

Effect of Lycopene on Formation of Low Density Lipoprotein-Copper Complex in Copper Catalyzed Peroxidation of Low Density Lipoprotein, as *in vitro* Experiment

Mohammad Ali Ghaffari* and Taibeh Ghiasvand

Dept. of Biochemistry, School of Medical, Ahwaz Jondi Shapour University of Medical Sciences, Ahwaz, Iran

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ABSTRACT

Background: A great deal of evidence has indicated that oxidatively modified LDL plays a critical role in the initiation and progression of atherosclerosis. Antioxidants that can prevent LDL oxidation may act as antiatherogens. Copper is a candidate for oxidizing LDL in atherosclerotic lesions. The binding of copper ions to LDL is usually thought to be a prerequisite for LDL oxidation by copper. The aim of this study was to investigate effect of lycopene on copper bound to LDL and also effect of this binding on the susceptibility of LDL to oxidative modification. **Methods:** In this study, LDL was isolated from EDTA-plasma by ultracentrifugation using a single- step discontinuous gradient. Then lycopene was added to LDL and oxidizability of LDL was estimated by thiobarbitoric acid reactive substances (TBARS) after CuSO₄ addition. Finally, the effect of lycopene on formation of LDL-copper complex by gel filtration was studied. **Results:** Our results showed that lycopene (as dose dependently) was suppressed the formation TBARS and LDL-copper complex. The lycopene with concentrations of 10 μM, 50 μM and 100 μM was reduced susceptibility of LDL to oxidative modification approximately by 31, 67 and 71 percent, respectively. Furthermore, the addition of lycopene to the mixture containing LDL and copper before incubation was prevented the formation LDL-copper complex, approximately by 38 percent. **Conclusion:** The results of this investigation show that lycopene with inhibition of binding of copper to LDL may decrease the susceptibility of LDL oxidation to this ion and thus may have a role in ameliorating atherosclerosis. *Iran. Biomed. J. 10 (4): 191-196, 2006*

Keywords: Low density lipoprotein (LDL), Oxidation, Lycopene, Atherosclerosis, Copper

INTRODUCTION

High blood cholesterol level is a major risk factor for atherosclerosis and it is associated with high blood concentration of low density lipoprotein (LDL). Excessive LDL deposits in the arterial wall leading to a blockage of blood flow to the heart and a consequent heart attack. Epidemiological and clinical studies have shown that cholesterol lowering therapy can reverse progression of atherosclerosis and reduce coronary heart disease [1]. However, at any given plasma concentration of LDL, there is a great diversity in the extent of atherosclerosis and in the expression of clinical disease. On the other hand, continuing to reduce LDL cholesterol did not abolish atherosclerosis, suggesting that not only the quantity

but also the quality of LDL may be associated with atherosclerosis [2]. A great deal of evidence has indicated that oxidatively modified LDL plays a critical role in the initiation and progression of atherosclerosis [3].

Oxidatively modified LDL is a potent ligand for the scavenger receptors on macrophages, contributing to the formation of lipid laden foam cells [4]. While it remains to be clarified how LDL is modified *in vivo*, it is known that oxidative modification of LDL by cultured vascular cells is dependent upon media contained transition metal ions such as copper or iron [5]. The mechanism by which copper initiates LDL oxidation has been partially defined. Copper binding to and reduction by LDL are required for LDL oxidation [6]. Antioxidant compounds provide resistance to this

*Corresponding Author;

process and have been suggested to lower atherogenicity [7]. Lycopene is a carotenoid without provitamin A activity, that found almost exclusively in tomatoes and tomato products. It accounts for about 50% of carotenoids in human serum. Among the common dietary carotenoids, lycopene has the highest singlet oxygen quenching capacity *in vitro*. Considerable evidence suggests that lycopene has a significant antioxidant potential *in vitro* and may play a role in preventing pathogenesis of atherosclerosis [8]. Therefore, the goal of the present study was to determine effect of lycopene on LDL-copper complex and thus affect it on the susceptibility of LDL to oxidative modification in a cell free system.

MATERIALS AND METHODS

Lycopene, EDTA, copper sulfate and sephadex G-25 were obtained from Sigma (St. Louis, Mo, USA). Dimethyl sulfoxide (DMSO), potassium bromide, bovine serum albumin, agarose and 2-thiobarbituric acid were purchased from Merck (Darmstadt, Germany). All reagents were freshly prepared and copper solutions were diluted from a freshly prepared 10 mM stock solution.

Isolation of LDL. EDTA-plasma (1mg EDTA/ml blood) was prepared from blood (40 ml) of healthy male donor (30 years and fasting for 14 hours). LDL (density 1.019-1.063g/ml) was isolated from EDTA-plasma by ultracentrifugation using a single-step discontinuous gradient [9]. EDTA solution (4.5 ml of 1 mg/ml), pH 7.4 was placed in 10-ml centrifuge tubes. Using a long needle this solution was under laid with dense plasma solution. The dense plasma solution was prepared by dissolving 0.632 g of solid potassium bromide in 2 ml of defrosted EDTA-plasma. The centrifuge tubes were ultracentrifuged in a Damon B-60 ultracentrifuge at 40,000 ×g for 2 hours. LDL band was removed through the side of the centrifuge tube with a syringe needle. For remove traces of transition metals (especially of copper), LDL solution was dialyzed in dialysis tubing (10 mm flat width) with a molecular weight cutoff of 12 to 14 kDa at 4°C for 24 hours against PBS, pH7.4, consisting of NaCl, 140 mM; Na₂HPO₄, 8.1 mM; NaH₂PO₄, 1.9 mM and EDTA, 100 μM. Again, LDL was dialyzed at 4°C for 12 hours against the phosphate buffer described above but without EDTA to remove EDTA [10]. LDL solution was assayed for protein and lipids content. The protein

content was measured by the method of Lowry *et al.* [11], using BSA as standard. Lipid concentrations (triglyceride, total cholesterol, HDL and LDL-cholesterol) were measured using commercially available Kits (Pars Azmon Kits, Iran). The purified LDL was examined by electrophoresis carried out at pH 8.6 in 0.05 M barbital buffer on 0.8% agarose gel. Gels were stained with Sudan Black B Stain [12]. LDL was sterilized by filtration (0.45 μm Millipore filter), stored in darkness under nitrogen gas at 4°C and was used within 4 weeks.

Oxidation of LDL and assay for products of lipid peroxidation. For preparation of oxidized LDL, LDL was incubated with CuSO₄ [13]. Briefly, 50 μl LDL (50 μg protein/ml) was added to eight pre-acid washed test tubes. Then 200 μl CuSO₄ with concentrations of different copper (0 to 50 μM) was added to the series of tubes and the volumes were made up 1 ml with PBS, pH 7.4, without EDTA. The solutions were then mixed vigorously by vortexing and left at 37°C for up to 3 hours. Lipid peroxidation of LDL was assessed by thiobarbituric acid reactive substances (TBARS) [14]. In this method, samples were incubated with 0.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% aqueous solution of thiobarbituric acid. After heating at 100°C for 20 minutes, the reaction mixtures were centrifuged at 2000 ×g for 5 minutes. The red pigment in the supernatant fractions was estimated by absorbance at 532 nm. The concentration of TBARS was calculated using the extinction coefficient of 165,000 mol/cm and expressed as nmol of malondialdehyde equivalents per mg LDL protein using a freshly diluted 1, 1, 3, 3-tetraethoxy propane for the standard curve.

Effect of lycopene on oxidation of LDL. To examine the effect of lycopene on oxidized LDL, two processes were followed. In first process, 50 μl LDL (50 μg protein/ml) was added to eight test tubes. Then, 200 μl lycopene (10 μM) and 200 μl DMSO of 10% (vol/vol) was added to the series of tubes. The lycopene was dissolved in 10% DMSO in PBS, pH 7.4. The solutions were incubated at 37°C for 1 hour. Finally, CuSO₄ (200 μl) with concentrations of different copper (0 to 50 μM) was added to the tubes and the volumes were made up 1 ml with PBS, pH 7.4. The solutions incubated at 37°C for 3 hours. Same procedure was repeated for concentrations of 50 μM and 100 μM of lycopene. In second process, 50 μl LDL (50 μg protein/ml)

was added to four pre-acid washed test tubes. Then, lycopene (200 μ l) with concentrations of 0, 10 μ M, 50 μ M and 100 μ M and 200 μ l DMSO was added to the series of tubes. The solutions were incubated at 37°C for 1 hour. Then, 200 μ l CuSO₄ with concentration of copper 10 μ M was added to the tubes and the volumes were made up 1 ml with PBS, pH 7.4. The solutions were mixed vigorously by vortexing and left at 37°C for up to 3 hours. Finally, lipid peroxidation of LDL in samples was assessed by TBARS as described previously.

Gel filtration of oxidized LDL. After oxidation of LDL (1.5 mg protein/ml) with 50 μ M CuSO₄ with or without subsequent dialysis against two changes of at least 100 volumes of the PBS, pH7.4, consisting of NaCl, 140 mM; Na₂HPO₄, 8.1 mM and NaH₂PO₄, 1.9 mM at 4°C for 24 hours, 1 ml of sample was diluted with 1 ml of PBS, pH 7.4 and loaded onto a column of sephadex G-25 (K9, Pharmacia, 600 \times 9 mm) and eluted with the same buffer [15]. Fractions (2 ml) were collected and assayed for protein and copper content. Protein content was measured by the method of Lowry *et al.* [11] and the copper concentration was determined by atomic absorption spectrophotometry using a Varian Model 220 [16].

Same method was repeated in the presence of lycopene (1 mM). First LDL (1.5 mg protein/ml) was incubated with 1 mM lycopene at 37°C for 1 hour and then 50 μ M CuSO₄ was added and incubated at 37°C for 3 hours. Finally, samples with or without subsequent dialysis were diluted with PBS, pH 7.4 and loaded on a column of sephadex G-25.

Statistical analysis. Results were expressed as mean \pm SD. Statistical significance was evaluated by the student's *t*-test. Differences were considered significant at $P \leq 0.05$.

RESULTS

The isolation of LDL from plasma using ultracentrifugation was assessed by measurement of lipid concentrations (Table 1). As shown in this Table, cholesterol lipid and LDL-cholesterol amounts were increased in LDL fraction by 62% and 72%, respectively. This measurement can confirm the isolation of LDL from plasma. The isolation was confirmed by agarose gel electrophoresis (Fig. 1). This Figure shows that band seen for fraction separated from plasma (Fig.1, lane 1 and 2) is

Table 1. Comparison of between lipid concentrations in plasma pool and LDL fraction.

Lipid	Plasma Pool (mg/dl)	LDL fraction (mg/dl)
Total Cholesterol	199 \pm 1.6	520 \pm 1.9
Triglyceride	90 \pm 0.9	87 \pm 0.8
LDL-Cholesterol	135 \pm 1.2	470 \pm 1.7
HDL-Cholesterol	43 \pm 0.5	23 \pm 0.3

Values are mean \pm SD of duplicate determinations on plasma and separated LDL. LDL, Low density lipoprotein; HDL, High density lipoprotein.

correspond to LDL band in plasma, as control (Fig.1, lane 3 and 4). After incubation at 37°C for 1 hour with various concentrations of lycopene (10, 50 and 100 μ M), LDL (50 μ g protein/ml) were exposed to various concentrations of copper (0 to 50 μ M). Three hours after copper addition, the extent of LDL oxidation was estimated from TBARS measurements as shown in Figure 2. At absence of lycopene (as control), various concentrations of copper (0 to 50 μ M) increased LDL oxidation. According to this study, 10 μ M copper had highest effect on copper induced LDL oxidation (Fig. 2A). TBARS production was decreased in presence 10 μ M, 50 μ M and 100 μ M of lycopene concentrations. As shown in Figure 2B, this reduction was dependent on lycopene concentrations. We also investigated the effect of lycopene on copper binding to LDL. Following incubation of LDL (1.5 mg protein/ml) with 50 μ M copper in absence and presence of lycopene (1 mM), 1 ml sample was diluted with 1 ml PBS, pH 7.4, and applied to the sephadex G-25 column. The elution patterns of protein and copper is shown in Figure 3A. Two peaks of copper were observed, the first was observed in the fractions containing LDL while second was seen in aqueous phase. These observations suggest that the copper ions exist not

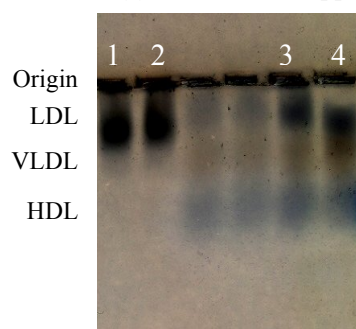


Fig. 1. Electrophoresis analysis of LDL fraction (1, 2) and plasma (3, 4) on 0.8% agarose gel. LDL, Low density lipoprotein; VLDL, Very low density lipoprotein; HDL, High density lipoprotein.

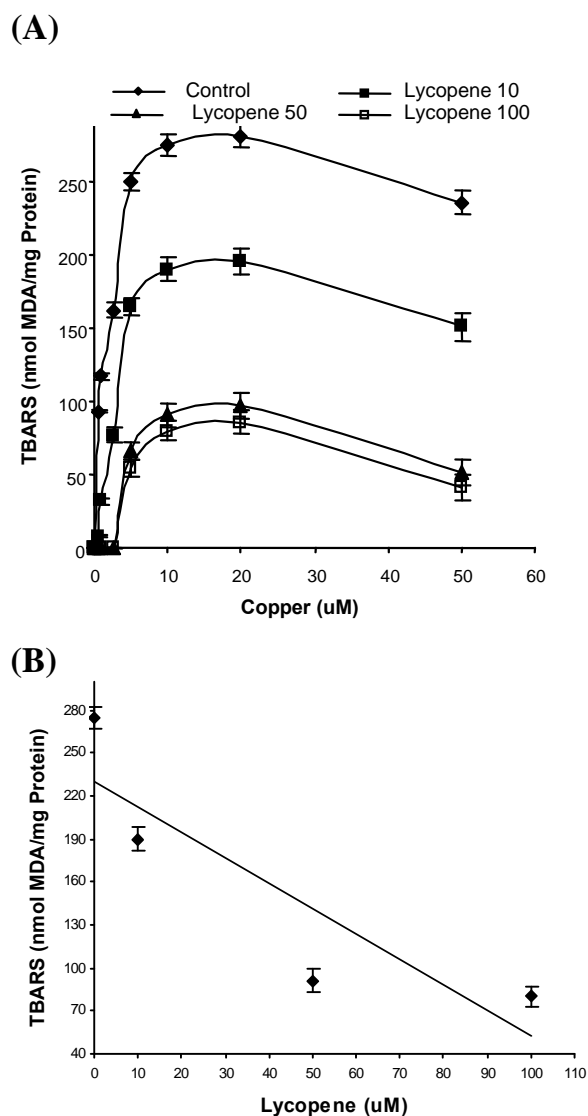


Fig. 2. (A) Effect of various concentrations of copper (0-50 μM) on oxidation of LDL (50 μg protein/ml) in absence (\diamond) and presence of 10 μM (\blacksquare), 50 μM (\blacktriangle) and 100 μM (\square) lycopene. (B) Effect of various concentrations of lycopene (0-100 μM) on oxidation of LDL (50 μg protein/ml) by copper (10 μM). Values represent the means.

only in a free form but also bound to the LDL particle. Following incubation of LDL with copper, in absence and presence of lycopene, the samples were dialyzed at 4°C for 24 hours against PBS, pH 7.4, and then applied to the column. As shown in Figure 3, copper disappeared from the aqueous phase and was only detected in the fractions containing LDL, suggesting that dialysis had removed the free copper ions not bound to LDL. The addition of lycopene before incubation of LDL with

copper (CuSO_4) was increased peak of free copper in aqueous phase (Fig. 3B). As shown in Figure 3B and Figure 4, in presence of lycopene was decreased binding of copper ions to LDL.

DISCUSSION

Over the 15 past years, the oxidation of LDL has been widely studied in order to understand better the role of LDL oxidation *in vivo* in pathological situations, such as atherogenesis [17]. For this purpose, *in vitro* models have been developed including the oxidation of LDL by copper [13, 18]. However, the exact mechanisms relating copper redox change to the lipid peroxidation in LDL are

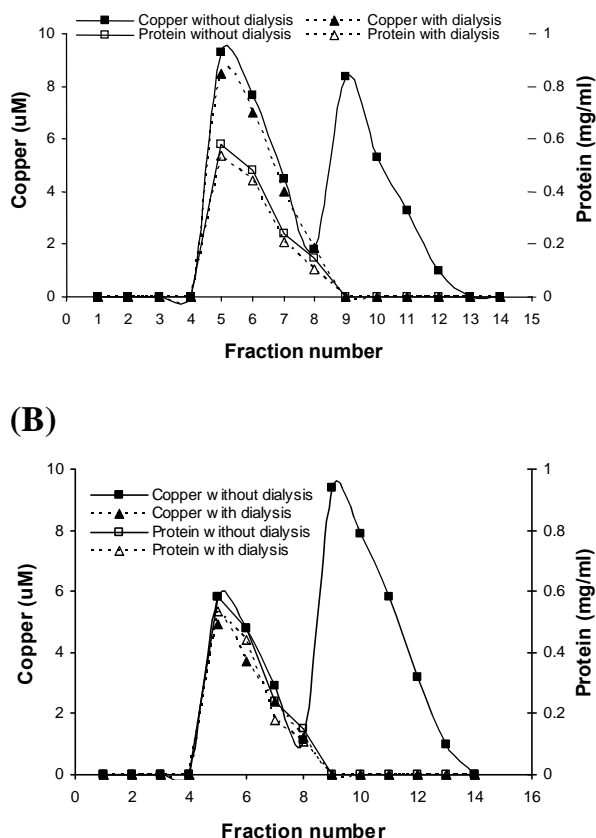


Fig. 3. Elution pattern of copper in absence (A) and presence (B) of lycopene. LDL (1.5mg protein/ml) was incubated in PBS, pH 7.4 with 50 μM CuSO_4 for 3 hours at 37°C (\blacksquare) and subsequently dialyzed against PBS, pH 7.4 for 24 hours at 4°C (\blacktriangle). Samples were then diluted with PBS, pH 7.4 (1:1, v/v) and 2 ml of aliquot was applied to sephadex G-25 column (600 \times 9 mm) and eluted with PBS. Fractions of 2ml were collected and assayed for copper (\blacksquare , \blacktriangle) and protein (\square , \triangle). The flow rate was 1 ml/min.

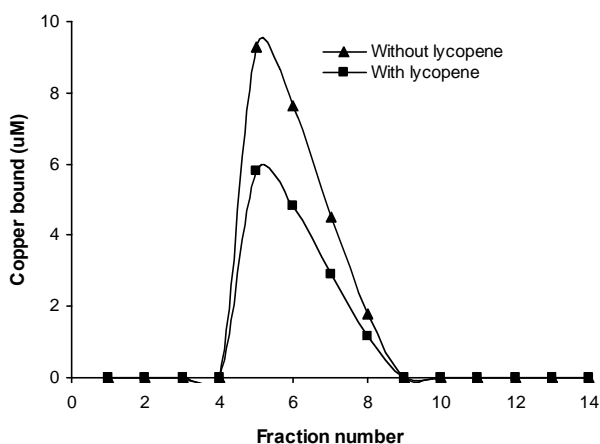


Fig. 4. Effect of lycopene (1 mM) on binding of copper to LDL. This Figure was obtained by Figure 3 data.

The main goal of this study was to investigate the effect of lycopene on the copper induced oxidation of LDL. The antioxidant properties of lycopene have been widely studied, which suggests this carotenoid has a role in preventing cardiovascular disease [8, 20, 21]. A peculiar attention has been paid to the antioxidant effect of lycopene towards the oxidation of LDL whose oxidative modification is thought to be a key process in atherogenesis. Lycopene was chosen in this study as it is rather universally found in plant foods (tomato, watermelon, pink grapefruit, papaya and apricot) [22]. In this study, we first performed experiments with various concentrations of copper (0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and 50 µM) on LDL oxidation. The oxidation of LDL was increased in the presence of 0.5 µM to 10 µM of copper concentrations; however in rather high copper concentrations (20-50 µM), LDL oxidation was almost constant. Our results were shown that copper with concentration of 10 µM has most effect on oxidation of 50 µg protein/ml of LDL concentration. In agreement with these observations, Gieseg and Esterbauer [23] reported that the rate of LDL oxidation increased with increasing copper concentrations. Our data also demonstrated a marked protective effect of lycopene on LDL oxidation. Lycopene concentrations used were 1, 5 and 10 times of 10 µM copper concentration. The presence of lycopene with concentrations of 10 µM, 50 µM and 100 µM was reduced significantly ($P < 0.05$) susceptibility of LDL to oxidative modification were approximately 31, 67 and 71 percent, respectively. This finding is in agreement with previous reports. According to these reports β-carotene, lycopene,

lutein [24], folate [25], quercetin [26] and ascorbate [27] efficiently inhibit LDL oxidation by copper. Thus, our and others results, support the suggestion that lycopene plays an important role in prevention of LDL oxidation mediated by copper ions *in vitro*. Despite early promise from initial studies, little is known regarding the specific biological mechanisms responsible for this lycopene-associated reduction LDL oxidation.

Lycopene is one of the most potent singlet oxygen quenchers, which suggests that it may have comparatively stronger antioxidant properties than other major plasma carotenoids [24]. Kuzuya *et al.* [15] demonstrated that copper binds immediately to LDL particle and that the content of copper bound to LDL increased during incubation with the addition of increasing concentrations of copper to the LDL preparation. They showed that most of the copper ions bound to apoprotein in LDL particle. The copper bound to lipoprotein has a redox potential in the LDL-copper complex. This observation implied that copper bound to LDL can catalyze the peroxidation of LDL even in the absence of free copper [15]. Therefore, copper bound to LDL involves in the peroxidation of LDL. The present study also showed that copper binds to LDL during incubation. Measurement of the copper content in both the lipoprotein and aqueous phases demonstrated that most of the copper bound to LDL. In this study, we showed that presence of lycopene in mixture containing LDL and copper before incubation was decreased the formation of LDL-copper complex approximately by 38 percent. Lycopene is an acyclic isomer of β-carotene. This β-carotene is a 40-carbon atom, open chain polyisoprenoid with 11 conjugated double bonds [24]. Therefore, we suggest lycopene, may with reduce of Cu^{+2} to Cu^{+1} , decreased the affinity of copper to LDL, and thus preventing copper induced oxidative.

In summary, the results presented here on the copper(II) induced oxidation of LDL show that lycopene, as other antioxidants, can protect LDL oxidation against the oxidant activity of copper(II). It is suggested that lycopene, because of its appropriate redox potential can generate Cu^{+1} by reducing the Cu^{+2} . The copper(I) is low affinity to LDL [15], thus decrease binding of this ion to LDL and also decrease the susceptibility of LDL oxidation to copper ions. Therefore, this mechanism may be a reason for lycopene effect in atherosclerosis prevention, but the detailed mechanisms deserve further investigation.

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