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Low density lipoprotein is saturable by pro-oxidant copper

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Abstract

The oxidative resistance of low density lipoprotein (LDL) can be experimentally described by the length of time during which no significant lipid peroxidation is observed in a pro-oxidant environment. This period of inhibited oxidation, termed the 'lag phase', is partially due to the radical scavenging reactions of the anti-oxidants contained in the LDL particle. We have shown that the LDL lag time decreases with increasing copper concentration, leveling out at a relatively high copper-to-LDL ratio. This behaviour demonstrates the existence of a finite number of saturable pro-oxidant copper binding sites within the LDL particle. The relationship is described by the equation, lag time = $[Cu]^{-1} \cdot K \cdot t_{min} + t_{min}$ where the constant, K, is the negative reciprocal of the x-axis intercept of the graphed function, and t_{min} is given by the y-axis intercept. By this definition of the constant, K is the amount of copper that will produce a lag time of twice t_{min}, while t_{min} is the minimum time a particular LDL will resist oxidation at a maximum copper concentration.

Key words: Low density lipoprotein; Copper oxidation; Free radical; Copper binding; Kinetics

1. Introduction

The peroxidation of low density lipoprotein (LDL) is considered to be a major initiating event in atherogenesis and therefore much effort has gone into the development of standardized in vitro models for assessment of oxidation resistance of LDL [1]. One such model that has become very popular is copper-mediated oxidation, usually carried out in phosphate-buffered saline or Hams F10 cell culture medium [2,3]. This model of LDL oxidation is highly reproducible [4,5] and produces an oxidised LDL sharing many structural and functional properties with LDL oxidized by cells [3] or LDL extracted from arterial atherosclerotic plaques [6]. Moreover, pro-oxidative copper and iron ions are present in the arterial wall and plaque extracts have been shown in vitro to oxidise LDL [7].

Copper-mediated LDL oxidation begins first with a lag phase during which protective endogenous anti-oxidants are consumed by initiating free radical species. After the consumption of all endogenous anti-oxidants, a lipid radical-propagated peroxidation chain reaction begins in which the polyunsaturated fatty acids contained in the LDL are rapidly oxidised to lipid hydroperoxides.

Although a considerable amount of work has been performed on this model of LDL oxidation little progress has been made in understanding the mechanism by which copper ions (Cu^{2+}) actually lead to the formation of lipid radicals. Copper ions are known to bind readily to various sites within proteins, usually forming co-ordination complexes with a histidine residue(s) [8,10]. Even though this bound copper is in close proximity to oxidisable amino acid residues, little or no reaction will occur in most proteins without the presence of hydrogen peroxide [9,10]. This is different to the situation with copper-mediated oxidation of liposomes and erythrocyte membranes where peroxidation can occur in the absence of hydrogen peroxide [11]. The oxidising species in this reaction appears to be a copper–oxygen complex as the addition of various free radical scavengers had little or no effect of the rate of peroxidation. In Hams F10, LDL oxidation by macrophages has been shown to require the presence of a reducing agent such as cysteine [12]. Therefore the reduction of copper(II) to copper(I) may be an important rate-limiting step in the initiation of LDL oxidation. Alternatively the formation of lipid peroxyl and alkoxyl radicals by copper-mediated decomposition of trace amounts of lipid hydroperoxide on the LDL has been implicated in peroxidation initiation [13].

Although the exact identity of the radical species involved in copper-mediated LDL oxidation is still undetermined, the rate of their formation should depend on the copper concentration and, hence, copper concentration should affect the length of the lag-phase and the rate of propagation. Previous studies from our laboratory [5,14] and others [4] have shown that lag decreases with increasing copper concentration. We show here that this relationship can be quantitatively described by an equa-

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Abbreviations: EDTA, ethylenedinitrilo tetraacetic acid disodium salt; LDL, low density lipoprotein; PBS, phosphate buffered saline.

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tion, which has the same functional form as the Michaelis–Menten equation for enzyme kinetics.

2. Materials and method

Reagents used were of AR grade or better and obtained from Merck, Germany, or Sigma, USA. All solutions were prepared from ion-exchanged laboratory water filtered through a Millipore Norganic cartridge.

EDTA-plasma was prepared from blood drawn from healthy male and female donors (age 25–35 years). The EDTA-plasma was either used immediately to prepare LDL or combined to make a plasma pool using plasma prepared from 10 blood donors. The plasma preparations were frozen at −80°C in 0.6% sucrose for up to 1 month.

LDL was prepared by ultracentrifugation using a single-step discontinuous gradient in a Beckman NVT65 rotor. 9 ml of 1 mg/ml EDTA solution pH 7.4 was placed in 14 ml Beckman Quick-Seal centrifuge tubes. Using a long needle this solution was underlaid with dense plasma solution. The dense plasma solution was prepared by dissolving 1.264 g of solid potassium bromide in 4 ml of defrosted EDTA-plasma. The centrifuge tubes were ultracentrifuged at 60,000 rpm for 2 h in a Beckman L-70M ultracentrifuge with acceleration and deceleration settings of nine. The LDL band was removed through the side of the centrifuge tube with a syringe needle and stored under argon gas in the dark at 4°C for a maximum of 2 weeks.

The LDL concentration was determined by enzymatic cholesterol determination using the Chol MPR 2 kit supplied by Boehringer-Mannheim. The LDL concentration is expressed as μM, assuming an LDL molecular weight of 2.5 MDa and with a cholesterol content of 31.6%.

The LDL was desalted into phosphate-buffered saline (PBS; 160 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.4, de-oxygenated by bubbling with argon gas) by gel-filtration in Econo columns supplied by Bio-Rad. The PBS solution had been previously stirred with Chelex-100 resin supplied by Bio-Rad to remove contaminating transition metal ions usually contained in sodium phosphate.

LDL at a concentration of 0.1 μM in PBS equal to 79 μg cholesterol/ml or 0.25 mg LDL total mass/ml, or 50 μg protein/ml, was oxidised with varying concentrations of copper sulphate at 30°C. The oxidation was monitored continuously by measuring the increase in absorbance at 234 nm due to conjugated diene formation as previously described [15].

Linear regression analysis was performed using the Linest function of the Microsoft Excel spread sheet program.

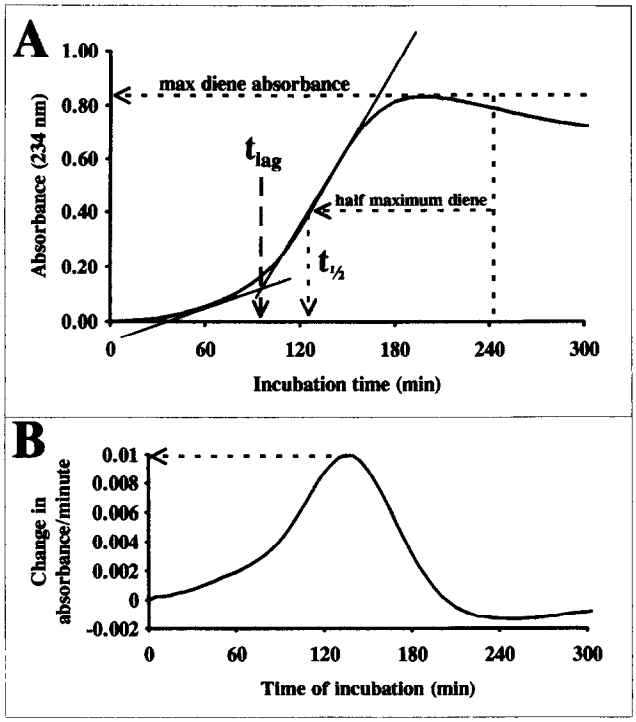

Fig. 1. Continuous monitoring of Cu^{2+}-mediated oxidation of LDL by diene absorption. 0.1 μM LDL in PBS was supplemented with 1.67 μM $CuSO_4$; the 234 nm absorption was recorded in intervals of 5 min in 1 cm cuvettes at 30°C. The absorption data were stored on a personal computer and then used to graph the diene curve (A) by subtracting from all data the initial absorbance using the Microsoft Excel spread sheet program. (B) The first derivative, i.e. change of rates of oxidation as a function of time, which was also calculated and plotted using the spread sheet program. The data has been smoothed by performing a moving average calculation (average change in absorption over 30 min) to reduce the noise in the plot. The value of t_{lag} was determined graphically as indicated; the values of $t_{1/2}$, v and $diene_{max}$ were computed by the Excel program. The values for the four oxidation parameters deduced from the experiment shown are as follows: t_{lag} = 95 min, $t_{1/2}$ = 130 min, v = 0.33 $\mu M \cdot min^{-1}$, $diene_{max}$ = 28 μM.

3. Results

As previously described [15] the kinetics of LDL oxidation can readily be followed by measurement of the increase of the 234 nm diene absorption; this absorption is due to the formation of lipid peroxides containing conjugated CC double bonds. Fig. 1A shows a typical experiment performed under standardized conditions with 0.1 μM LDL (equal to 50 μg LDL protein/ml) in PBS and 1.6 μM Cu^{2+}.

There are several parameters additional to the lag-time which can be obtained from such diene vs. time profiles. The value of the lag-time (t_{lag}) is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. The other parameters are the time required for reaching half maximum dienes ($t_{1/2}$), and the maximum velocity (v) of lipid peroxidation given by the peak of the first derivative, i.e. change of A_{234} as a function of time (Fig. 1B). For example change in absorbance of 0.01 per min corresponds to a velocity of diene production of 0.3389 μM/min. Finally, the maximum increase of the 234 nm absorption

Table 1
Comparison of LDL isolated from a plasma pool with and without storage at −80°C

	LDL from fresh plasma[a]	LDL from plasma stored at −80°C[b]
t_{lag} (min)	91 ± 2	95 ± 8
$t_{1/2}$ (min)	117 ± 3	122 ± 10
v ($nM \cdot min^{-1}$)	337 ± 8	362 ± 30
$diene_{max}$ (μM)	25 ± 0.6	28 ± 2

Data were obtained under the conditions shown in Fig. 1. The values are the mean ± S.D.

[a] Values obtained from three oxidation assays performed in parallel.

[b] Values obtained from two separate experiments performed 1 week apart from the same LDL preparation which had been prepared from plasma stored at −80°C for 15 days.

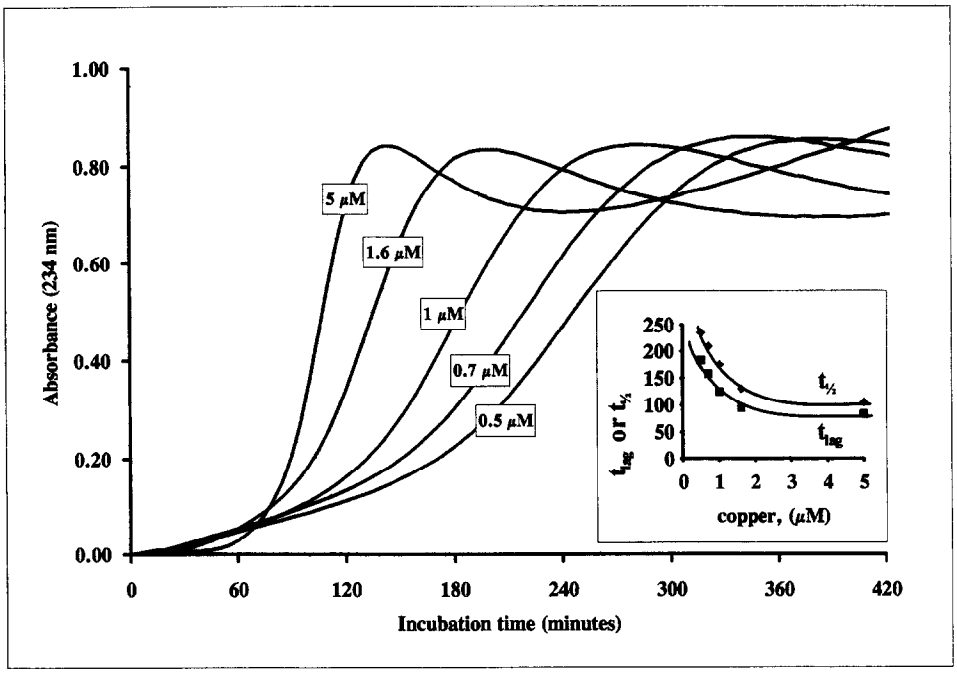

Fig. 2. Effect of copper concentration on kinetics of oxidation of LDL. The experiment was performed as described in Fig. 1. The LDL (0.1 μM) used was prepared from the pool of frozen plasma. Copper concentrations were 0.5, 0.7, 1.0, 1.67 and 5 μM. The diene curves were measured simultaneously in a Shimadzu UV1202 instrument with an auto-cell holder for six cuvettes. The insert shows the change of t_{lag} and $t_{1/2}$ with copper concentration.

gives the peak diene concentration ($diene_{max}$); an absorption increase of 1.0 corresponds to 33.9 μM dienes. The conversion of absorption into concentrations is based on a molar absorptivity of ε 29,500.

For the subsequent set of experiments it was important to have, over a longer period of time, an LDL sample with constant oxidation properties at hand. We therefore prepared a plasma pool from 10 donors. An aliquot was used immediately to prepare LDL and to measure the oxidation parameters. The remaining plasma was supplemented with 0.6% sucrose and stored in 32 ml batches at −80°C for up to 1 month prior to isolation of LDL.

As can be seen from Table 1, LDL prepared from the frozen plasma gave values for the oxidation parameters that were within experimental error of those obtained with LDL prepared from fresh plasma. This indicates that a sucrose-supplemented plasma frozen at −80°C can be used as a source of LDL with reproducible oxidation properties.

The investigation of the effect of copper concentration on the kinetics of LDL oxidation was carried out by measuring the diene vs. time profiles at a fixed LDL concentration of 0.1 μM and a copper concentration ranging from 0.5 to 5 μM. Fig. 2 shows the diene vs. time profiles for 0.5, 0.7, 1.0, 1.6 and 5 μM copper for an LDL isolated from frozen pooled plasma (15 days at −80°C).

It can be seen that an increase in copper concentration caused a significant decrease in t_{lag} and $t_{1/2}$, whereas the velocity, v, of propagation significantly increased. The maximum diene concentration, however, remained virtually constant. A plot of t_{lag} and $t_{1/2}$ against copper concentration shows that both tend towards a plateau of a minimum (Fig. 2, insert); on the other hand the velocity of propagation (v) approaches a maximum value with increasing copper concentration (Fig. 3). This suggests that some radical-generating property of the system can be saturated by copper ions. This relationship is clearly emphasized by the hyperbolic function obtained by replotting the data as $1/t_{lag}$ or $1/t_{1/2}$ against copper concentration (Fig. 4, insert). These hyperbolas can be mathematically described by the Eq. 1, where t is t_{lag} or $t_{1/2}$, respectively, and t_{min} is the associated value for the minimum t_{lag}, or minimum $t_{1/2}$. The hyperbolas function has the same mathematical form as the Michaelis–Menten equation.

$$\frac{1}{t} = \frac{1}{t_{min}} \cdot \frac{[Cu^{2+}]}{K + [Cu^{2+}]} \quad (1)$$

This can be demonstrated by the fact that a strict linear equation (corresponding to the Lineweaver–Burk plot) is obtained if data are plotted in a double reciprocal manner, i.e. t_{lag} or $t_{1/2}$ vs. 1/Cu (Fig. 4), which can be described by Eqs 2a and 2b.

$$t_{lag} = K \cdot t_{lag\,min} \cdot \frac{1}{[Cu^{2+}]} + t_{lag\,min} \text{ (for } t_{lag} \text{ determination)} \quad (2a)$$

$$t_{lag} = K \cdot t_{1/2\,min} \cdot \frac{1}{[Cu^{2+}]} + t_{1/2\,min} \text{ (for } t_{1/2} \text{ determination)} \quad (2b)$$

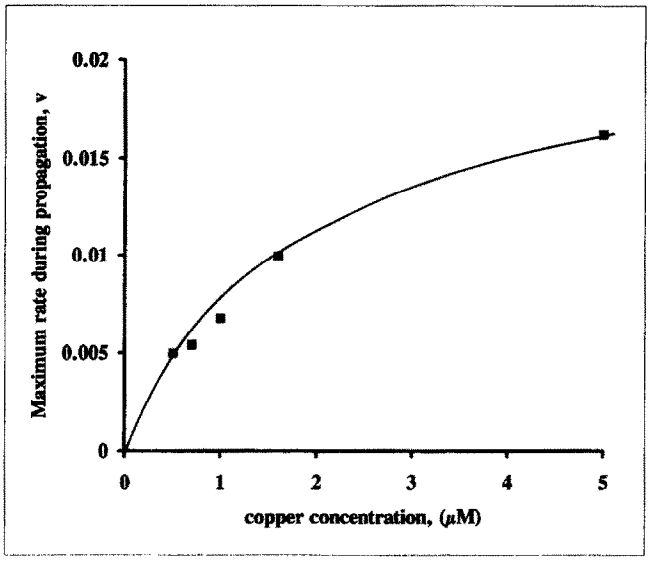

Fig. 3. A plot of the maximum propagation rate, v, vs. copper concentration demonstrating that the rate asymptotically approaches a maximum. Data points were obtained by calculating the first derivative of the data shown in Fig. 2.

This mathematical relationship allows the description of an LDL preparation's t_{lag} and $t_{1/2}$ parameters at any copper concentration in terms of the two parameters ($t_{lag\ min}$, $t_{1/2\ min}$), given by the y-intercept, and the constant, K, given by the negative reciprocal of the x-axis intercept. The values, $t_{lag\ min}$ and $t_{1/2\ min}$ are the minimum t_{lag} and $t_{1/2}$ values theoretically possible for a particular LDL oxidation at an infinite copper concentration, and the value of K gives the copper concentration in μM producing a t_{lag} and $t_{1/2}$ of twice the minimum values. Linear regression analysis showed a very high correlation ($r^2 = 0.99$) between the experimental data points shown in Fig. 4. The plot of t_{lag} gave a K value of 0.9 μM and a $t_{lag\ min}$ value of 66 min. The plot of $t_{1/2}$ gave a K value of 0.84 μM and a $t_{1/2\ min}$ value of 90 min.

Virtually the same values for $t_{lag\ min}$ and $t_{1/2\ min}$ (coefficient of variation < 5%) were obtained when the experiments were repeated 5 days later with the same LDL preparation. We have found, however, that the K value is easily affected by any agents which affect the amount of available copper in the oxidation incubation. Serum albumin contaimination of LDL preparations, inaccurate pipetting or poor mixing of the oxidation mixture can cause variation in the values obtained for the K constant.

To investigate whether K and the minimum value of t_{lag} and $t_{1/2}$ vary between different LDL samples, two other LDL preparations from individual donors (donors A, B) were examined for this relationship between copper concentration and kinetics of oxidation. Both LDL preparations showed a strong correlation ($r^2 > 0.99$) between t_{lag} and the reciprocal of the copper concentration (Fig. 5a,b); again the t_{lag} plot gave a similar x-intercept as the $t_{1/2}$ plot. From these plots the values of K and the minimum values of t_{lag} and $t_{1/2}$ were obtained (Table 2). The comparison of the three investigated LDL samples (pooled plasma, donor A, donor B) shows significant differences; the value of K ranged from 0.5 to 0.9 μM, $t_{lag\ min}$ ranged from 52 to 81 min and $t_{1/2\ min}$ ranged from 62 to 99 min. It appears that $t_{lag\ min}$ and $t_{1/2\ min}$ and K vary independently of each other. Re-examination of donor A showed that LDL prepared from blood drawn 3

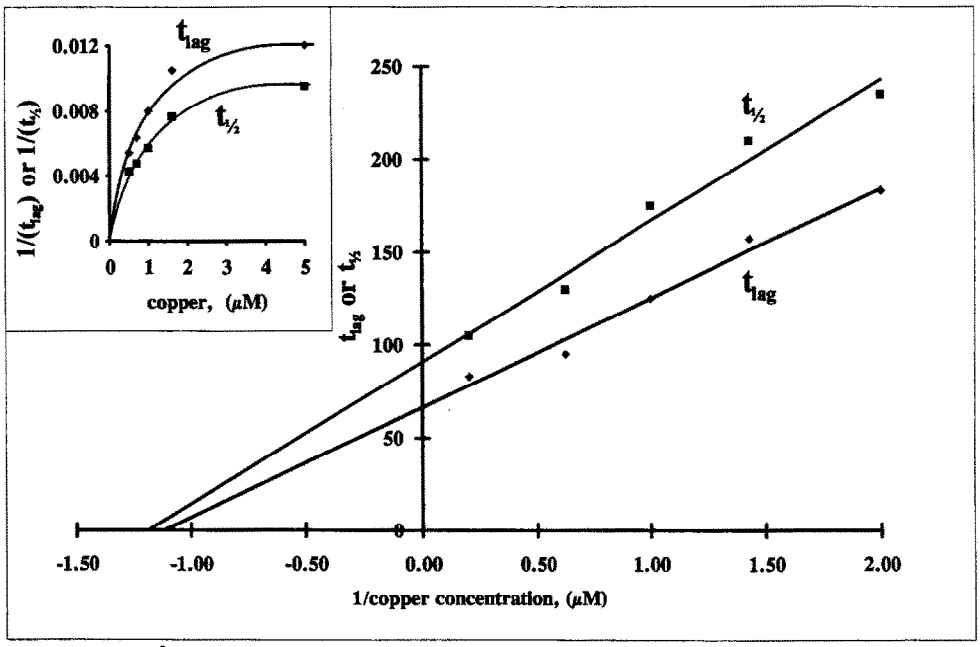

Fig. 4. A plot of t_{lag} and $t_{1/2}$ vs. $1/[Cu^{2+}]$ illustrating the graphical determination of the constant, K (= negative reciprocal of the x-intercept) and the minimum values of t_{lag} and $t_{1/2}$ (y-intercepts). The insert shows the hyperbolic function of the plots, $1/t_{lag}$ and $1/t_{1/2}$, vs. Cu^{2+}. Data points were obtained from Fig. 2.

months after the preparation shown in Fig. 5a had the same minimum value of t_{lag} and $t_{1/2}$ (52 min, 62 min, respectively) but the value of K had changed from 0.5 to 1.9 μM and 2.5 μM for t_{lag} and t_{min}, respectively. This suggests that $t_{lag\,min}$ and $t_{1/2\,min}$ are donor-specific values, whereas the quantity of K varies and is perhaps influenced by the diet or life style.

4. Discussion

Oxidation of LDL by Cu^{2+} ions is frequently used to assess effects of anti-oxidants and other nutritional factors on resistance of LDL to oxidative stress. The chemical mechanism of initiation of lipid peroxidation as a result of copper ion addition to an LDL solution is largely unknown. This is the first detailed investigation on the inter-relationship between copper concentration and kinetics of LDL oxidation as measured by the diene vs. time profile (Fig. 1). Our studies demonstrate that LDL has a finite number of pro-oxidative copper binding sites. Saturation of these sites by copper results in a limiting value for several oxidation parameters (t_{lag}, $t_{1/2}$, v_{max}; Figs. 2, 3 and 4).

A characteristic index of LDL oxidation is the lag time (t_{lag}), which is precedes the period of rapid lipid peroxidation termed the propagation phase. During the lag-time, lipid peroxidation is inhibited by anti-oxidants contained in LDL, but once the anti-oxidants are consumed the lipid peroxidation proceeds via a lipid radical-propagating chain reaction. As has been shown in other studies [16,17], the length of the lag-time is directly proportional to the initial anti-oxidant content and inversely proportional to the rate of initiation, R_i, by which free radicals, X$^\bullet$, initiate lipid peroxidation according to Eq. 3, where LH is an oxidizable polyunsaturated fatty acid;

$$LH + X^\bullet \xrightarrow{R_i} L^\bullet + XH; \quad t_{lag} = \frac{n\,[\text{anti-oxidant}]}{R_i} \qquad (3)$$

In our experiments on copper dependence of t_{lag}, the initial anti-oxidants, as well as the stoichiometric factor, n (i.e. number of peroxyl radicals scavenged by each molecule of anti-oxidant) can be regarded as invariable for a particular LDL and, hence, t_{lag} is a measure of the rate of initiation, R_i. To understand these findings it is appropriate to bring Eq. 1 into a new form by substituting $1/t$ and $1/t_{min}$ by R_i and $R_{i\,max}$, respectively.

$$R_i = R_{i\,max} \cdot \frac{[Cu^{2+}]}{K + [Cu^{2+}]} \qquad (4)$$

This new equation has the same (operational) form as the Michaelis–Menten equation and indicates that the rate of initiation, R_i, is saturable. The equation immediately explains that constant K gives the quantity of the copper concentration, at which half of the pro-oxidative copper binding sites are occupied, and therefore the rate of initiation is half the maximum. The maximal rate of initiation, $R_{i\,max}$, occurs when the pro-oxidative copper binding sites are entirely saturated. The K values determined in this study for the different LDL samples (Table 2 and text) ranged from 0.5 to 1.9 μM. With the LDL concentration used in experiments (i.e. 0.1 μM) these values indicate that the rate of initiation is half-maximal, with 5–19 mol copper ions per mol LDL. These values are close to the number of strong copper binding sites found by Kuzuya et al. [18] by dialysis experiments; an LDL sample incubated with $CuSO_4$ in a molar ratio of 1 : 6 and 1 : 33 contained, after dialysis, 3.5 and 9.5 mol copper per mol LDL, with more than 80% bound to apo B.

Polarographic studies [19] and electron spin resonance measurements [20] have also shown that LDL can form complexes with copper. It is reasonable to assume that only a subset of the bound copper is in a redox-active state and capable of producing initiating free radicals.

The nature of these binding sites and the reason for their variation is presently not clear. Mechanistically, we hypothesize that they are located in close approximation to reducing components, thus Cu^{2+} can be reductively activated by a net transfer of one electron ($Cu^{2+} + e \rightarrow Cu^+$). Cuprous ion ($Cu^+$) is a strong pro-oxidant, which probably rapidly forms the ultimate initiating radical, X$^\bullet$, capable of abstracting a hydrogen atom from a polyunsaturated fatty acid. The finding that nearly identical K values were obtained, irrespective of whether t_{lag} or $t_{1/2}$ was plotted (Figs. 4 and 5), strongly suggests that the rate of initiation, R_i, remains constant, at least until

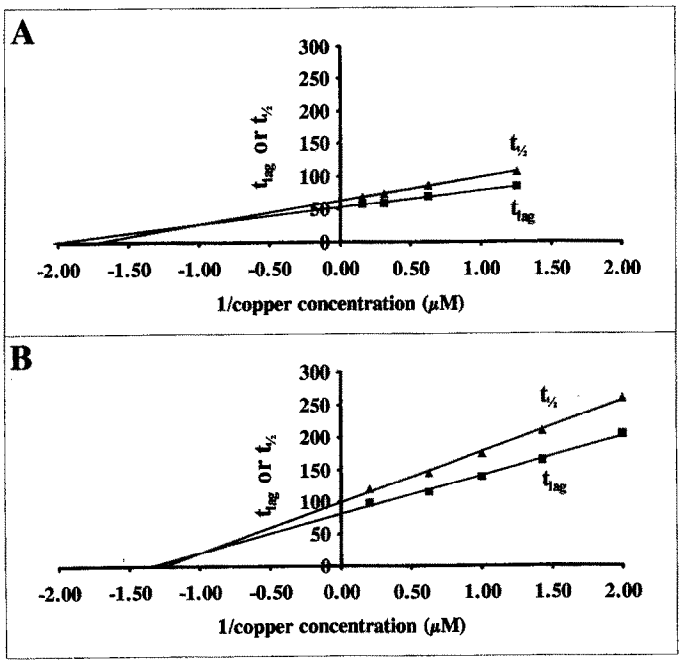

Fig. 5. Examination of copper-dependent oxidation of LDL from two individual donors. The values of t_{lag} and $t_{1/2}$ were determined by measuring the dienes vs. time curves at different copper concentrations as shown in Fig. 2. The graph shows the plots of t_{lag} and $t_{1/2}$ vs. $1/Cu^{2+}$ for donor A (A) and donor B (B). The quantities for K and the minimum values of t_{lag} and $t_{1/2}$ are given in Table 2.

oxidation of LDL, and, as measured by the diene profile, is half-maximal. This shows indirectly that chain branching caused by decomposition of lipid hydroperoxides to initiating radicals (e.g. $LOOH + Cu^{2+} \rightarrow LOO^{\bullet} + H^{+} + Cu^{+}$) is negligible.

Eq. 3 implicity shows that the quantity of the minimum lag-time, which is obtained by extrapolation, as shown in the plots of Figs. 4 and 5, is inversely related to the maximum rate of initiation, $R_{i\,max}$. A conversion of the numerical values of t_{lag} into the numerical values of R_i affords knowledge of the concentration of chain breaking anti-oxidants and their stoichiometric factors. On a molar basis the main anti-oxidant in LDL is vitamin E. Although many other LDL compounds (e.g. carotenoids, ubiquinol, plasmalogens) may have anti-oxidant activity and thereby contribute to the lag-time, we assume here for the sake of simplicity that vitamin E is quantitatively the most important one. The vitamin E content of LDL is on average 7 mol per mol LDL, therefore, an LDL concentration of 0.1 μM as used in this study equals 0.7 μM vitamin E. For vitamin E the values of n is 2 since both vitamin E and vitamin E radical (tocopheroxyl radical) trap $LOO^{\bullet}$ and thereby interrupt the propagating lipid peroxidation chain. A simple calculation, based on Eq. 3 and $t_{lag\,min}$ values of Table 2 gives a maximum rate of initiation, $R_{i\,max}$ of 21.2, 26.9 and 17.2 $nM \cdot min^{-1}$ for pooled LDL, donor A and donor B, respectively. As with the turnover number in enzyme kinetics, the value of $R_{i\,max}/[LDL] = k_{cat}$ provides a measure of the efficiency of LDL in producing initiating radicals. The quantity of k_{cat} gives the number of initiating radicals produced by each LDL particle within every minute. The k_{cat} values obtained from the three sets of experiments shown in Table 2 are 0.212, 0.269 and 0.172 min^{-1}, or, in other words, at copper saturation one initiating radical can be generated every 4.7, 3.7 and 5.8 min per LDL particle. This calculation brings to light an important kinetic parameter. The rate of initiation in copper-mediated oxidation is not at all a fast process, on the contrary, it is a rather slow process with an upper limit of about 1 hit per LDL particle every 5 min.

Further examination of the hyperbolic function of R_i given in Eq. 4 shows that the rate of initiation only slightly changes when $[Cu^{2+}] \geq K$. For example, it can be calculated for the LDL oxidation experiment shown in Fig. 2, where K was 0.9 μM, that copper concentrations of 5, 1.6, 1.0, 0.7 and 0.5 μM produced one radical hit every 5.8, 6.8, 8.9, 10.9 and 12.8 min, compared to the upper limit of 1 hit every 4.7 min.

Table 2
Comparison of $t_{lag\,min}$, $t_{1/2\,min}$ and K constant values for pooled and Donor A and B LDL preparations

	Pooled	Donor A	Donor B
$t_{lag\,min}$ (min)	66	52	81
$t_{1/2\,min}$ (min)	90	62	99
K based on t_{lag} (μM)	0.89	0.48	0.74
K based on $t_{1/2}$ (μM)	0.85	0.55	0.79

Numerous recently published studies have employed lag-time measurements at a single copper concentration to correlate oxidation resistance of LDL with other parameters, such as severity of myocardial infarction [21], predominance of more dense LDL subclasses [22,23], anti-oxidant status [16], composition of dietary fats, [24] and various diseases [25]. Our findings clearly show that a single lag-time measurement may be misleading since a high oxidation resistance of a particular LDL can result either from a high constant K or a low radical-generating capacity of the bound copper ($R_{i\,max}$). To fully characterize the resistance of LDL to copper oxidation the quantity of both these parameters is needed.

We would finally like to point out that the relationship described here between copper and LDL lag-time may only apply for radical fluxes in the range of one radical hit per LDL particle every 3–30 min. It would be unwise to extrapolate our findings directly to much lower radical fluxes where other determinants of LDL oxidation (e.g. initiation of lipid peroxidation by peroxide decomposition products [13] or by tocopheroxyl radicals [26]) may become significant. An additional problem is that experiments below a certain rate of initiation ($Cu \leq 0.2$ μM) became increasingly prone to error, e.g. due to copper binding by traces of contaminants such as albumin or EDTA.

In conclusion, the importance of the findings described here is that they show for the first time the existence of a finite number of copper binding sites in LDL which can initiate lipid peroxidation, and that this number can vary considerably between different LDL preparations.

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