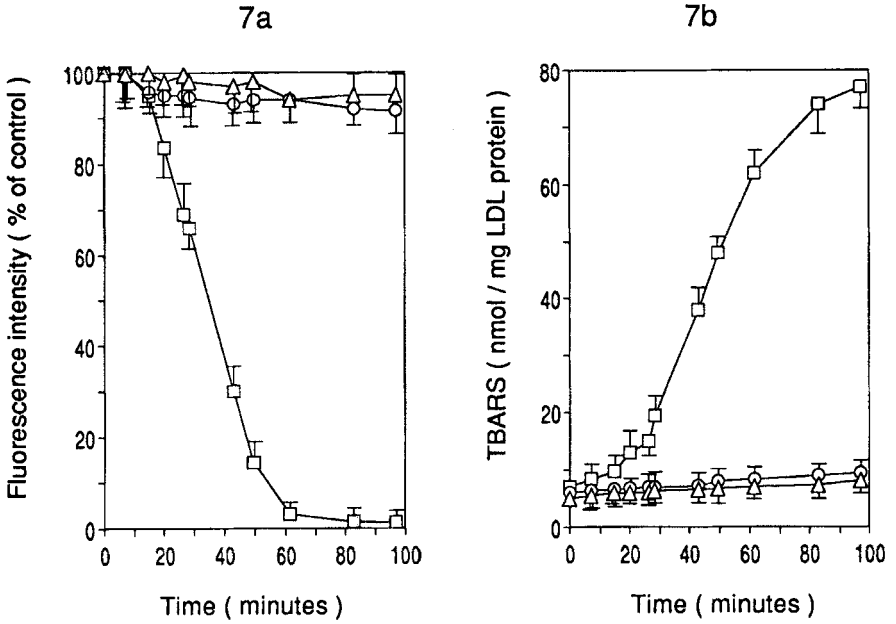

FIGURE 7 Effect of probucol and flunarizine on LDL oxidation induced by Cu^{2+} ions. Experimental conditions were the same as those specified in Figure 3 legend. The drug and the copper ion concentrations were 5×10^{-5}M, whereas the LDL concentration was 20 μg/ml. Figure 7a: decay of fluorescence at 432 nm as a function of time; figure 7b: TBARS formation (nmol/mg LDL protein) as a function of time. □: no drug; △: probucol; ○: flunarizine. Results are means of 6 experimental values ± S.D.

measurements show that about 90% of the probe initially incorporated into the particle is destroyed during the course of LDL oxidation. Moreover, the inhibition by vitamin E, probucol or flunarizine of the decrease in the DPH fluorescence induced by cupric ions is in good agreement with results obtained by more classical methods such as TBARS measurement or evaluation of the relative electrophoretic mobility of the LDL particle in agarose gel (21,22).

In our experiments, the lag phase for the onset of the DPH consumption ranged from 10 to 20 min. and from 25 to 40 min. at Cu^{2+} concentrations of 5×10^{-5} and 5×10^{-6} M, respectively, for LDL originating from various donors. The amount of TBARS formed also varied with the donor from 40–50 to 60–80 nmol./mg LDL protein. These large variations are in accordance with previous observations on the susceptibility of LDL from different donors to autooxidation (19,23,24). However, in all the cases, it must be stressed that under our experimental conditions, the lag phase observed before the decrease in the fluorescence of DPH (and the appearance of TBARS) was very short as compared to those described in the literature, which may range from 60 to 180 min. under usual conditions (3). This is probably due to the low concentration of LDL protein used in our experiments (0.02 mg/ml LDL protein). As a result, for the same concentration of cupric ions, the rate of LDL autooxidation is readily increased. This allows to reduce the time needed for complete LDL peroxidation and provides a rapid test for the evaluation of the susceptibility of the LDL particle to oxidation, or for testing the protective effect of antioxidant drugs.

DPH is a fluorescent lipophilic probe which has been mainly used for studies concerning the structural parameters of cell membranes (25,26), liposomes (27) and

also lipoproteins (28). To our knowledge, this is the first time that the decrease in the DPH fluorescence emission is used for monitoring LDL peroxidation. It is of note that previous works have demonstrated that the fluorescence of DPH is reduced in an exponential manner in erythrocyte membranes submitted to ionizing radiations (29). These results, as well as our observations on LDL peroxidation and DPH destruction, suggest that DPH may be attacked either directly by free radicals, or by lipid radicals generated during lipid peroxidation. It must be underlined that under our experimental conditions, DPH behaves as a neutral probe, neither inhibiting nor accelerating LDL fatty acid peroxidation (Figure 6). This is probably due to the low amounts of probe used. Indeed, assuming that PUFA represent about 50% of LDL fatty acids (3), calculation shows that a 0.02 mg/ml LDL protein corresponds to about 5×10^{-5}M PUFA. Thus, for a final concentration of DPH of 5×10^{-7}M used in our experiments, the molar ratio DPH/PUFA was about 1% or less. As a consequence

i it can be assumed that the LDL structure is not markedly modified by the probe, and
ii the small amounts of probe are not sufficient to influence the course of PUFA peroxidation, as demonstrated by results in Figure 6.

Moreover, experiments with micelles of diarachidonoyl-phosphatidyl-choline (DA-PC) or dioleoyl-PC (DO-PC) labeled with DPH showed that the DPH fluorescence was decreased in DA-PC/DPH micelles exposed to cupric ions, while it remained stable in DO-PC/DPH micelles (data not shown). Since DO-PC is not susceptible to autooxidation (only 1 double bond in oleic acid), this result strongly suggests that PUFA peroxidation is a prerequisite for DPH destruction.

It must also be noted that the wavelengths used in our experiments (excitation of DPH at 354 nm, emission at 432 nm) are very close to that used by various authors for monitoring LDL oxidation by measurement of fluorescent products (excitation at 360 nm, emission at 430 nm, see ref. 3,11,15,). However, there was apparently no significant influence of the LDL autofluorescence on the decrease of the fluorescence of DPH at 430 nm. This may be easily explained by the very high fluorescence obtained after labeling LDL with DPH as compared to the autofluorescence of the particle.

In conclusion, the method herein described is non-destructive for the LDL, and, like the conjugated diene method described by Esterbauer *et al.* (14), allows continuous monitoring of the oxidative process. It is very reproducible, with S.D. lower than 10% for the same LDL preparation (see for example results in Table I). Conjugated diene measurement offers the advantage of not using an external probe, and is very convenient in most cases. However, the very high ratio signal/noise of the DPH fluorescence allows to work with extremely small amounts of LDL, and it can be assumed that the sensitivity of the method is much higher than that of the most sensitive methods previously described. Indeed, even if the present work has been carried out using in most cases 0.020 mg/ml LDL protein, reliable data were obtained with 0.005 mg/ml LDL protein or even less, and the lower limit of the method seems to be around 0.001 mg/ml LDL protein (data not shown). For comparison, with 0.005 mg/ml LDL protein and under similar conditions, the conjugated diene methods gives a differential O.D. at 234 nm of about 0.07. Moreover, as discussed above, if DPH is destroyed during the course of LDL autooxidation, we demonstrated that the probe behaves only as a tracer of lipid peroxidation, and does not significantly perturb the lipid peroxidation end product formation. A very low concentration of LDL in the spectrofluorometer cuvette also allows to achieve complete oxidation of LDL within a very short time. Although

the method does not give an absolute measurement of the lipid peroxidation products generated during LDL oxidation, it could be of interest for the comparative evaluation of the effect of a wide variety of drugs against copper-induced peroxidation with the same LDL preparation, because of the very small amount of LDL required for each assay. This is of importance considering the great variability in the sensitivity of LDL originating from different donors to copper-induced peroxidation (19,23,24). Finally, the extremely high sensitivity of the method could provide a useful tool for studies on lipid peroxidation in new experimental models.

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