Free Rad. Res. Comms., Vol. 6, No. 1, pp. 67-75
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CONTINUOUS MONITORING OF *IN VITRO* OXIDATION OF HUMAN LOW DENSITY LIPOPROTEIN

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(Received November 9, 1988; in final form November 15, 1988)

The kinetics of the oxidation of human low density lipoprotein (LDL) can be measured continuously by monitoring the change of the 234 nm diene absorption. The time-course shows three consecutive phases, a lag-phase during which the diene absorption increases only weakly, a propagation phase with a rapid increase of the diene absorption and finally a decomposition phase. The increase of the dienes is highly correlated with the increase of MDA or lipid hydroperoxides. The duration of the lag-phase is determined by the endogenous antioxidants contained in LDL (vitamin E, carotenoids, retinylstearate). Water-soluble antioxidants (ascorbic acid, urate) added in micromolar concentrations prolong the lag-phase in a concentration-dependent manner. The determination of the lag-phase is a convenient and objective procedure for determining the susceptibility of LDL from different donors towards oxidation as well as effects of pro- and antioxidants.

KEY WORDS: Human low density lipoprotein, LDL, lipid peroxidation, diene conjugation, atherosclerosis.

ABBREVIATIONS: LDL: low density lipoprotein; o-LDL: oxidized low density lipoprotein; apoB: apolipoprotein B; TBA: thiobarbituric acid; EDTA: ethylenediaminetetraacetic acid; BHT: butylated hydroxytoluene; MDA: malondialdehyde.

INTRODUCTION

Oxidative modification of human low density lipoprotein (LDL) is considered to be an important event in atherogenesis.[1-11] *In vitro*, oxidized LDL (o-LDL) shows diminished affinity to the LDL receptor and increased affinity to the macrophage scavenger receptor, the uptake of o-LDL by macrophages is not downregulated by internalized LDL-cholesterol and leads to lipid loading of these cells.[1,2] o-LDL is cytotoxic towards vascular cells,[4] exerts chemotactic activity towards monocytes, but inhibits migration of resident macrophages.[9] These and several other properties support the assumption of a pathophysiological importance of o-LDL. *In vitro*, oxidation of LDL can be mediated by cells[1,2,3,5] or in cell-free medium, traces of transition metal ions strongly catalyze the oxidating.[2,12-14] The properties of cell-oxidized LDL are very similar if not identical to LDL oxidized in cell-free medium[11] and indeed most biochemical and biological studies were performed with LDL oxidized by traces of iron or copper-ions. It has been clearly shown that the oxidation of LDL is a lipid peroxidation process[2,6,15] in which the polyunsaturated fatty acids of LDL are successively degraded to a variety of products. These lipid peroxidation

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products and their interactions with the apo B are likely the cause for the altered functional properties of o-LDL.[6,15-17]

The rate and degree of the oxidation of LDL is most frequently measured with the thiobarbituric acid assay (TBA assay) which measures the amount of malonaldehyde and/or malonaldehyde-like substances formed by the lipid peroxidation process. The limitations of this assay have been repeatedly discussed and it is therefore more than dissatisfying when important conclusions regarding biological properties of o-LDL are based on an unreliable index for oxidation. Other possibilities of determining the stage of LDL oxidation are the measurement of the decrease of lipophilic antioxidants and fatty acids[6,18] or the measurement of the increase of the protein and lipid fluorescence,[15,19,20] peroxides[21] and aldehydic lipid peroxidation products (for review see[8,18]). All these methods have a number of weak points besides their strength, i.e. large sample size, and they are discontinuous as well as tedious.

We describe here a procedure which allows to follow the oxidation of LDL continuously by monitoring the increase of the 234 nm absorbance. This absorption develops in LDL during oxidation through the formation of ocnjugated fatty acid hydroperoxides. This continuous measurement allows the investigation of the effects of pro- and antioxidants in a quantitative and reproducible manner and has significant advantages over other methods in LDL oxidation studies.

MATERIALS AND METHODS

Fresh whole blood was obtained by venipuncture from healthy human volunteers under 25 years of age and supplemented immediately with EDTA (1 mg/ml) and BHT (4.4 μg/ml). LDL was prepared by step-wise ultracentrifugation within a density gradient of 1.020–1.050 g/cm^3. EDTA and BHT were present throughout all the steps of the isolation. The EDTA/BHT containing stock solution (15–30 mg LDL/ml) was stored at 4°C in the dark in a nitrogen atmosphere until use, but never longer than two weeks.

Before oxidation experiments the LDL stock solution was dialyzed in the 100-fold volume of 0.01 M phosphate buffer pH 7.4, 0.16 M NaCl, 0.1 μg/ml chloroamphenicol, which was made oxygen-free by vacuum degassing following by purging with nitrogen. The buffer was changed four times. This EDTA- and BHT-free LDL stock solution was used for all oxidation studies. The stock solution was stored not longer than 24 h at 4°C.

For performing the oxidation experiments, the EDTA- and BHT-free LDL stock solution was diluted with oxygen-saturated 0.01 M phosphate buffer pH 7.4, 0.16 M NaCl and the oxidation was initiated by the addition of a freshly prepared aqueous $CuCl_2$ solution. The final conditions were in all experiments: room temperature, 0.25 mg LDL/ml and 1.66 μM $CuCl_2$.

Vitamin E and cartenoids were determined by HPLC as described in (18). For determination of retinylstearate 4 ml of the LDL solution (0.25 mg LDL/ml) was spiked with 30 μl of 3.63 μM retinylpalmitate in hexane and extracted 4 times with each 2 ml $CHCl_3$:MeOH = 2:1. The pooled $CHCl_3$-phase was evaporated under a stream of N_2 and the residue was dossolved in 30 μl 2-propanol. 20 μl of this solution was separated by HPLC on a Spherisorb S5 ODS 2 column with MeOH:2-propanol = 3:1 as an eluent and fluorimetric detection with excitation/emission at 350/

510 nm. Lipid hydroperoxides were determined as described,[21] MDA was measured by the TBA assay[23]

RESULTS AND DISCUSSION

1. Formation of conjugated dienes during oxidation of LDL

The primary products of lipid peroxidation are lipid hydroperoxides of the general structure -CH=CH-CH=CH-CHOOH- with an absorption maximum around 234 nm. Since o-LDL is, like native LDL, fully soluble in buffer, the generation of such conjugated lipid hydroperoxides can directly be measured by recording the UV spectrum of the aqueous LDL solution. This is different from most other biological

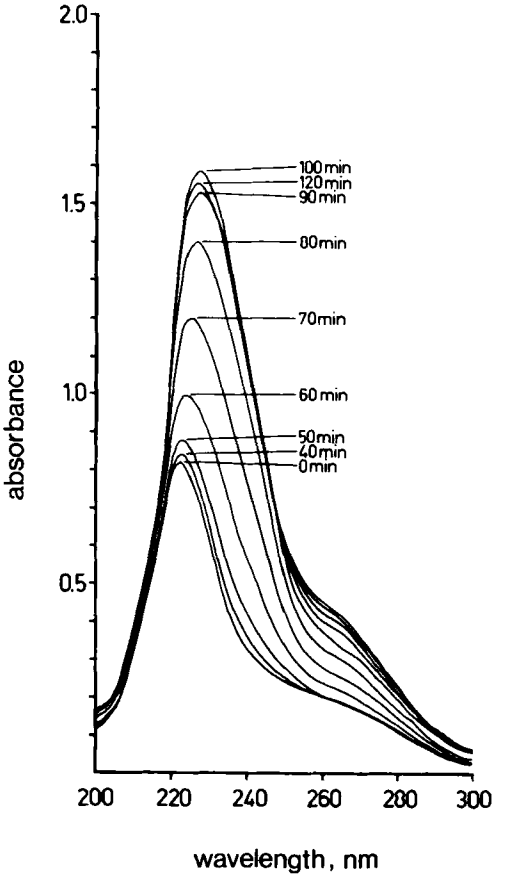

FIGURE 1 Change of the UV spectrum of an LDL solution during oxidation. LDL (0.25 mg/ml) in 0.01 M phosphate buffer pH 7.4, 0.16 M NaCl was supplemented with 1.66 μM $CuCl_2$ as prooxidant. The UV spectrum of the solution was recorded at the time points indicated in a 1 cm quartz cuvette. The reference cuvette contained the phosphate buffered saline, 1.66 μM $CuCl_2$, yet no LDL.

systems (microsomes, mitochnodria, cells, tissue homogenate) where lipid peroxidation was studied. In such cases the conjugated dienes can only be obtained by analyzing the lipid extracts.

Figure 1 shows the UV spectrum of an LDL solution measured at different time points after the addition of Cu^{2+} as a prooxidant. Native LDL showed a maximum at 222 nm, which is probably due to the absorption of the apo B. With increasing oxidation time the absorbance in the 220–230 nm region increased and the maximum shifted bathochrome towards 227 nm. If the spectral changes occurring during LDL oxidation were measured against the same LDL in which oxidation was inhibited by EDTA it became clearly evident that the newly formed chromphore had the absorbance maximum at 234 nm as expected for conjugated lipid hydroperoxides (Figure 2).

To prove that the 234 nm absorbance is associated with the lipid domain of the LDL, native and oxidized LDL were delipidated and the UV spectra of the apoB (in 0.15% aqueous SDS) and lipids (in hexane) were measured and compared (data not shown). No difference existed between the spectra of the apoB whereas the spectrum of the lipids extracted from the o-LDL showed a strong absorbance maximum at 234 nm. The shape of the spectrum was very similar to that measured in the difference spectrum of the complete LDL (Figure 2). To further substantiate the association of the diene absorption with the degree of the oxidation of LDL, we have simultaneously measured the MDA values by the TBA assay, the peroxide value by a newly developed simple iodometric assay [21] and the diene absorption by the different UV spectrum as shown in Fig. 2. Up to the time point of the maximum oxidation i.e. the maximum of diene absorption, an excellent correlation ($r = 0.99$) was found between the amount of conjugated dienes and MDA or lipid hydroperoxides (Figure 3). This good correlation gradually disappeared again after a prolonged oxidation beyond the

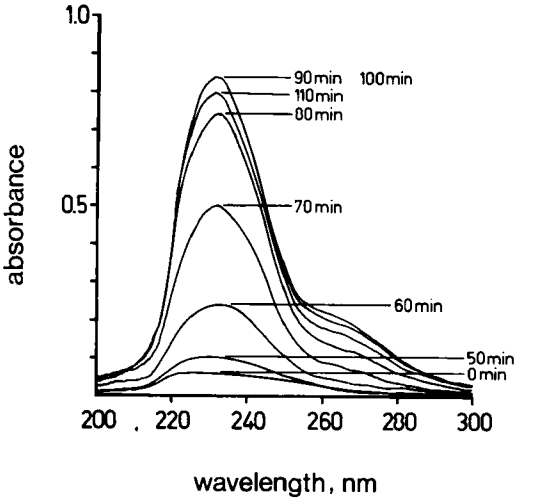

FIGURE 2 Change of the UV difference spectrum of an LDL solution during oxidation. The experimental conditions were as in Figure 1, except that the reference cuvette contained LDL (0.25 mg/ml) in phosphate buffered saline supplemented with 2.5 mM EDTA to prevent oxidation, no $CuCl_2$ was added to the reference.

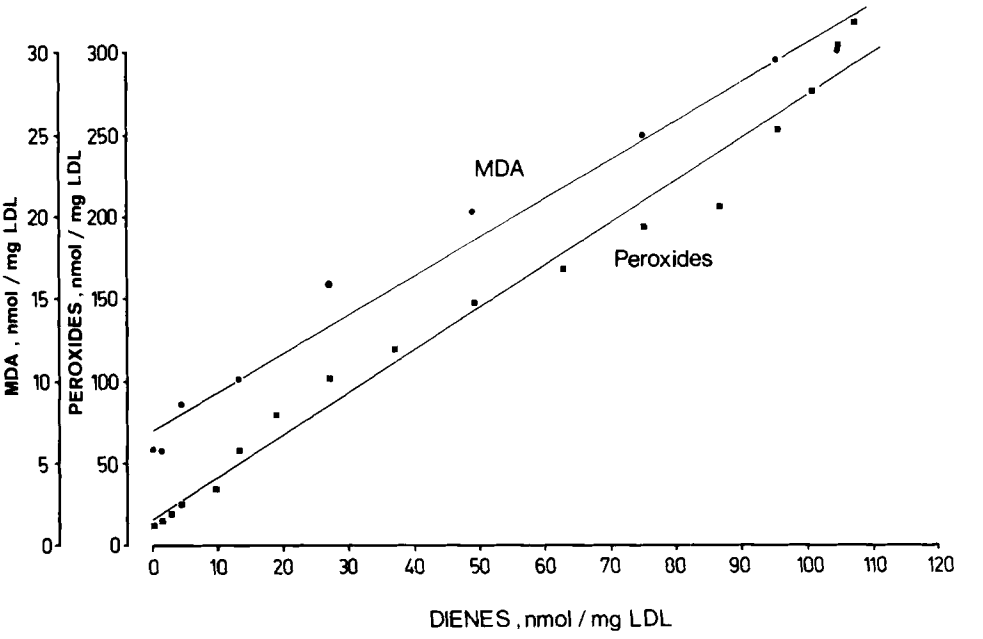

FIGURE 3 Correlation between the amount of conjugated dienes, lipid peroxides and MDA in oxidized LDL. Experimental conditions as in Figure 1, the amount of dienes was calculated from the increase of the absorbance at 234 nm; MDA was determined by the TBA assay and peroxides by the iodometric method described in[21].

time point where the lipid hydroperoxides or conjugated dienes reach their maximum value.

2. *Continuous monitoring of oxidation of LDL*

The fact that the formation of conjugated dienes in the LDL particle can be measured directly i.e. without prior extraction of the lipids, offers the possibility to continuously monitor the time course of their formation. An example for such an experiment is shown in Figure 4. For that a 1 cm quartz cuvette with the LDL solution supplemented with Cu^{2+} as a pro-oxidant was placed in single-beam UV-spectrophotometer, the initial absorbance at 234 nm was set to an arbitrary value and then the increase was recorded over a time period of 3 h. The 234 nm wavelength was chosen because this is the maximum of the conjugated diene band as shown by the difference spectra (Figure 2). The kinetics of the diene formation i.e. the change of the absorbance vs. time can be clearly divided into three phases. A first phase during which the dienes do not or only very slowly increase, a second phase during which the dienes very rapidly increase to a maximum value, and a third phase during which the dienes strongly decrease again. In analogy to other kinetic studies on lipid peroxidation, as for example in liposomes,[22] the first two phases can be termed as lag-phase and propagation phase. During the lag phase (or induction phase), the lipophilic antioxidants protect the polyunsaturated fatty acids against oxidation and thus prevent that

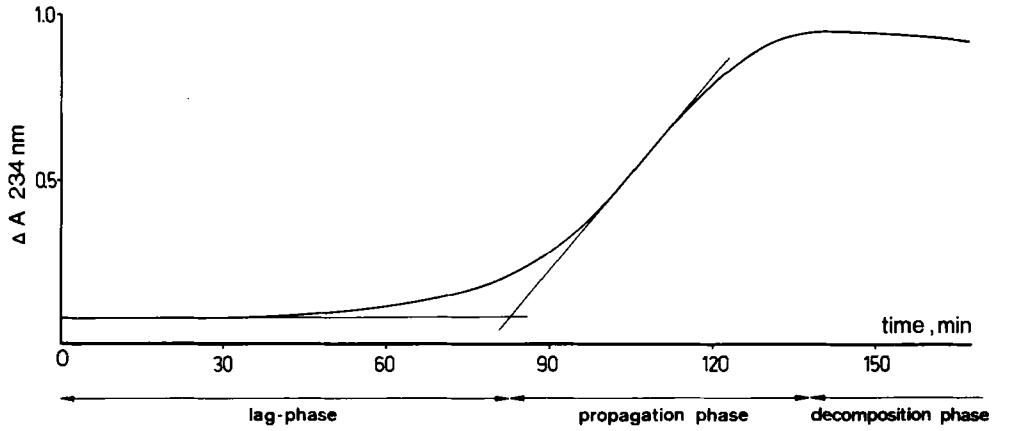

FIGURE 4 Measurement of the kinetics of oxidation of LDL by continuous monitoring the change of the absorbance at 234 nm. The experimental conditions were as in Figure 1. The initial 234 nm absorbance of the LDL solution was set to 0.1 and then the increase was recorded over 3 h. No blank cuvette was used in this experiment.

the lipid peroxidation process can enter into the propagating chain reaction. As shown in the experiment of Figure 5, the protective action of the antioxidants progressively decreases since they are inactivated and consumed if they scavenge free radicals. If the LDL particle is depleted of its antioxidants, the lipid peroxidation process enters the propagation phase in which the polyunsaturated fatty acids are

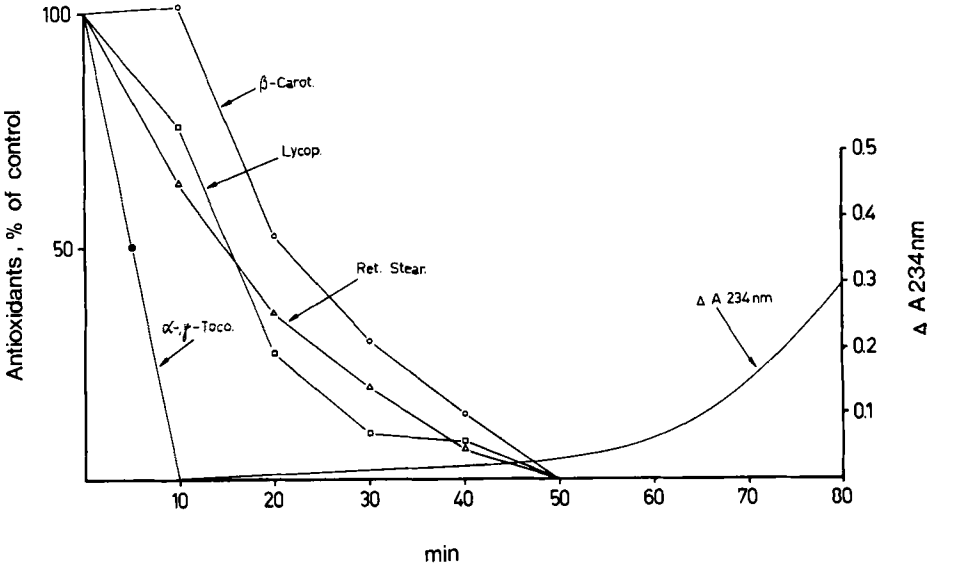

FIGURE 5 Time-course of the consumption of anitoxidants and formation of conjugated dienes during oxidation of LDL. Experimental conditions as in Figure 1, the diene increase was measured by continuous monitoring the increase of the 234 nm absorbance.

rapidly converted to conjugated lipid hydroperoxides as indicated by the increase of the 234 nm absorbance. The transition from the lag phase to the propagation phase is not abrupt, but continuous. Nevertheless, the rates of the diene formation during the two phases differ widely enough to obtain from the curve the length of the lag phase. We define as the end of the lag phase the intercept of the two straight lines as shown in Figure 4. In addition to the length of the lag phase the curve also allows to determine the maximum rate of oxidation and the maximum amount of conjugated dienes formed in the LDL. In the experiment shown in Fig. 4 the maximum rate ΔA_{234}/min is 0.0181 and the maximum increase ΔA is 0.87. Based on the molar absorptivity of conjugated lipid hydroperoxides ($\epsilon_{234} = 29{,}500\,M^{-1}\,cm^{-1}$) and on our experimental conditions (0.25 mg LDL/ml) this corresponds with 2.4 nmole dienes/-min., mg LDL (maximal rate) and 118 nmole dienes/mg LDL (maximal concentration).

The curve in Figure 4 shows that the 234 nm absorbance slowly decreases again after reaching its maximum value. This is not unexpected since it is well known that lipid hydroperoxides are labile and decompose in a number of consecutive reactions to a variety of products. To further substantiate the behavior of the 234 nm absorbance, we have followed it up to time periods of 16 hours. After reaching the maximum, the 234 nm absorbance decreased for about 2 h and then slowly and steadily increased again. We conclude that the last phase of the LDL oxidation is characterized by decomposition of the lipid hydroperoxides formed during the propagation phase. These decomposition reactions are extremely complex and can lead to many compounds showing UV absorbance in the 210–240 nm range; for example, 2-alkenals or 4-hydroxyalkenals, typical products of lipid peroxidation, absorb at 220–225 nm region. The second increase of the 234 nm absorbance is therefore certainly not due to a second phase of the formation of conjugated lipid hydroperoxides but should be seen as "decomposition phase". The UV spectrum of an LDL sample oxidized for 16 h showed a maximum at 220 nm, the difference spectrum had a maximum at 232 nm, but in the difference spectrum of the lipid-extracts there was no maximum at 234 nm.

3. Application of the procedure for determining the protective effects of antioxidants

The measurement of the lag phase by continuous monitoring of the LDL oxidation offers the possibility of studying the effects of pro- and antioxidants. Fig. 6 shows as an example the effect of ascorbic acid on the oxidation of LDL stimulated by copper(II)ions. It is clear from this experiment that ascorbic acid prolongs the lag phase in a concentration-dependent manner and therefore can retard the oxidation of LDL most likely due to the fact that it reactivates vitamin E.[22] Even more effective on a molar base is urate which increased the lag phase from 76 to 200 min at a concentration of 1.66 μM (Table 1). We have also tried to study the effects of alpha-tocopherol and retinylstearate, the major lipophilic antioxidants in LDL (Table 1). These were dissolved in ethanol (alpha-tocopherol) or DMSO (retinylstearate) and added to the LDL solution prior to the initiation of the oxidation of LDL by $CuCl_2$. The effect of the lipophilic antioxidants on the lag phase appears to be much less pronounced than compared to the water-soluble antioxidants urate or ascorbate (Table 1). This, however, is probably due to the experimental conditions and does not reflect the effectiveness of endogenous alpha-tocopherol or retinylstearate contained in native LDL. It is very likely that the greater part of the added lipophilic antiox-

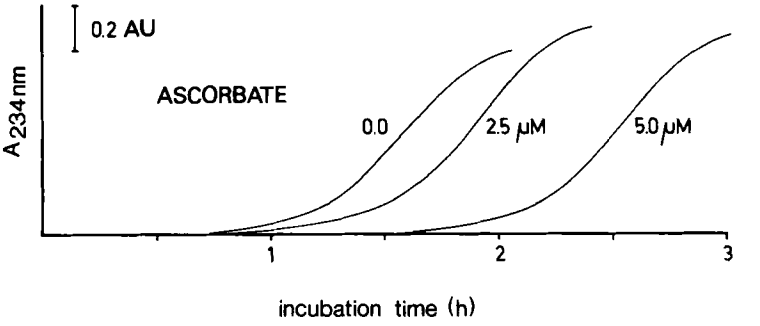

FIGURE 6 Effect of ascorbate on the lag-phase of LDL oxidation as measured by the increase of the diene absorption. The LDL solution was supplemented with 0, 2.5 and 5.0 μM ascorbate prior to addition of $CuCl_2$, other conditions as in Figure 1 or 4.

TABLE 1

Effect of antioxidants on the lag phase of LDL oxidation. The incubation mixtures (1 ml) contained 0.25 mg LDL/ml, 0.01 M phosphate buffer pH 7.4, 0.16 M NaCl. Oxidation was initiated by addition of 1.66 μM $CuCl_2$. Alpha-tocopherol was added in 10 μl ethanol, retinylstearate was added in 10 μl dimethylsulfoxide.

System	lag phase (min)
Ascorbate	
control LDL, 1.26 nmole α-tocopherol/mg LDL, 0.77 nmole retinylstearate/mg LDL	72
+ 5 μM ascorbate (= 20 nmole/mg LDL)	129
+ 10 μM ascorbate (= 40 nmole/mg LDL)	200
Urate	
control LDL, as for ascorbate experiment	76
+ 0.83 μM urate (= 3.32 nmole/mg LDL)	129
+ 1.66 μM urate (= 6.64 nmole/mg LDL)	200
Alpha-tocopherol	
control LDL, 2.32 nmole α-tocopherol/mg LDL, 0.20 nmole retinylstearate/mg LDL, + 10 μl EtOH	47
+ 0.58 μM tocopherol (2.3 nmole/mg LDL)	51
+ 1.16 μM tocopherol (4.6 nmole/mg LDL)	50
+ 2.32 μM tocopherol (9.2 nmole/mg LDL)	56
+ 4.64 μM tocopherol (18.4 nmole/mg LDL)	76
Retinylstearate	
control LDL, as for α-tocopherol experiment, + 10 μl DMSO	20
+ 0.26 μM retinylstearate (1.04 nmole/mg)	26
+ 0.52 μM retinylstearate (2.08 nmole/mg)	33
+ 1.04 μM retinylstearate (4.16 nmole/mg)	61

idants was not incorporated in the LDL particle but remained suspended in the aqueous buffer. In the case of retinylstearate which was added as DMSO solution, it must be considered that 1% DMSO by itself significantly reduced the lag phase probably due to destructive effects on the LDL structure.

Experiments are now in progress in our laboratory in which the plasma is supplemented by lipophilic antioxidants prior to the isolation of LDL. Preliminary

results indicate that this technique allows to incorporate lipophilic antioxidants, at least to a certain extent, into the LDL. In our ongoing study on the oxidation of LDL, we have also repeatedly observed that the oxidizibility of LDL from different donors varies in a wide range. The measurement of the lag phase as described here could be a highly promising routine method for measuring the total antioxidant status of LDL and its susceptibility towards oxidative conditions.

Acknowledgements

This work was supported by the Association for International Cancer Research (U.K.) and by the Austrian Science Foundation, project no. P6176B.

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Accepted by Prof. H. Sies