Mechanistic aspects of the relationship between low-level chemiluminescence and lipid peroxides in oxidation of low-density lipoprotein

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Received 30 June 1999; received in revised form 29 August 1999

Abstract In this study oxidation of low-density lipoprotein (LDL) induced by different Cu^{2+} concentrations was investigated. Lipid peroxidation was assessed by monitoring low-level chemiluminescence (LL-CL), conjugated diene hydroperoxide (CD) and α-tocopherol (TocOH), the major lipophilic antioxidant in LDL. At high Cu^{2+} concentration, LDL oxidation was characterised by CD formation, LL-CL emission and TocOH consumption. At low Cu^{2+} concentration, CD formation was independent of LL-CL and occurred in the presence of TocOH. Thus, two different mechanisms lead to lipid peroxide formation in LDL. The combination of CD assay and LL-CL monitoring makes it possible to distinguish the autocatalytic mechanism of CD formation and that associated with TocOH, found at a high and a low rate of initiation, respectively.

Key words: Low-density lipoprotein; Lipid peroxidation; Low-level chemiluminescence; Tocopherol

1. Introduction

Several lines of evidence suggest that oxidation of low-density lipoprotein (LDL) may be involved in the early phase and during progression of atherogenesis [1–3]. In general, the evaluation of LDL oxidation is fraught with difficulties [4]. Recently, we reported that oxidation of isolated LDL was associated with the emission of low-level chemiluminescence (LL-CL), which is the basis of a new method to monitor oxidation [5].

Although the basic principles of lipid peroxidation are relatively simple and established, including an initiation, a propagation and a termination phase [6–8], their application to lipoproteins has raised a number of unresolved issues. One of the most intriguing points is that the mechanism of LDL oxidation may be in part different at low and high initiation rates [9]. This may be the reason for the apparently ambiguous role of α-tocopherol (TocOH), the most abundant lipophilic antioxidant in LDL. At first, it was taken for granted that significant oxidation of polyunsaturated fatty acids to lipid peroxides (LOOH) would occur only after the complete consumption of TocOH [10]. However, subsequent studies demonstrated that when sufficiently low concentrations of Cu^{2+} or peroxyl radicals were used, the oxidation of LDL occurred also in the presence of endogenous TocOH [11–13]. These observations led to the formulation of the TocOH-mediated peroxidation model, according to which $TocO^{\bullet}$ could attack a polyunsaturated fatty acid, contributing significantly to oxidation at low radical flux, thus acting as a prooxidant [11,12].

In our study LDL oxidation at various initiation rates was monitored by LL-CL, conjugated diene (CD) hydroperoxides, and TocOH. The relationships between these oxidation parameters are illustrated and two pathways leading to lipid peroxides are proposed. The use of Cu^{2+} as an oxidation catalyst is justified because it is possibly involved in LDL oxidation in vivo, as suggested by the observation that transition metal ions (mainly copper and iron) were found in atherosclerotic lesion in free [14] and protein-bound form [15] and the presence of metal ions (even as a contaminant) is a prerequisite for cell-mediated LDL oxidation [16].

2. Materials and methods

2.1. Materials

All reagents were of AR grade and were obtained from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

2.2. Preparation of LDL

LDL was isolated from plasma of normolipidaemic healthy volunteers of both sexes (age 25–35 years) after an overnight fast by ultracentrifugation in a single-step discontinuous gradient [5]. 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 Esterbauer and Ramos [6].

2.3. LDL oxidation

Before oxidation, LDL was freed of EDTA by gel filtration [5]. 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 0.2 μM LDL corresponded to 0.5 mg/ml total mass and 158 μg/ml total cholesterol [17].

LDL, freed of EDTA, was oxidised with $CuSO_4$ at various concentrations (0.2, 0.5 and 1.6 μM) in PBS at 37°C [5]. In our incubation mixtures biocides were not added because they might interfere with LDL oxidation by several mechanisms, including free radical scavenging and metal binding activity. Several approaches were taken to

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Abbreviations: LDL, low-density lipoprotein; CD, conjugated diene hydroperoxide; LL-CL, low-level chemiluminescence; TocOH, α-tocopherol; LOOH, lipid peroxides; $LOO^{\bullet}$, lipid peroxyl radical; LH, reactive methylene group of a polyunsaturated fatty acid; $L^{\bullet}$, carbon-centred lipid radical; $LC = {}^3O$, triplet carbonyl compounds; ${}^3O_2$, triplet oxygen; PBS, phosphate-buffered solution

PII: S0014-5793(99)01217-X

prevent the growth of contaminating microorganisms in our oxidation mixtures, which include the use of autoclaved materials (cuvettes, pipettes), of filtered PBS and LDL solutions (pore size 0.2 μm). At the end of the incubation time the incubation mixtures were clear and UV-Vis spectra did not show evidence of Tyndall light scattering due to microbial growth. Moreover, aliquots of LDL incubation mixtures at the end of incubation were plated on various agar media and the absence of microbial growth was observed.

2.4. Assessment of LDL oxidation

CD hydroperoxides were monitored by recording the increase of absorbance at 234 nm ($A_{234\,nm}$) and estimated with a molar absorption coefficient of 29 500 M^{-1} cm^{-1} [18]. As kinetic index of LDL oxidation, lag time was determined graphically [17,18]; maximum rate of oxidation was calculated as the highest value of the first derivative of CD vs time profile. TocOH was measured by HPLC chromatography using a reverse-phase C18 column and spectrophotometric detection at 292 nm; an external standard was used for calibration [19]. In our preparations, TocOH content of LDL was 5 ± 0.3 mol/mol LDL.

2.5. Chemiluminescence measurement

LL-CL 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 source of LL-CL is the decay to the ground state of triplet carbonyl compounds (LC = ^{3}O) produced by recombination of lipid peroxyl radicals to non-radical products [5].

3. Results

The kinetic profiles of LDL oxidation were monitored at different Cu^{2+} concentrations by both CD and LL-CL. At high Cu^{2+} concentration (1.6 μM) (Fig. 1A,B), the kinetic profiles exhibited an inhibited phase (lag time) which was followed by a propagation phase characterised by the fast increase of CD and LL-CL. The time courses of CD and LL-CL were quite similar. The CD concentration at the end of incubation was 58 ± 6 μM (about 290 mol CD/mol LDL); the maximum rate of oxidation was reached 240 min after the start of incubation and was 0.7 ± 0.06 μM CD/min.

With decreasing Cu^{2+} concentration, the CD profile showed increasing complexity. At 0.5 μM Cu^{2+} (Fig. 1C), it was S-shaped and three phases could be distinguished: a preoxidation phase (from 0 to about 150 min), a lag phase (from 150 to 400 min) and a propagation phase (from 400 to 600 min). The maximum rate of oxidation was reached at 530 min of

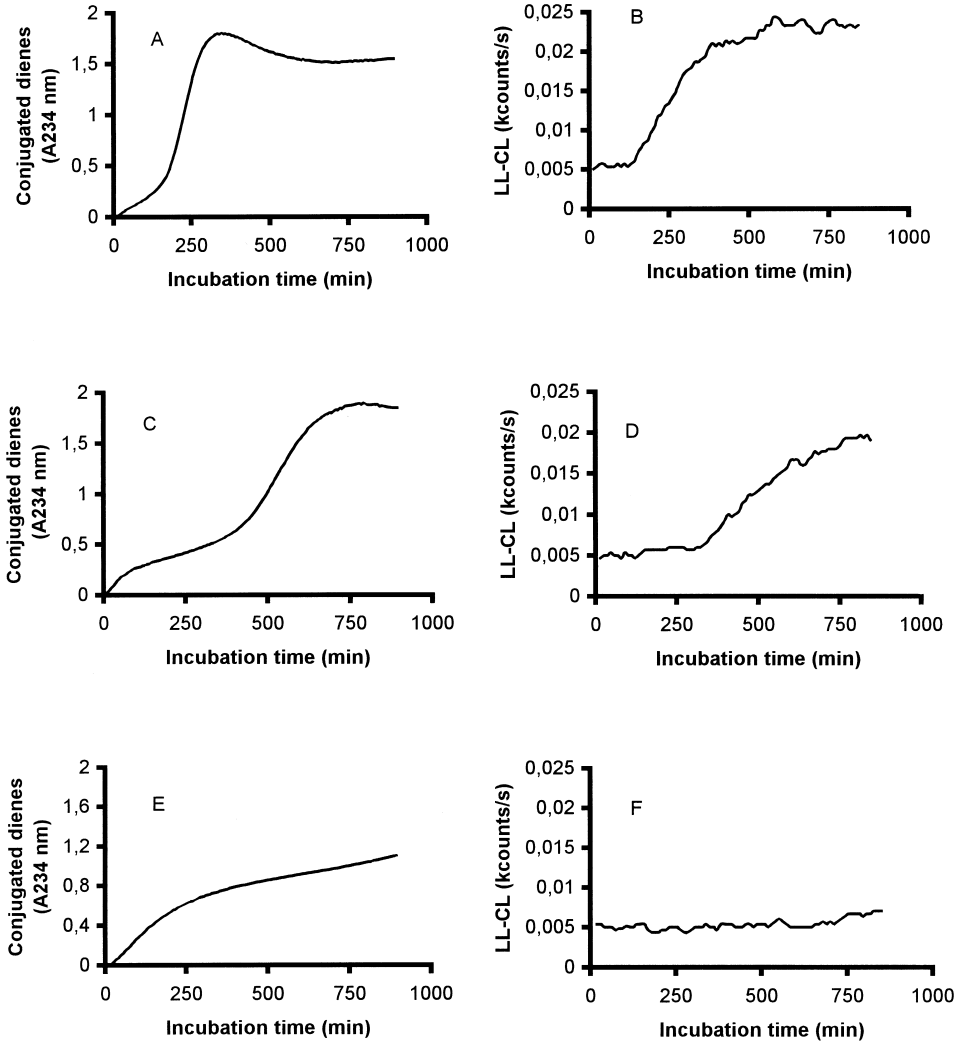

Fig. 1. Kinetic profile of LDL oxidation induced by Cu^{2+}. 0.2 μM LDL was incubated at 37°C in PBS. Oxidation was monitored by recording CD formation and LL-CL. Cu^{2+} concentrations were 1.6 μM (A, B), 0.5 μM (C, D), 0.2 μM (E, F). The results are representative of four independent experiments.

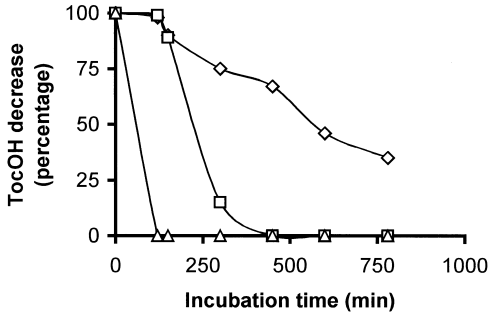

Fig. 2. Time course of TocOH consumption during LDL oxidation. 0.2 μM LDL was incubated at 37°C in PBS; Cu^{2+} concentrations were 1.6 μM (△), 0.5 μM (□), and 0.2 μM (◇). Appropriate aliquots of the oxidation mixture were withdrawn at the time points indicated and the reaction was stopped by addition of EDTA solution (1 mg/ml, final).

incubation and was 0.18 ± 0.01 μM CD/min. At the end of the incubation, the CD concentration was similar to that found at 1.6 μM Cu^{2+}. The LL-CL profile maintained the typical biphasic shape, with an inhibited and a fast increase at 0.5 μM Cu^{2+} (Fig. 1D). Interestingly, the rise of LL-CL was associated with the propagation phase of CD but not with the preoxidation phase, i.e. the initial formation of CD after Cu^{2+} addition.

At 0.2 μM Cu^{2+}, the CD formation exhibited an increase after Cu^{2+} addition and then it slowed down progressively (Fig. 1E). The maximum rate of oxidation was reached at 70 min of incubation (much earlier than at 1.6 and 0.5 μM Cu^{2+}) and was 0.12 ± 0.02 μM CD/min. At the end of incubation, the CD concentration was 35 ± 4 μM (Fig. 1E), about 60% of the value found at higher Cu^{2+} concentrations. For 0.2 μM Cu^{2+}, virtually no LL-CL could be detected (Fig. 1F). Thus, with a decreasing rate of initiation, differences between CD and LL-CL profiles were found.

As a further index of oxidation, TocOH was assayed during LDL oxidation at the different Cu^{2+} concentrations used (Fig. 2). Complete consumption of TocOH was obtained after 120 min of incubation at 1.6 μM Cu^{2+}, thus before formation of significant amounts of CD and LL-CL (Fig. 2 and see Fig. 1A,B). TocOH was consumed at about 380 min at 0.5 μM Cu^{2+}, after concomitant formation of CD during preoxidation and lag phase (about 17 μM, i.e. 85 mol CD/mol LDL) (Fig. 2 and see Fig. 1C) but before the rise of LL-CL (see Fig. 1D). At 0.2 μM Cu^{2+}, TocOH was still present at the end of the incubation at a concentration of 2 mol/mol LDL (Fig. 2). These data indicate that at lower rates of initiation lipid peroxides were formed in the presence of lipophilic antioxidants.

4. Discussion

Our results show direct evidence that the mechanism of LDL oxidation changes depending on Cu^{2+} concentration, which is a major determinant of the initiation rate of lipid peroxidation. The modifications of CD time course with decreasing Cu^{2+} concentration are in substantial agreement with previous findings [9,13], although it was not generally agreed that this could have any mechanistic bearing. The monitoring of oxidation by LL-CL provides new insights into this issue, following the consideration that the source of LL-CL is the termination reaction between two peroxyl radicals, $LOO^{\bullet}$ [5,20,21]:

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOH + LC{=}{}^{3}O + {}^{3}O_2 \quad (1)$$

where $^3O_2$ and $LC{=}{}^3O$ indicate triplet oxygen and triplet carbonyl compounds, respectively.

At high Cu^{2+} concentration (1.6 μM), LL-CL and CD profiles were essentially parallel and oxidation occurred through the main propagating reaction:

$$LOO^{\bullet} + LH + O_2 \rightarrow LOOH + LOO^{\bullet} \quad (2)$$

where LH indicates the reactive methylene group of a polyunsaturated fatty acid. This reaction ensues after depletion of TocOH by initiating radicals during lag phase and yields a high concentration of lipid peroxyl radicals, which can be detected by LL-CL. This model interprets lipid peroxidation in LDL at high Cu^{2+} concentrations as a free radical-mediated autocatalytic reaction, in agreement with conventional knowledge [6,8]. As lipid peroxidation proceeds, various breakdown products of lipid peroxides (including biologically active aldehydes) are formed which covalently modify apo B-100, increasing the affinity of LDL for macrophage scavenger receptor [2,6].

The formation of lipid peroxides at low radical flux in the presence of TocOH and the absence of LL-CL can be explained by a different model, which takes into account the chemical reactivity of TocOH [7] and in particular its ability to scavenge lipid peroxyl radical and the possibility that it may attack a polyunsaturated fatty acid, acting as a chain transfer agent and being regenerated, as first described by Bowry and Stocker [11]:

$$TocOH + LOO^{\bullet} \rightarrow LOOH + TocO^{\bullet} \quad (3)$$

$$TocO^{\bullet} + LH \rightarrow L^{\bullet} + TocOH \quad (4)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (5)$$

As shown by our measurements, the cycle proposed does not generate LL-CL, because the high affinity of TocOH for $LOO^{\bullet}$ prevents reaction 1.

Our study implies an intriguing function for TocOH. The argument that TocOH acts as antioxidant at high rate of initiation is widely accepted [6–13] and is in agreement with our findings. The role of TocOH in LDL oxidation at low Cu^{2+} appears more problematic. In a number of studies [11–13] an exclusively prooxidant role was ascribed to TocOH, when LDL oxidation was induced at low radical flux. Alternatively, on the basis of our results, TocOH may be involved in a set of reactions which have an antioxidant (Eq. 3) as well as a prooxidant effect (Eq. 4), as already proposed in a mechanistic study [22]. The result of this balanced mechanism is a slow formation of lipid peroxides without emission of LL-CL, explaining the low amount of lipid peroxides formed at the lowest Cu^{2+} concentration employed.

Hence, LL-CL makes it possible to distinguish the formation of lipid peroxides in LDL via the autocatalytic, propagative mechanism from that via a TocOH-regenerating mechanism, which are typical of a high and a low rate of initiation, respectively.

If our results are extrapolated to the physiological situation, the LDL oxidation pathway occurring at low radical flux might be particularly insidious because lipophilic antioxidants, such as TocOH, might not prevent the formation of lipid peroxides. Through this mechanism, LDL might be 'seeded' with lipid peroxides and become highly susceptible to the conventional autocatalytic peroxidation. This sequence of events might explain how LDL is oxidised in interstitial fluid of arterial wall, which contains a high concentration of plasma antioxidants. The evidence that even minute amounts of Cu^{2+} are able to oxidise LDL suggests the possible prooxidant role of transition metals, which may be released from damaged plasma transport protein and cellular debris in atherosclerotic lesion [8]. On the basis of the present knowledge, it is not possible to estimate the level of oxidative stress which LDL undergoes in arterial wall; however, it is conceivable that both the autocatalytic mechanism of LDL oxidation and that associated with TocOH may be effective in vivo, as suggested by findings that both heavily [23] and mildly [24] oxidised LDL are present in arterial wall.

Acknowledgements: This work was supported by the Austrian Science Foundation, Project F709, and by MURST (60% and 'Cofinanziamento di Progetti di Ricerca di Interesse Nazionale') of Italy.

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