ORIGINAL ARTICLE

KINETIC STUDY OF LOW DENSITY LIPOPROTEIN OXIDATION BY COPPER

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ABSTRACT

Oxidation of Low Density Lipoprotein (LDL) is regarded as a key event in the development of atherosclerosis. The aim of this study was to investigate effect of various copper concentrations on LDL oxidation kinetic profile as a mechanism in atherosclerosis process. LDL was isolated from plasma and its oxidation with copper was investigated by monitoring the formation of conjugated dienes. Based on time course of the formation of conjugated diene was observed at concentrations of 0.5 to 10 μ M copper, represented the conventional kinetics of LDL oxidation with an inhibition period followed by a propagation phase. In contrast, at concentrations of 20 to 50 μ M copper, LDL oxidation proceeded after a negligibly short lag-time followed by a distinct propagation phase. At lower copper concentrations of about 0.5 μ M, LDL oxidation can be combined in 4 consecutive oxidation phase. The increasing copper concentration (to 10 μ M) lowered the first propagation and shortened the second inhibition period until they melted into one apparent kinetic phase. But in copper concentrations of about 20 to 50 μ M, increasing copper concentration increased the first propagation and the second inhibition but lowered the second propagation phase. The results of this investigation on the copper dependence of the oxidation kinetics suggest that LDL contains two different copper binding sites. Copper bound to the low affinity binding sites with molar ratio of 200 to 500 of copper / LDL. These ions bound to the high affinity binding sites with molar ratio of copper / LDL of 5 to 100.

KEY WORDS

Kinetic profile, LDL oxidation, Atherosclerosis, Copper.

INTRODUCTION

The susceptibility of low density lipoprotein (LDL) to oxidation is believed to be an important factor in the process responsible for foam cell formation and atherosclerosis (1). The common evaluation methods of LDL susceptibility to oxidation *in vitro* are based on LDL oxidation induced either by transition metals (usually copper) or by organic free radical generators (2-3). Although the relevance of oxidation induced by either of these "prooxidants" to *in vivo* oxidation is questionable, the simplicity and reproducibility of the copper induced oxidation assay make it attractive for both routine and research assays (4). 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 oxidized to lipid hydroperoxides (5). Copper is redox active metal that can participate in electron transfer reactions with the consequent production of oxidant species capable of oxidizing cell components. Copper can catalyze the formation of the highly reactive hydroxyl radicals from hydrogen peroxide via the Haber-Weiss reaction and decompose lipid peroxides to peroxy and alkoxy radicals, which favor the propagation of lipid peroxidation (6). Alternatively the formation of lipid peroxy and alkoxy radicals by copper mediated decomposition of trace amounts of lipid hydroperoxide on the LDL has been implicated in peroxidation inhibition. Although considerable amounts of work has been performed on this model of LDL oxidation, but the mechanism by which copper initiates

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oxidation in LDL is not fully clear. Therefore, in this study we examined interaction of copper with LDL using spectrophotometric titration and also investigated kinetic of LDL oxidation by various range of copper concentrations.

MATERIALS AND METHODS

The reagents were obtained from Sigma (St. Louis, Mo, USA) or Merck (Damstadt, Germany). All reagents were freshly prepared and copper solutions were diluted from a freshly prepared 10 mM stock solution. Phosphate buffered saline (PBS) was 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride. The PBS solution had been stirred with Chelex-100 resin supplied by Bio-Rad to remove contaminating transition metal ions usually contained in sodium phosphate. Deionized water was used throughout this study for washing and preparing the solutions to minimize metal contamination. Laboratory glassware were soaked overnight in 10% HNO₃ and then thoroughly rinsed with distilled and deionized water.

Blood samples (~30 ml) were obtained by venipuncture from six men healthy volunteers (age 25 – 30 years) fasted overnight and collected in glass tubes containing 1 g/L of EDTA. After 10 minutes at room temperature, plasma was separated by centrifugation at 3000 rpm for 20 minutes at 20°C. For preparation of pooled LDL, plasma from subjects was mixed. LDL ($d = 1.019$ to 1.063) were isolated by single step discontinuous gradient ultracentrifugation at 4°C using potassium bromide (KBr) as density adjustment reagent (7). Samples were ultracentrifuged in a Damon B-60 ultracentrifuge using a 70.1 rotor at 60,000 rpm (400,000 g) for 3 hours. LDL, which appeared as a yellow layer in the center of the tube, was removed with syringe needle. LDL containing fraction was dialyzed in the dark for 24 hours at 4°C against 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 100 μ M EDTA. The protein concentration of LDL was measured by the method of Lowry using bovine serum albumin as a standard (8). Purity of LDL preparation was checked by agarose gel electrophoresis on 0.8% gel (9). Isolated LDL was kept in darkness under nitrogen at 4°C and used within 2 to 3 weeks. Just before experiments, LDL samples were further dialyzed twice for 16 hours against the phosphate buffer described above but without EDTA, to remove EDTA. In this study the LDL concentration is expressed as μ M, assuming an apo-B₁₀₀ molecular weight of 513 KD (10).

Interaction of copper with LDL was carried out using spectrophotometric titration technique (11). Briefly, 50 μ l LDL at a concentration of 0.1 μ M in PBS equal to 50 μ g protein /

ml was added to eight pre-acid washed test tubes. Then 200 μ l copper sulphate (CuSO₄) with various concentration of copper (0 to 100 μ M) was added to the series of tubes and volumes were made up 1 ml with PBS, pH 7.4, without EDTA. The solutions were mixed vigorously and left at 37°C for up to 3 hours. The absorption spectrum of the samples at 200 to 300 nm was recorded using a Perkin-Elmer, Lambda 2, UV spectrophotometer with intervals of 10 nm.

LDL oxidation kinetics was monitored by following the conjugated diene (CD) formation (12). Briefly, 50 μ l LDL in PBS and 200 μ l CuSO₄ were pipetted into the tubes and volumes were made up 1 ml with PBS, without EDTA. Concentration of LDL was 0.1 μ M, final concentrations of copper ranged from 0.5 to 50 μ M, giving a molar ratio of copper / LDL of 5 to 500. Recording of CD formation at 234 nm was started immediately after the addition of CuSO₄ with intervals of 5 minutes up to 450 minutes. The temporal change in absorbance at 234 nm is divided into three phases, a lag phase, a propagation phase and a decomposition phase. Concentrations of CD were calculated using $\epsilon_{234} = 29500 \text{ M}^{-1} \text{ cm}^{-1}$ (10). In this study the oxidation indices (R_1 , R_2 , CD_{max} , t_{lag} and CD_{lag}) were also determined (13).

RESULTS AND DISCUSSION

The oxidation of LDL has been widely studied in order to understand better the role of LDL oxidation *in vivo* in pathological situation, such as atherogenesis (1). For this purpose, *in vitro* models have been developed including the oxidation of LDL by copper (14). However, the exact molecular mechanisms by which copper initiates oxidation in LDL are

Fig 1: Electrophoresis analysis of LDL fraction (1, 2) and plasma (3, 4, 5) on 0.8% agarose gel. LDL=Low density lipoprotein, VLDL=Very low density lipoprotein, HDL=High density lipoprotein

not yet clearly established. Therefore, we used spectrophotometry technique for investigate the copper interaction to LDL.

In this study isolation of LDL from plasma was confirmed by agarose gel electrophoresis (Fig 1). This Figure shows that band seen for fraction separated from plasma (Fig 1, Lanes 1 and 2) is corresponding to LDL band in plasma, as control (Fig 1, Lanes 3, 4 and 5).

Study of absorption spectrum of concentrations 0.01 μM to 0.16 μM of LDL (equal to 5 to 80 μg protein / ml) was showed in Figure 2. LDL absorption spectrum has one peak at 208 nm (Fig 2A), that is dose dependent (Fig 2B). The absorption spectrum of 0.1 μM LDL in presence of 0.5 μM to 100 μM

Fig 2: (A) Absorption spectrum of LDL at 200 to 300 nm. (B) Absorbance of various concentration of LDL (0.01 – 0.16 μM) at 208 nm

Fig 3: (A) Absorption spectrum of LDL (0.1 μM) at presence of different copper concentrations (0.5 – 100 μM). (B) Spectrophotometric titration of LDL (0.1 μM) with different copper concentrations (0.5 – 100 μM) at 237 nm

copper also showed two peaks at 208 and 237 nm (Fig 3A and Fig 3B). Thus absorption spectra obtained from LDL and copper-LDL complex indicated two maximum of absorbance at 208 nm and 237 nm, respectively. According to the previous reports, the maximum absorbance for copper-LDL complex is 234 nm (12), which is comparable to our findings.

The oxidation kinetics followed by continuously recording the formation of conjugated diene (CD) at 234 nm. Figure 4A shows how different copper concentrations (0.5 – 50 μM) affect the pattern of the CD curves for LDL preparation. The oxidation with copper concentrations of 0.5, 2.5 and 10 (Fig 4B, Fig 4C and Fig 4D) gave the well know kinetic profile with an initial inhibited period followed by a period of rapid oxidation. In this

Fig 4: Effect of different copper concentration (0.5 – 50 μM) on oxidation kinetics of 0.1 μM LDL (A); The oxidation kinetics of LDL (0.1 μM) by copper concentrations of 0.5 μM (B); 2.5 μM (C); 10 μM (D); 20 μM (E) and 50 μM (F)

study, these two periods were termed as lag-phase and propagation phase. The oxidation kinetics changed markedly at copper concentrations of 20 and 50 μM (Fig 4E and Fig 4F). This oxidation profile showed a negligibly short period of inhibition (lag-phase) followed by rapid oxidation (propagation phase). Low copper concentration (for example 10 μM copper) produced the more conventional oxidation profile demonstrated by a symmetrical increase and decrease in rates during propagation phase (Fig 5A). In contrast, high copper concentration (for example 50 μM copper) displayed the characteristic asymmetric rate profile (Fig 5B). Copper concentrations of lower than 10 μM (0.5 and 2.5 μM copper), conjugated diene profile displaying two propagation phases (Fig 4B and Fig 4C). To better visualizing these differences, the 0.1 μM LDL oxidation curve in presence 0.5 μM copper is plotted as a dashed line (Fig 6). Figure 6 shows a very short inhibition period, which was followed by a propagation phase. In such curves, denote phases as first inhibition, first propagation, second inhibition and second propagation, respectively. Note that lag-time at these oxidations refers to the end of the second inhibition period and the onset of the second propagation, respectively (Fig 6A). At low copper concentrations (0.5, 2.5 and 10 μM copper), oxidation proceeds during the second propagation phase exhibiting a peak maximum R_2 (R_2 is the maximal rate during the second propagation phase) as shown in Fig 7, curves of A to C. This Figure shows at lower copper concentrations, maximal rate during the first propagation phase (R_1) began slowly to decline again. As R_1 become zero at 10 μM copper. In contrast, increasing copper concentration leads to a decrease in R_2 and to an increase in R_1 (Fig 7D and Fig 7E). In agreement with many previous studies (5, 10, 15), we found that at low

Fig 5: The first derivative (change of rates of oxidation as a function of time) conjugated dienes formation kinetics mediated by 10 μM copper (A) and 50 μM copper (B). These Figures were obtained by Fig. 4D and Fig. 4F data's, respectively

Fig 6: Overview on principles for determining the oxidation indices. (A part) Shows a representative diene curve for oxidation of $0.1 \mu\text{M}$ LDL in presence of $0.5 \mu\text{M}$ copper with four consecutive time periods. The concentration of conjugated dienes (CD) at the peak is CD_{max} , the concentration of CD at the end of lag-time is CD_{lag} . Note that lag-time at this oxidations refers to the end of second inhibition period and the onset of second propagation. (B part) Shows associated rate curve of diene formation. R_1 and R_2 are the maximal rates during the first and second propagation, respectively.

Fig 7: Concentrations effect of $0.5 \mu\text{M}$ (A), $2.5 \mu\text{M}$ (B), $10 \mu\text{M}$ (C), $20 \mu\text{M}$ (D) and $50 \mu\text{M}$ (E) copper on maximal rates during first propagation (R_1) and second propagation (R_2) of $0.1 \mu\text{M}$ LDL

Fig 8: (A) Effect of various copper concentrations (0 – 50 μM) on lag-time of 0.1 μM LDL oxidation. (B) A plot of lag-time versus 1/copper concentration, data points were obtained from Fig 8A

molar ratios of copper/LDL (at least 25), propagation of oxidation occurs during a long initial inhibited period (lag-time). The increase in conjugated diene during the propagation phase was reported to be mainly due to the formation of cholesteryl linoleate hydroperoxides (16). The rate of this type of propagation is characterized by a symmetrical profile. In contrast, at high molar ratios of copper/LDL (200 or 500 μM), LDL becomes fully oxidized after a negligibly short inhibited period and a propagation phase which shows an asymmetrical profile. The increase in conjugated diene during this propagation is associated with the formation of cholesteryl linoleate hydroperoxides and substantial amounts of cholesteryl linoleate hydroxides (3). In the range of approximately 0.5 to 10 μM copper, LDL oxidation begins with a negligibly short first inhibition phase (1st inhibition) followed by a first propagation phase (1st propagation) displaying an

asymmetrical rate of conjugated diene formation. This is followed by a long second inhibition phase (2nd inhibition) and a second propagation phase (2nd propagation), displaying a symmetrical rate. Such oxidation kinetics suggests the copper dependence for the rate of the second propagation phase (R_2). In concentration of 0.5 to 10 μM copper, R_2 increased with increasing copper concentration, as R_2 approaches a maximum at 10 μM copper. In parallel, the rate of the first propagation phase (R_1) progressively decreased in presence 0.5 to 10 μM copper. In contrast, 20 μM and 50 μM copper promote the first propagation phase (R_1). This copper mediated LDL oxidation kinetics support the hypothesis that the first and second propagation phases present two different oxidation mechanisms which are mediated by different states

Fig 9: Effect of 0.5 to 50 μM copper on the extent of 0.1 μM LDL oxidation by determine maximal conjugated diene value (CD_{max}) (A) and conjugated diene value at lag-time (CD_{lag}) (B)

of copper or copper binding sites. This finding also suggests that LDL contains low affinity binding sites responsible for the 1st propagation and high affinity binding sites responsible for the 2nd propagation. We assume that half saturation of the high affinity sites occurs in range of 5 to 100 copper/LDL, while half saturation of the low affinity binding sites requires much higher copper, in range of 200 to 500 copper/LDL. The assumption of two copper binding sites is consistent with previous investigation by Kuzuya et al (17). The rapid acceleration of the second propagation phase can be explained by the decomposition of hydroperoxides by copper ions (18). This finding indicates that the second propagation phase can be regarded as a conventional lipid peroxidation phase. The substantial formation of cholesteryl ester linoleate hydroxides during the first propagation phase may be due to the rapid formation and decomposition of hydroperoxides. The redox active copper complexes, which yield a high radical flux, may be involved in the decomposition of hydroperoxides. Approximately 80% of total copper ions are likely to be bound to the apo-B₁₀₀ protein, possibly to ligands in close vicinity to the LDL lipids (18). Aside from histidine and / or cysteine which are known to readily form copper complexes, preferred binding sites have been identified at or near tryptophan residues (19). The early oxidation of apo-B₁₀₀ tryptophan residues and the formation of kynurenine and formylkynurenine suggest a localized formation of protein radicals by copper at specific apo-B₁₀₀ complexes (20). Interestingly, the preoxidation of LDL leads to a decrease in the first propagation as initiated by intermediate copper concentration in absence of lipid hydroperoxides (21). This can be partly caused by modification of the redox properties of apo-B₁₀₀, for example, due to the loss of thiol group, as well as by depletion of α -tocopherol (22). According to results this study we suggest probably at concentration of 10 μ M copper, copper (II) ions may be bound to high affinity sites. Whereas at copper concentrations of lower and / or higher than 10 μ M, copper (II) ions are bound to high as well as to low affinity sites. At lower concentrations of 10 μ M copper (0.5 and 2.5 μ M) these ions higher are bound to high affinity sites, but at higher concentrations of 10 μ M copper (20 and 50 μ M), copper ions higher are bound to low affinity sites.

Next we investigated the relationship between copper concentration and duration of lag-time (t_{lag}). The term lag-time or t_{lag} originally refers to the inhibited period preceding the onset of the propagation phase. In oxidation curves that exhibiting two propagation phase, the term lag-time is not accurate in the classical sense but instead refers to the onset second propagation phase (Fig 6A). This study shows that increasing copper from 0.5 μ M to 50 μ M concentration led to

a rapid decline of t_{lag} from about 280 minutes to less than 10 minutes (Fig 8A). A plot of t_{lag} versus 1/copper concentration should give a straight line in which the y-intercept is the minimum lag-time. The Fig 8B shows such a plot describes the copper dependence of lag-time over a very wide copper concentration range (0.5 to 50 μ M). The strong influence of copper concentration on the lag-time clearly indicates that at high copper concentration of about 20 μ M (~200 copper/LDL) and 50 μ M (~500 copper/LDL) would have the advantage of given lag-times very close to the minimum lag-time, which can be considered as a copper independent characteristic for LDL oxidation. However, for such conditions one must accept that the effect of antioxidants becomes small (23). On the other hand, lower concentrations of copper in the range of 5 – 100 copper/LDL would give the more complex kinetic characteristics for LDL oxidation

Finally, we investigated the relationship between copper concentration and extent of oxidation. The maximal oxidation is given by CD_{max} , corresponding to the maximum value of the conjugated diene curve (as shown in Fig 6A). The proportion of LDL oxidized by copper was determined by the 234 nm absorbance ratio (Fig 9). Figure 9A demonstrates the extent of the fractional oxidation with increasing copper concentration. The copper dependence for CD_{lag} (this CD is conjugated diene concentration at the end of the lag-time) also shown in Figure 9B. This plot shows that CD_{lag} decreases to a minimal value with increasing copper concentration (Fig 9B).

Our study indicates that copper at low concentrations (≤ 10 μ M) bound to low and high affinity sites of LDL and to cause increase the susceptibility of LDL to oxidation. Therefore, this study is as a probably mechanism for copper effect on LDL oxidation and atherosclerosis formation. However, the detailed mechanisms *in vivo* deserve further investigation.

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