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Oxygen Availability as a Possible Limiting Factor in LDL Oxidation

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Kinetic studies of copper-induced LDL peroxidation commonly assume that the availability of molecular oxygen in the reaction media is not a limiting factor. The present study reveals that this assumption is valid only at low LDL concentrations. At high LDL concentrations, accumulation of oxidation products, as monitored spectroscopically under conditions of various oxygen concentrations in the medium, comes to a halt when the oxygen concentration in the solution, as measured by an oxygen electrode, decreases to near zero levels. Bubbling of the oxygen into the solution results in resumption of peroxidation. These results are important with respect to the *ex vivo* assaying of lipoprotein peroxidation because many previous studies have been conducted with LDL concentrations that corresponded to polyunsaturated fatty acid concentrations in access of the concentration of molecular oxygen. The possible pathophysiological significance of the results of this study has yet to be evaluated.

Keywords: LDL oxidation; Copper; Oxygen; Lipoprotein

Abbreviations: LDL, low density lipoprotein; PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acid; A, absorbance

INTRODUCTION

The postulated role of lipid peroxidation in atherosclerosis invoked much interest in the mechanisms responsible for lipid oxidation *in vivo* and in the possibility of evaluating the individual "oxidative stress" *ex vivo*.[1,2] The development of spectroscopic assays for continuous monitoring of the kinetics of oxidation[3] resulted in a large number of investigations on the susceptibility of lipoproteins to oxidation (commonly referred to as the "oxidizability"). In each of these studies, lipoproteins have been exposed to an inducer of peroxidation, most often copper ions, and the resultant accumulation of peroxidation products (mostly conjugated dienic hydroperoxides and oxysterols) have been continuously monitored at 230–250 nm, usually at 234 nm.[3]

A typical kinetic profile of copper-induced LDL oxidation exhibits three discrete phases:[3] First, lipoprotein-associated antioxidants are being consumed and hydroperoxides accumulate slowly. This "lag phase", ends when a sufficiently large fraction of the lipoproteins becomes oxidized into hydroperoxides. Autoacceleration subsequently yields relatively rapid propagation, which continues until almost all the oxidizable lipids become oxidized. Thereafter, decomposition of hydroperoxides becomes faster than their continuing production, resulting in a decrease of the level of hydroperoxides. Yet, production of oxysterols continues, resulting in subsequent increase of the absorption of UV radiation.

Recently, we have shown[4] that within the range of 0.025–0.15 μM, the lag preceding oxidation depend only on the occupancy of the LDL's copper binding sites, i.e. on the bound copper/LDL ratio, and not on the concentrations of either copper or LDL. Nonetheless, at LDL concentrations above 0.15 μM, these attributes of the concentration-dependence

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of the kinetic factors were observed only at relatively low molecular ratios of bound copper/LDL. At copper concentrations that corresponded to about 50% occupancy or more,[4] commonly used in *in vitro* studies of LDL oxidation, the kinetics of oxidation depended not only of the occupancy but also on the total LDL concentration.[4] Under these conditions, increasing the concentration of LDL resulted in distortion of the kinetic profiles whereas A_{max} increased only slightly.

The most straightforward explanation for this apparent "saturation" is that under conditions of relatively high concentrations of LDL and copper, the limiting factor is the availability of molecular oxygen in the solution. As expected, several investigations have demonstrated that the consumption of soluble molecular oxygen, is about equal to the formation of oxidation products.[5,6] Under normal conditions, the initial concentration of molecular oxygen in the aqueous solution (prior to oxidation) is in the range of 200–250 μM, depending on temperature.[7] This means that for 0.15 μM LDL, containing about 200 μM PUFA,[3,8] the initial oxygen content of the solution is sufficient to oxidize all the PUFA, independent of replenishment of oxygen from the air to the solution.

This is not the case for higher LDL concentrations. Thus far, the kinetics of oxygen replenishment from the air (in systems open to air) during peroxidation have not been investigated in samples containing LDL concentrations higher than 0.15 μM.[9,10] Although most kinetic studies have been conducted with about 0.1 μM LDL, many other studies, including several recent investigations,[11–17] used higher LDL concentrations, either to overcome the limited sensitivity of the methods of product analyses and/or with the intention to appropriately model the *in vivo* situation. The initial oxygen concentration in several of these studies was lower than the concentration of PUFA.[13,14] Hence, the availability of oxygen might have limited peroxidation, unless replenishment of oxygen from the air was faster than peroxidation.

The present study shows that under conditions of relatively high concentrations of LDL and copper, the availability of molecular oxygen in the solution is indeed rate limiting. We do not know whether this, often ignored, finding carries any pathophysiological significance. It should, however, be kept in mind when planning "oxidizability studies" or relating to previous results on LDL peroxidation.

MATERIALS AND METHODS

$CuCl_2$ was purchased from Merck (Darmstadt, Germany).

LDL was isolated as previously described.[4,14] Prior to being diluted for oxidation studies, the LDL samples were stored at 4°C for up to four days.

Cholesterol concentration was determined with Boehringer–Mannheim's kit, Germany. Protein concentration was assayed according to Lowry.[18]

Oxidation of lipoproteins (0.09–0.9 μM), was monitored at 37°C by continuous recording of the absorbance at four wavelengths (234, 245, 250 and 268 nm) using a Kontron (Uvikon 933) double-beam spectrophotometer equipped with a 12-position automated sample changer. Measurements were carried out in quartz cuvettes containing a PBS solution (pH = 7.4; final volume of 1.0 ml, optical pathway of 1 cm) containing 150 mM NaCl, 10 mM phosphate buffer and different concentrations of $CuCl_2$, as indicated for each experiment. The kinetics was monitored spectroscopically at 245 nm instead of the more usual 234 nm, to increase the linear range of the spectrophotometeric measurments.[19] The spectroscopic results are given in terms of absorbance (A_{245}) assuming that the increase of recorded optical density in the time course of peroxidation is due to the absorbance of peroxidation products. The kinetics of oxidation were derived from the time course of A_{245} and analyzed as previously described.[3,20]

Oxygen concentration was measured using an Inter-Medical Co. PO_2 monitor, Nagoya Japan, and is given in terms of oxygen concentration (in μM units).

Unless otherwise stated, air was bubbled into the solution (as in most previous studies) for 5 min before adding copper to the LDL solution used for peroxidation kinetic studies. In several experiments, other gases were bubbled into the solution for 5 min at room temperature, to vary the initial oxygen concentration in the reaction mixture. After bubbling gas through each of these solutions, it was equilibrated at 37°C for 5 min before the addition of $CuCl_2$. The oxygen concentration as measured after bubbling various gases is given in Table I.

TABLE I Oxygen concentrations in solutions containing 0.72 μM LDL, following 5 min of bubbling the solutions with different gases, at room temperature and subsequent equilibration at 37°C for 5 min

Gases	Initial PO_2 (mmHg)	$A_{e.p.}$
Air	155 (≈0.23 mM)	1.05
Argon	66 (≈0.10 mM)*	0.62
Oxygen (4.5%) Nitrogen (95.5%)	90 (≈0.13 mM)	0.75
Oxygen (95%) CO_2 (5%)	320 (≈0.48 mM)†	–

Ae.p. denotes the absorbance values at the end of propagation phase, as described in Fig. 4. *Bubbling of argon for periods longer than 5 min resulted in lower oxygen concentrations and in complete inhibition of the oxidation. †Bubbling the mixture of oxygen (95%) and CO_2 (5%) into the solution lowered the pH of the solution from 7.4 to 6.8. According to Patterson *et al.*[21] this may result in prolongation of the lag preceding copper-induced LDL oxidation, but is not expected to influence either the maximal rate or the maximal accumulation of oxidation products.

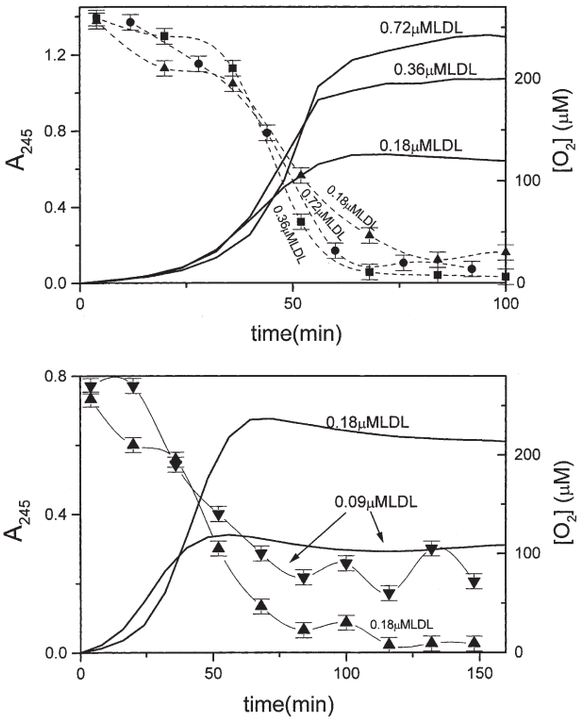

FIGURE 1 Time-course of copper-induced LDL oxidation at 37°C, as monitored by continuous recording of the absorbance of oxidation products at 245 nm (left hand scales) and by measurements of the oxygen concentration (depicted by different symbols and the right hand scales). The kinetic profiles given in both panels are of reactions with four different LDL concentrations, as given in the figure. Oxidation was induced by the addition of $CuCl_2$. The final concentration of Cu^{+2} in the solution was 8.1 μM for 0.09 μM LDL, 9 μM for 0.18 μM LDL, 10.5 μM for 0.36 μM LDL and 13.5 μM for 0.72 μM LDL. These concentrations were chosen so as to achieve a constant ratio of bound copper to LDL (Cu/LDL = 14 ± 1) according to the affinity of copper for its binding sites, as described in Ref. [4]. Error bars depict the SEM of oxygen measurements.

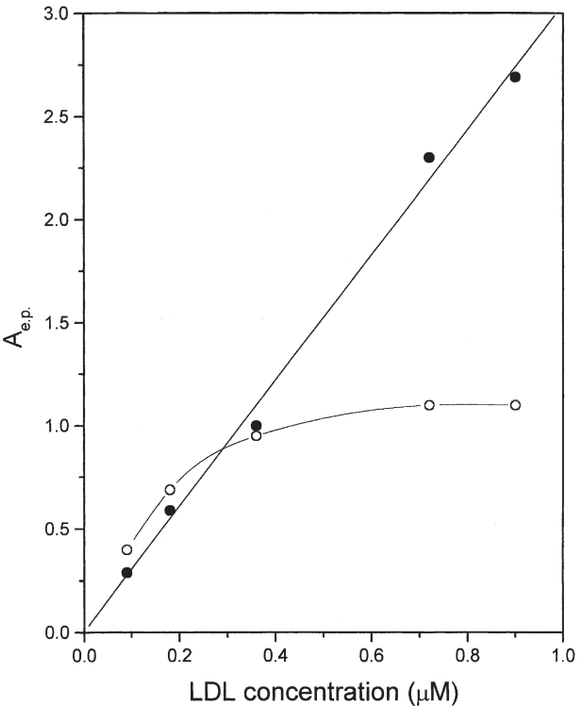

FIGURE 2 Dependence of the maximal accumulation of oxidation products at the end of the propagation phase on LDL concentration. In the experiment described by the filled circles, oxygen (95%) was bubbled into the solution for 20 s every 12 min. The open circles describe a control experiment in which no gas was bubbled into the solution. Copper concentrations were calculated for each LDL concentration so that the ratio of copper bound to LDL will be equal to 14 ± 1 at all LDL concentrations. Data points represents two different experiments.

RESULTS AND DISCUSSION

The major goal of this study was to gain understanding of our previous observation[4] that under conditions of a relatively high copper concentration, when most of the copper binding sites on LDL are occupied, the kinetics of copper-induced peroxidation was not only a function of the bound copper/LDL ratio but also of the absolute LDL concentration. This observation is shown in Fig. 1, in which the absorbance of oxidation products is described as a function of time after the addition of $CuCl_2$ to LDL. The four kinetic profiles depicted in this figure are of four $CuCl_2$–LDL mixtures of different concentrations of $CuCl_2$ and LDL but high and approximately equal molar ratio of bound copper to LDL (bound copper/LDL = 14 ± 1). The most pronounced observation seen in this figure is that increasing the LDL concentration from 0.36–0.72 μM (upper panel) has only a minor effect on the maximal accumulation of oxidation products. Accordingly, A_{245} is a "saturating function" of the LDL concentration (empty symbols in Fig. 2)

We propose that this apparent "saturation" is a result of complete consumption of oxygen dissolved in the solutions containing high LDL concentrations. In other words, we think that when the consumption of dissolved molecular oxygen is rapid (due to rapid peroxidation), the availability of oxygen becomes rate limiting, because replenishment of the solution with oxygen, via diffusion of oxygen into the solution (from the air) is slower than the consumption of oxygen through lipoprotein oxidation.

One evidence for this hypothesis comes from direct measurement of the kinetics of oxygen consumption, as shown in Fig. 1.: The lower panel of this figure shows that at LDL concentrations below 0.18 μM the maximal accumulation of oxidation products is an apparently linear function of LDL concentration and that after the absorbance approached its maximal level, the oxygen concentration remained apparently constant. At 0.18 μM LDL, the oxygen approached near zero level whereas at 0.09 μM LDL, the oxygen concentration decreased to about 30% of its initial concentration, as expected from stoichiometric consideration and in agreement

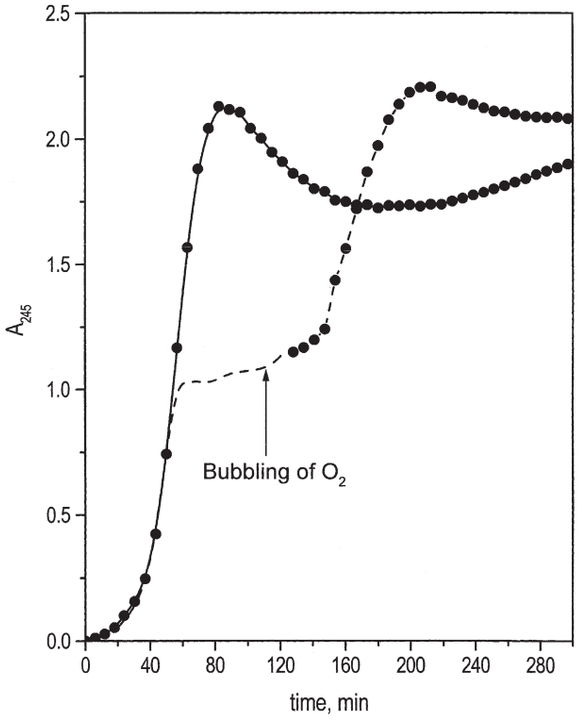

FIGURE 3 Time dependency of the accumulation of oxidation products (A_{245}) during oxidation of LDL (0.72 μM) by $CuCl_2$ (22.5 μM). Oxidation was initiated at 37°C either without bubbling gas (control, no symbols, dashed line), or with bubbling of 95% oxygen for 20 s every 12 min (filled circles, solid line). At the time indicated by the arrow, a similar bubbling protocol was applied to the control sample (filled circles, dashed line).

with the results of other recent studies.[9] By contrast, at higher LDL concentrations, A_{max} exhibits a "saturation dependency" on LDL concentration and when the absorbance approached its maximal level, the oxygen concentration in the solution was very low (Fig. 1 upper panel)

Another evidence for our hypothesis is presented in Fig. 2. In the experiment depicted by the filled-in symbols, we have periodically bubbled an oxygen-rich mixture of gases into the solution and recorded the optical density continuously. In this experiment, the observed dependence of A_{max} on LDL concentration was about linear up to the limit of linearity of the spectrophotometric measurements (achieved at 0.9 μM LDL). This dependence accords with the notion that when oxygen is not bubbled into the solution (as in the experiment described by the empty symbols), the replenishment of oxygen via diffusion is slower than the consumption of oxygen during peroxidation. As a consequence, the initial concentration of soluble oxygen (about 200 μM at 37°C) is sufficient for oxidation of only 0.15 μM LDL (which contains about 200 μM PUFA). Hence, when the accumulation of peroxidation products approached an apparent plateau prior to complete peroxidation of PUFA, supposedly due to shortage of oxygen, bubbling of oxygen into the solution induced further accumulation of peroxidation products (Fig. 3).

Further support for the above notion is that at the end of propagation the concentrations of oxygen in 0.4 μM LDL solution, not bubbled with oxygen, was only 81 ± 0.7 μM (not shown). By contrast, in the same solution, bubbled with oxygen, the oxygen concentration increased to 360 μM probably due to accumulation of oxygen during the constant bubbling.

When oxygen was not bubbled into the reaction mixtures during peroxidation, and the solution contained a higher concentration of oxidizible lipids than of oxygen, the phase of rapid oxidation ended when the soluble oxygen became consumed and peroxidation continued at a much slower rate, presumably determined by the rate of diffusion of molecular oxygen. This is shown in Fig. 4, in which the kinetics of oxidation is presented for three mixtures. All these mixtures contained equal (and high) concentrations of LDL (0.72 μM) and $CuCl_2$ (22.5 μM; $Cu_B/LDL = 15.6$) but different initial oxygen concentrations. At the points denoted $A_{e.p.}$ (absorbance at the end of the propagation phase) peroxidation was markedly slowed down, probably because diffusion of oxygen from the air into the solution (and within the solution) became rate limiting. As a consequence, $A_{e.p}$ increased with the initial oxygen concentration (Table I) whereas the rate of peroxidation at later times was apparently independent of the initial oxygen concentration (Fig. 4).

Notably, in most studies of LDL peroxidation, the solution is aired (at room temperature) prior to the addition of copper, to ensure saturation of the solution with oxygen, and then equilibrated at 37°. In our experiments, bubbling of oxygen-rich mixture of gases into the solution for 5 min at room temperature yielded an oxygen-rich solution (480 μM). However, diffusion of oxygen from the solution at 37°C resulted in a decrease of the oxygen concentration to 270 μM in less than 7 min (result not shown). This is in contrast to the much slower diffusion of oxygen into a solution depleted of oxygen. Thus, a solution bubbled for 5 min with argon and heated to 37°C contained only about 130 μM O_2 and 30 min later the oxygen concentration remained about equal. Bubbling of either air or oxygen into the LDL solution prior to peroxidation experiments appears to be quite ineffective.

The present study provides evidence that during *in vitro* peroxidation of high lipoprotein concentrations (higher than 250 μM PUFA), oxygen availability may indeed be a limiting factor. Most peroxidation studies conducted thus far employed lower lipid concentrations. However, many previous studies,[11–16] including several recent

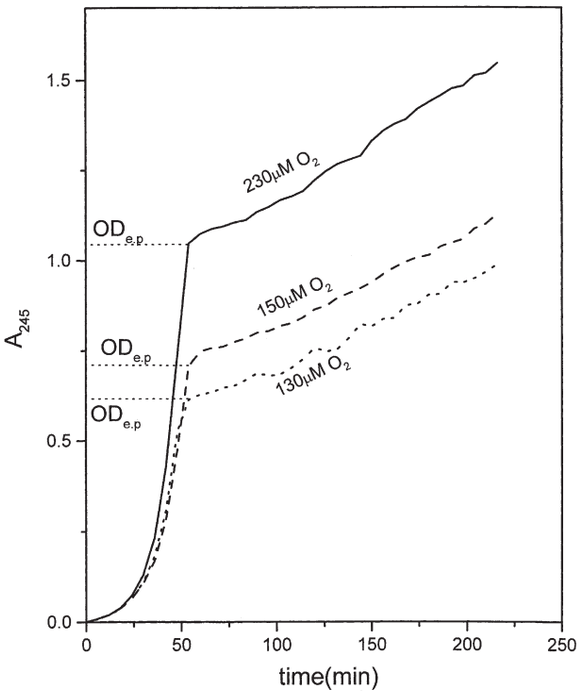

FIGURE 4 Time dependencies of the accumulation of oxidation products (A_{245}) during copper-induced oxidation of LDL (0.72 μM). In order to vary the initial oxygen concentration in the reaction mixture, prior to the addition of $CuCl_2$ (22.5 μM), we have bubbled different gases into the oxidation mixtures. Following 5 min at room temperature and subsequent equilibration at 37°C for another 5 min, the oxygen concentrations were as given in Table I and in the figure.

investigations, employed higher concentrations. In view of our study, the results of the latter investigations are quite questionable, at least with respect to the observed maximal accumulation (A_{max}) and maximal rate (V_{max}). In their recent review, Hogg *et al.*[22] have already noted that "The end of the propagation phase occurs as a result of depletion of either substrate or oxygen". The possibility that oxygen availability is the limiting factor should not be ignored.

In view of the rapid oxygenation in blood, oxygen availability is not likely to be a limiting factor in lipoprotein oxidation in the circulation. Nonetheless, it may become rate limiting in the intima of atherosclerotic arteries. Specifically, the LDL concentration in the intima of atherosclerotic arteries is in the range micro-molars range, i.e. the concentration of PUFA is in the milli-molars range.[23] Although the latter concentration is almost two orders of magnitude higher than that of oxygen in the arterial wall,[24] in normal tissues the rate of replenishment of oxygen may be sufficiently rapid to enable continuing peroxidation. By contrast, the diffusion capacity in developing atherosclerotic lesions is impaired. This impairment is the basis of the "anoxemia theory of atherosclerosis"[25] but it may also limit further oxidative modification of lipoproteins and by that limit the continuing formation of foam cells. However, given the very slow rate of oxidation *in vivo*, this possibility is quite questionable. Altogether, much more research is needed to clarify the possible pathophysiological implications of the role of oxygen level in controlling lipid peroxidation *in vivo*.

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