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Monitoring of Low Density Lipoprotein Oxidation by Low-level Chemiluminescence

RICCARDO ALBERTINI[a,b] and PETER M. ABUJA[a,*]

[a] *Institute of Biochemistry SFB Biomembrane Research Center, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria;*
[b] *Laboratory of Clinical Chemistry, IRCCS Policlinico S. Matteo, P.le Golgi, 27100 Pavia, Italy*

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A method for monitoring low-density lipoprotein (LDL) oxidation by low-level chemiluminescence (LL-CL) is described in this study. The kinetic indices obtained with this procedure, in particular lag-time and *K* value (related to prooxidant activity of Cu^{2+} bound to LDL) are compared with those of the established UV-absorbing conjugated diene assay. The correlation of lag-time values obtained by LL-CL and conjugated diene assay was very high both in the case of Cu^{2+}- and peroxyl-radical-mediated oxidation ($r = 0.99$). By using the transient free radical scavenging activity of butylated hydroxytoluene, a calibration of LL-CL for lipid peroxyl radical and termination rate was obtained. The spectral analysis of LL-CL from oxidizing LDL shows a maximum peak between 420 and 500 nm, corresponding to the emission of triplet carbonyl compounds. LL-CL allows continuous and direct monitoring of LDL oxidation as extraction and derivatization of lipid peroxidation products are not required. Moreover, some limitations of UV spectroscopy such as by absorbing compounds need not be considered. Therefore, the present procedure represents a simple and convenient tool for continuous monitoring of LDL oxidation which may be applied to mechanistic and clinical studies.

Keywords: Low density lipoprotein, low-level chemiluminescence, lipid peroxidation

Abbreviations: LDL, low density lipoprotein; CD, conjugated dienes; LL-CL, low-level chemiluminescence; EDTA, ethylenediaminetetraacetate; BHT, butylated hydroxytoluene; AAPH, 2,2-azobis (2-amidinopropane hydrochloride); R_i, rate of initiation; R_{term}, rate of termination

INTRODUCTION

The growing interest in low density lipoprotein oxidation, related to its possible involvement in atherosclerosis, prompted several methods to be proposed for the investigation of such modifications.[1,2] As LDL oxidation is in principle a lipid peroxidation process driven by free radicals,[2] it is evident that the analytical techniques used to study peroxidation of polyunsaturated fatty acids were generally applied, with the necessary adaptations. In mechanistic and clinical studies the determination of several kinetic oxidation indices which can be conveniently obtained by continuous monitoring of LDL oxidation is useful. This may discourage the use of sensitive

*Corresponding author. Tel.: +43 316 3805486. Fax: +43 316 3809845.

and specific procedures committed to the identification of specific lipid peroxidation products[3] because they are not suited to continuous measurement and are generally rather sophisticated for routine work.

The established and widely employed UV detection of conjugated dienes (CD) in oxidizing LDL[4] has important limitations, such as the necessity to exclude UV-interfering compounds from the sample; moreover, the unfavorable ratio between the high volume of the solution in the cuvette and the air-exposed surface may limit oxygen supply for propagation under certain experimental conditions.

It is known that lipid peroxidation in various experimental system is associated with low-level chemiluminescence (LL-CL) in the visible and near UV regions.[5,6] Here a procedure is described which allows to monitor LDL oxidation continuously by recording LL-CL. The kinetic indices obtained with this method are compared with the ones from CD assay. Finally, we apply the present procedure to oxidation of LDL at high concentration. These experimental conditions are impossible to be realized in the UV assay of CD for the reasons outlined above.

MATERIALS AND METHODS

Materials

2,2′-Azobis (2-amidinopropane hydrochloride) (AAPH) was purchased from Polysciences (Warrington, PA, USA). Other reagents were of AR grade obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

Preparation of LDL

Ethylenediaminetetraacetate (EDTA)-plasma (1 mg/ml) was prepared from venous blood of normolipidemic healthy volunteers of both sexes (age 25–37) after overnight fast. Plasma samples were pooled and frozen at −80°C in 0.6% sucrose for up to 4 weeks. LDL was isolated by ultracentrifugation (Beckman L70) in a single step discontinuous gradient using a NVT 65 rotor (Beckman).[7] LDL was stored in a vial under argon at 4°C in the dark and used within 1 week after preparation. Chemical composition of LDL was normal according to.[3]

LDL Oxidation

Before oxidation, LDL was freed of EDTA by gel filtration with an Econo-Pac 10 DG column (Biorad, Hercules, CA, USA) using PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) as eluent. LDL concentration was determined by total cholesterol assay (CHOD-PAP kit, Boehringer Mannheim, Germany). Assuming a molecular mass of 2.5 MDa for LDL and a cholesterol content of 31%, a concentration of 0.1 μM LDL corresponded to 0.25 mg/ml total mass and 79 μg/ml total cholesterol.[7]

LDL, freed of EDTA, was oxidized with either $CuSO_4$ or AAPH in PBS at 37°C. Oxidation was followed by monitoring the increase in absorbance at 234 nm (A234 nm), due to the formation of conjugated diene hydroperoxide from polyunsaturated fatty acids.[4] When LDL oxidation was induced by AAPH which thermally decomposes to UV absorbing products, the absorbance at 234 nm of PBS solution containing only AAPH was subtracted from the sample. Lag-time was considered as index of LDL oxidation and was determined according to.[8]

Chemiluminescence Measurements

Low-level chemiluminescence was measured in a Lucy 1 luminometer (Anthos Labtech Instruments, Salzburg, Austria) equipped with a photon counting photomultiplier (sensitivity ranging from 300 to 700 nm). Integration time for each data point was set to 90 s. The assays were performed at 37°C in a white microplate.

The spectrum of LL-CL was measured with a Perkin-Elmer LS50B fluorimeter operating in the luminometer mode (excitation lamp was switched off). For the spectrum, 0.3 μM LDL

was incubated with 5 mM AAPH at 37°C and the spectrum was measured during 400 min of oxidation, summing up 100 scans over the indicated spectral range (300–700 nm, 100 nm/min).

RESULTS

Monitoring of Cu^{2+}-induced LDL Oxidation by Low-level Chemiluminescence

The LL-CL versus time profile of LDL oxidation by Cu^{2+} exhibited an inhibited period, without production of photoemissive species, followed by a considerable rise of light emission, indicating the oxidative modification of the particle (Figure 1). Thus, the time course of LDL oxidation by LL-CL resembled the one obtained by the assay of other lipid peroxidation products.[2,7] The peak value of light emission and the length of the inhibited period were directly and inversely dependent on Cu^{2+}-concentration, respectively (Figure 1). After reaching a maximum, the light signal is progressively decreasing over a period of several hours (Figure 1). The long duration of LL-CL after onset of propagation suggests that photoemissive species are generated from secondary rearrangement of lipid peroxides through metal-catalyzed reactions.

The addition of several antioxidants to oxidizing LDL at 180 min of incubation induced different effects. EDTA and butylated hydroxytoluene (BHT) caused a quick and almost complete quenching of LL-CL (Figure 2A and B), indicating that the presence of both redox active Cu^{2+} and peroxyl radicals is required for the formation of photoemissive species. Other antioxidants such as thiourea and NaN_3 only marginally affected the level of LL-CL, when added at the same concentration as EDTA and BHT (Figure 2A).

Interestingly, the quenching effect of BHT on the peak of light emission was not permanent; in fact after about 350 min after the addition of 10 μM BHT, i.e., after 530 min of incubation, LL-CL started to increase again (Figure 2B). If it

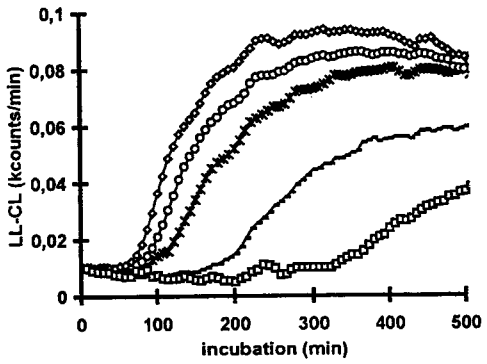

FIGURE 1 Monitoring of Cu^{2+}-induced LDL oxidation by low-level chemiluminescence. LDL (1 mg/ml total mass, equivalent to 0.4 μM) was incubated in PBS at 37°C with Cu^{2+} at concentrations of 0.5 μM (□), 1 μM (-), 2 μM (*), 3 μM (○) 4 μM (◇).

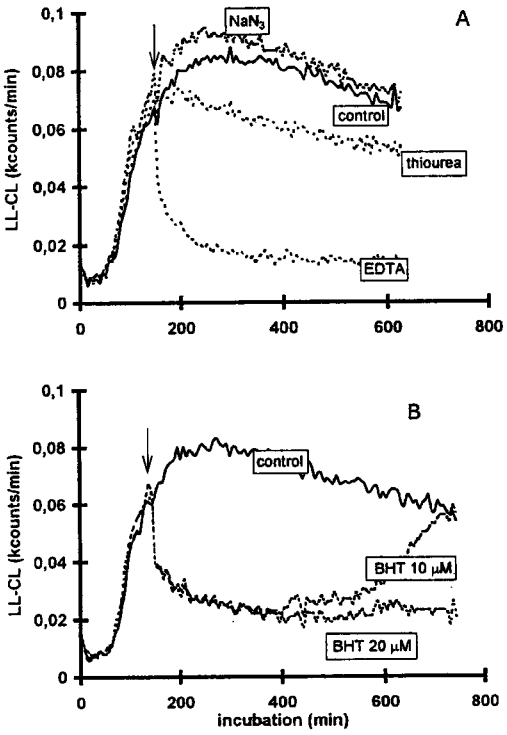

FIGURE 2 Effect of antioxidant addition on low-level chemiluminescence of oxidizing LDL. Panel A: 0.4 μM LDL was oxidized with 5 μM Cu^{2+} in PBS at 37°C; at the time point indicated by the arrow, antioxidants (thiourea, sodium azide, EDTA) were added at the final concentration of 20 μM. Panel B: BHT at the concentrations indicated was added under the same experimental conditions described above. One experiment representative of three is shown.

is assumed that the quenching effect of BHT is due to the lipid peroxyl radical scavenging properties of this lipophilic compound and that the reincrease of LL-CL indicates the complete consumption of BHT, a calibration of LL-CL for lipid peroxyl radicals and termination rate would be possible in principle. Nevertheless, the contribution of rearrangement of lipid hydroperoxide, catalyzed by transition-metal ions, to LL-CL discourages the estimation of rate of initiation (R_i) and peroxyl radical concentration from these oxidation profiles. However, it was feasible using peroxyl radical-mediated LDL oxidation (see below).

Since lag-time of LDL oxidation is regarded as an important index of oxidation resistance, useful in mechanistic and clinical studies, we compared the results of parallel experiments in which oxidation was monitored by UV detection of CD and by recording LL-CL. The correlation of lag-time values obtained by CD and LL-CL at varying Cu^{2+} concentrations was very high ($r = 0.99$) (Figure 3); the absolute values of lag-time obtained by LL-CL were higher by a factor of about 1.3 related to a temporal shift of LL-CL profiles as compared to CD.

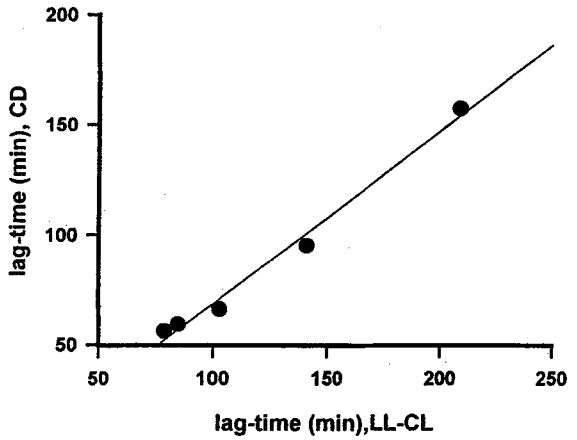

FIGURE 3 Correlation of lag-time of LDL oxidation mediated by Cu^{2+} as obtained by CD and LL-CL. LDL (0.2 μM) was oxidized with 0.5, 1, 2, 3 and 4 μM Cu^{2+} in PBS at 37°C; oxidation was monitored as CD and LL-CL. Lag-time values were determined graphically from CD and LL-CL profiles and plotted.

The relationship between lag-time as obtained from LL-CL profiles and Cu^{2+}-concentration was hyperbolic, obeying the equation

$$lag^{-1} = lag_{min}^{-1} \cdot [Cu^{2+}]/(K + [Cu^{2+}]),$$

where lag_{min} indicates the minimum value of lag-time, theoretically reached at infinite Cu^{2+}-concentration, in agreement with a previous report based on the CD assay[7] (Figure 4A). This may be interpreted assuming that LDL oxidation can be saturated, possibly as a consequence of the full occupancy of prooxidant Cu^{2+}-binding sites. We linearized this relationship by plotting lag-time as determined by CD and LL-CL assays as a function of the reciprocal of Cu^{2+}-concentration, in order to determine the value of the constant K. This is the Cu^{2+}-concentration corresponding to twice lag_{min} and to the half-saturation of the putative Cu^{2+}-binding sites on LDL, according to[7] (Figure 4B). The values obtained were 1.6 μM Cu^{2+} in case of the CD assay and 1.25 μM in case of LL-CL. This difference of results is likely to depend on the structural properties of LDL at the time points identified as lag-time by these two methods. As a consequence of the time-shift of the two techniques, CD lag-phase describes a condition with minimal chemical compositional changes of LDL, with the exception of depletion of lipophilic antioxidants; in contrast, LL-CL lag-phase describes a somewhat more advanced state of oxidation which might involve comparatively more extensive alterations in the protein and lipidic constituents of LDL leading to a different Cu^{2+}-binding ability.

Monitoring of AAPH-induced LDL Oxidation

We tested this method also in a case of metal independent oxidation, using AAPH as generator of aqueous peroxyl radicals. Preliminary experiments established that AAPH decomposition in PBS did not result in any LL-CL, which is a significant advantage in the design of experiments requiring high AAPH concentration

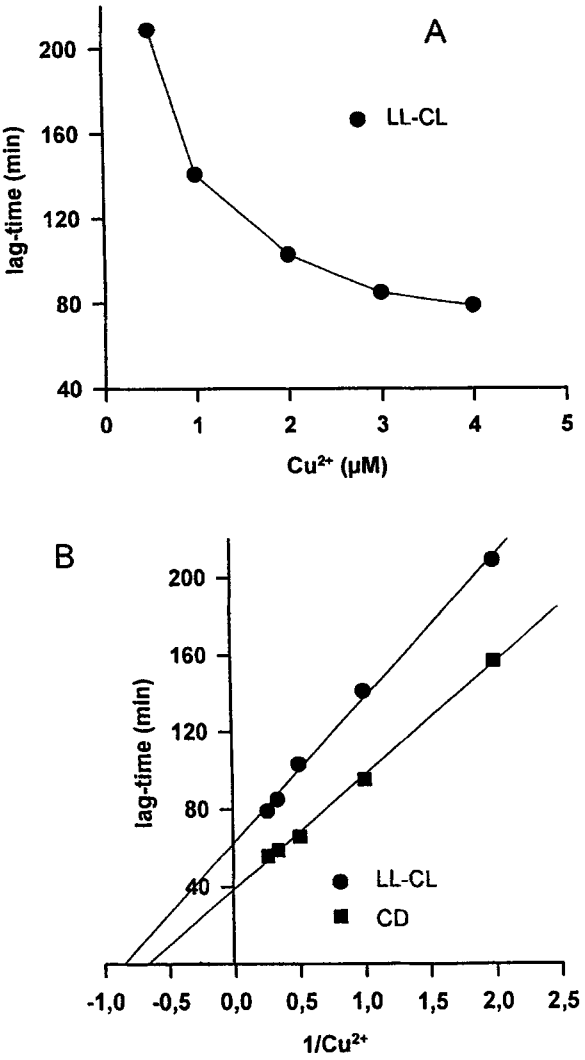

FIGURE 4 Kinetic study of LDL oxidation by CD and LL-CL method. Panel A: LDL was oxidized under the experimental conditions described in Figure 3. Lag-time values obtained by LL-CL were plotted against Cu^{2+}-concentration. Panel B: LDL was oxidized under the experimental conditions described in Figure 3. Lag-time values obtained by LL-CL and CD were plotted against the reciprocal of Cu^{2+}-concentration in order to determine the constant K (the intercept on x-axis), describing the half maximal activity of prooxidant Cu^{2+} binding sites of LDL.

(data not shown). Similarly to Cu^{2+} as prooxidant, with increasing AAPH concentrations the lag-times of LDL oxidation progressively decreased and the peak values of LL-CL increased (Figure 5). It is noteworthy that the peak level of

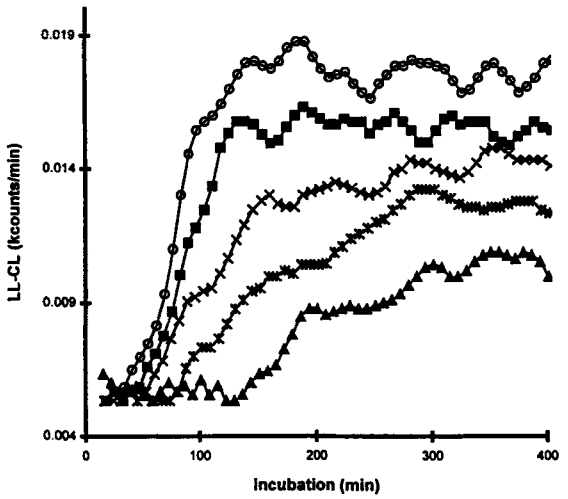

FIGURE 5 Monitoring of AAPH-induced LDL oxidation by low-level chemiluminescence. LDL (0.2 μM) was oxidized in PBS at 37°C with AAPH at the following concentrations: 0.5 mM (▲), 1.0 mM (*), 1.5 mM (×), 2.0 mM (■) and 2.5 mM (○).

LL-CL was generally lower in the case of AAPH-induced oxidation as compared to Cu^{2+}; this may partially depend on the absence of Cu^{2+}-catalyzed decomposition of lipid hydroperoxides contribution to LL-CL. Also, the rate of initiation provided by different AAPH concentrations directly influences the peak level of LL-CL, as this parameter is related to rate of recombination of $LOO^{\bullet}$, which in turn is equal to the rate of initiation in uninhibited lipid peroxidation.[9] Figure 5 shows that decreasing AAPH concentrations lead to decreasing peak levels of LL-CL, accordingly.

The spectrum of LL-CL emitted from oxidizing LDL shows a maximum between 420 and 500 nm, corresponding to the wavelength emitted by the decay of triplet carbonyl compounds (Figure 6).

LDL oxidation was monitored under the same experimental conditions by CD assay and by LL-CL, at AAPH concentrations ranging from 0.5 to 3 mM. The correlation of lag-time values obtained with these two methods was high ($r = 0.98$), as shown in Figure 7. The comparison of lag-time values as obtained from CD and LL-CL (Figure 7)

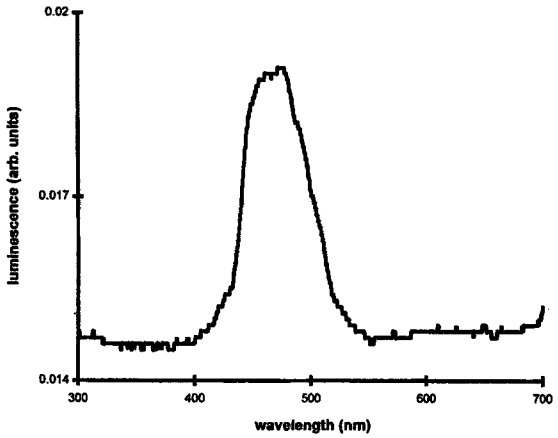

FIGURE 6 Spectral analysis of LL-CL from oxidizing LDL. LDL (0.3 mM) was oxidized in PBS at 37°C with 5 mM AAPH over 400 min and luminescence spectra were recorded on a fluorimeter. The spectrum is the sum of 100 scans over the spectral range shown.

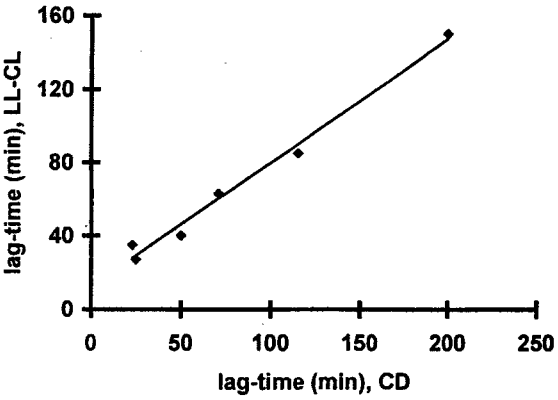

FIGURE 7 Correlation of lag-time of peroxyl-radical-induced LDL oxidation as assayed by CD and LL-CL. LDL was oxidized with AAPH as described in the legend to Figure 5; the oxidation was monitored as CD and LL-CL: lag-time values were determined graphically and plotted.

shows that there is no significant difference between lag-times of LL-CL and CD profiles.

Estimation of Peroxyl Radical Concentration from LL-CL

We used the transient scavenging of peroxyl radicals by BHT in AAPH-mediated LDL oxidation

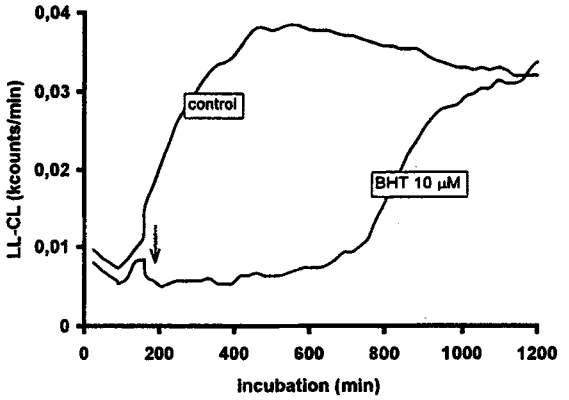

FIGURE 8 Effect of BHT on low-level chemiluminescence of oxidizing LDL. LDL (0.3 µM) was oxidized with 1 mM AAPH in PBS at 37°C. At the time point indicated by the arrow, BHT was added.

to estimate the correspondence between LL-CL, R_i and [LOO•] using the steady-state approximation for lipid peroxidation. As shown in Figure 8, at 1 mM AAPH, 10 µM BHT was able to quench LL-CL for 570 min after addition at 200 min (i. e. at beginning propagation), similar to Cu^{2+}-mediated oxidation. This indicates an R_i of $5.8 \times 10^{-10}\,M\,s^{-1}$ generated by 1 mM AAPH (one BHT scavenges two peroxyl radicals), which corresponds well to the radical flux of about $4.6 \times 10^{-10}\,M\,s^{-1}$ estimated from the 150 min required for the consumption of 7 mol TocOH/mol LDL, at 0.3 µM LDL. As R_i = rate of termination (R_{term})[9] we calculate that 0.0246 (= 0.0325 (quasi-steady-state maximum) − 0.00786 (basal level)) kcounts/min correspond to $R_i = 5.8 \times 10^{-10}\,M\,s^{-1}$. As a result it can be estimated that one kcount per minute (under our experimental conditions) equals an $R_i = R_{term}$ of $2.3 \times 10^{-8}\,M\,s^{-1}$.

To calculate the peroxyl radical concentration from

$$[LOO^{\bullet}]_{ss} = \sqrt{\frac{R_i}{2k_{term}}}$$

with $k_{term} = 1 \times 10^5\,M^{-1}\,s^{-1}$, we need to convert R_i, to the value for local reactant concentrations

($5.8 \cdot 10^{-7}\,M\,s^{-1}$) within LDL,[9] to arrive at [LOO•] of 1.7×10^{-6} M (concentration within LDL), corresponding to 0.0057 mol LOO•/mol LDL. The global [LOO•] for 0.3 μM LDL is thus 1.7×10^{-9} M.

Application to Oxidation of LDL at High Concentration

LDL at concentrations ranging from 0.2 to 0.5 μM was oxidized using Cu^{2+} as prooxidant agent. The peak level of chemiluminescence was increasing more or less proportionally with LDL concentration. Our data show that the relationship between Cu^{2+} and lag-time persisted to be hyperbolic and could be linearized at all LDL concentrations tested (Figure 9). Thus, LDL oxidation by Cu^{2+} is a saturable process also when high LDL concentrations are employed.

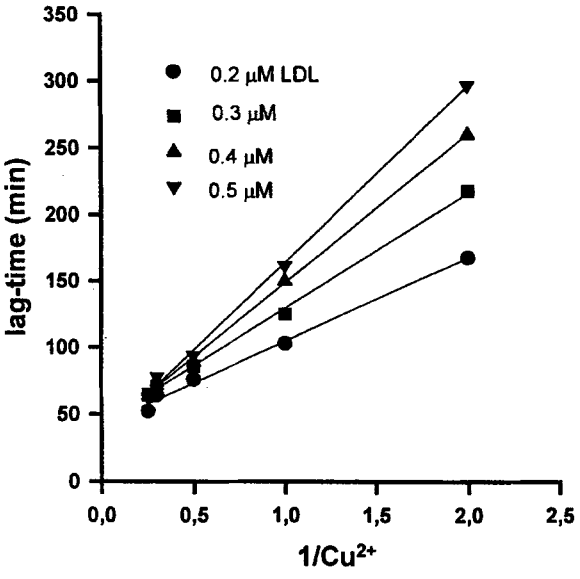

FIGURE 9 Oxidation of LDL at high concentration. LDL at the indicated concentrations was oxidized with Cu^{2+} in PBS at 37°C and the reaction was followed by LL-CL. Lag-time was determined and plotted against the reciprocal of Cu^{2+} concentration. It is noteworthy that UV absorption of LDL at concentrations higher than 0.2 μM did not lead to accurate readings by the spectrophotometer.

DISCUSSION

Our study describes a procedure for monitoring of metal-dependent and independent LDL oxidation which may be useful in basic research and clinical studies as well as in the characterization of prooxidants and antioxidants. Evident advantages consist of the lack of interference by UV-absorbing compounds, the use of disposable plastic multiwell plates with a favorable surface/volume ratio and the possibility of simultaneous multisample analysis. The possibility of calibration of LL-CL with lipid peroxyl radical flux by means of BHT is noteworthy. Indeed, LL-CL indicates a radical flux and not a lipid peroxidation product. The integration time for each data point can be adjusted on most instruments to adapt to the sensitivity of the photomultiplier and to various experimental conditions. This method allows continuous and direct monitoring of LDL oxidation because extraction and derivatization of lipid peroxidation products are not required.

The present procedure may be an alternative to CD or multiple wavelength UV absorption analysis[10] or oxygen uptake measurements[11] all of which are suited to obtain continuous profiles of lipoprotein oxidation; this is supported by the high correlation of lag-time values as obtained by LL-CL and CD assays. Other methods are available to assay lipoprotein oxidation. Although techniques like monitoring of free radicals by ESR spectroscopy,[12] chromatographic separation of products and detection by either UV[13] or chemiluminescence[14] or gas chromatography–mass spectrometry analysis[15,16] possess a high degree of sensitivity and specificity, most of these methods cannot provide for continuous monitoring, have elevated cost of instruments and reagents and require large sample volumes due to the fact that one aliquot is needed for each determination.

According to a series of previous studies,[5,6,17] the chemiluminescent species formed during lipid peroxidation are excited singlet oxygen and triplet carbonyl compounds, resulting from

the recombination of two lipid peroxyl radicals, formed by oxygenation of a carbon centered radical or by decomposition of lipid hydroperoxides, via a tetroxide intermediate, according to the following reactions:[17]

$$2\,LOO^{\bullet} \rightarrow LO + LOH + {}^{1}O_2 \qquad (1)$$

or

$$2\,LOO^{\bullet} \rightarrow {}^{3}O_2 + {}^{3}LO^{*} + LOH \qquad (2)$$

The dimol reaction of singlet oxygen, reverting to ground state, generates light of 634 and 703 nm, while triplet carbonyl leads to emission at 375–450 nm.[5] According to our spectral analysis, triplet carbonyl compounds are likely to be the main chemiluminescent species responsible for LL-CL from oxidizing LDL.

Other than in AAPH-mediated oxidation, in Cu^{2+}-induced LDL oxidation, lag-times as measured by LL-CL were longer by a factor of 1.3. The reason for this difference is presently not clear, it might be connected with a slow increase of $LOO^{\bullet}$ due to the autocatalytic process of transition-metal-ion-mediated decomposition of lipid hydroperoxides at onset of propagation. In contrast, AAPH-derived aqueous peroxyl radicals deliver a constant radical flux and start of propagation does not require the buildup of sufficient hydroperoxide concentrations. As the metal-dependent decomposition of LOOH also leads to light-emission, both processes together might be responsible for the delay.

The chemical basis of low-level chemiluminescence explains the long duration of light emission after onset of propagation during LDL oxidation and the lower intensity of LL-CL when LDL oxidation was induced by AAPH; in fact, the rearrangement and decomposition of lipid peroxides by Cu^{2+} catalyzed reactions, as well as their formation during propagation phase, yield photoemissive species. Under circumstances where such processes are not favored, i.e. in the absence of transition metal ions, it is thus possible to extract directly parameters like the rate of initiation, which equals the rate of termination in uninhibited oxidation and, eventually, the concentration of lipid peroxyl radicals. Using BHT to prevent oxidation by scavenging peroxyl radicals it was possible to estimate $R_i = R_{term}$ to be $5.8 \cdot 10^{-10}\,M\,s^{-1}$, for 1 mM AAPH and 0.3 μM LDL and to calibrate LL-CL for termination rate. By converting to local concentrations, it is possible to calculate [$LOO^{\bullet}$] from R_i. Thus, in addition to the extraction of oxidation indices, like lag-phase, this method allows for the non-invasive monitoring of low concentrations of peroxyl radicals over a wide range of experimental conditions.

Acknowledgments

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