

The Effect of α -Tocopherol on Copper Binding to Low Density Lipoprotein

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Abstract

The oxidative modification of low density lipoprotein (LDL) may play an important role in atherogenesis. Antioxidants that can prevent LDL oxidation may act as antiatherogens. Our understanding of the mechanism of LDL oxidation and factors that determine its susceptibility to oxidation is still incomplete. 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. Therefore we investigated the effect of α -tocopherol (as a major fat soluble antioxidant) on copper bound to LDL and furthermore effect of this binding on the susceptibility of LDL to oxidative modification.

In this study LDL was isolated from EDTA-plasma (1 mg EDTA/ml blood) by ultracentrifugation using a single-step discontinuous gradient. Then α -tocopherol was added to LDL and incubated for 1 h at 37° C. The oxidation rate of LDL was estimated by thiobarbituric acid reactive substances (TBARS) after CuSO₄ addition. Finally, the effect of α -tocopherol on formation of LDL-copper complex by gel filtration was studied.

Our results showed that α -tocopherol (dose dependently) suppressed the formation of TBARS and LDL-copper complex. The α -tocopherol with concentrations of 10, 50 and 100 μ M reduced susceptibility of LDL to oxidative modification approximately by 2, 13 and 21 percent, respectively. Furthermore, addition of α -tocopherol to the LDL and CuSO₄ mixture containing before hand prevented the formation of LDL-copper complex, approximately by 30 percent.

In conclusion we found that α -tocopherol with inhibition of copper binding to LDL may decrease the susceptibility of LDL oxidation to this ion and thus could play a role in prevention of atherosclerosis.

Keywords: Low density lipoprotein (LDL); Oxidation; α -tocopherol; Atherosclerosis, Copper.

Introduction

There is substantial evidence that the oxidation of lipoproteins, particularly, LDL plays

an important role in atherogenesis (1-2). The oxidation of LDL involves peroxidation of fatty acids and covalent modification of apolipoprotein B by fatty acid peroxidation breakdown products. Such modified LDL is a potent ligand for the scavenger receptors on macrophages, which can then be readily taken up by macrophages

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leading to formation of foam cells (3). Despite the substantial evidence linking LDL oxidation to atherosclerosis (4-5), our knowledge of the mechanisms of LDL oxidation and factors that affect its susceptibility to oxidative modifications is still incomplete. All four major cell types within atherosclerotic lesions (endothelial cells, smooth muscle cells, macrophages and lymphocytes) can oxidize LDL (6-7). The presence of catalytic amounts of transition metal ions such as copper may be necessary for these cell based oxidations. The mechanism by which copper initiates LDL oxidation has been partially defined. Binding of copper to LDL and copper reduction are required for LDL oxidation (8). This binding occurs, in part, to the histidine containing sites on apolipoprotein B-100, the protein moiety of LDL (9).

Antioxidant compounds provide resistance to this process and have been suggested to lower atherogenicity (10). Considerable epidemiologic (11), biochemical (12) and clinical (13) evidence has accumulated in support of this hypothesis. Alpha-tocopherol (α -tocopherol) is the major fat soluble antioxidant present in the LDL particle. On average 5 to 9 α -tocopherol molecules are carried by each LDL particle and are believed to protect LDL from oxidative damage (14). Several lines of evidence support an inverse relationship between α -tocopherol and atherogenesis. Low levels of α -tocopherol have been shown in epidemiological studies to be related to an increased frequency of cardiovascular disease mortality (15).

Therefore, the goal of the present study was to determine effect of α -tocopherol on copper binding to LDL and thus its effect on the susceptibility of LDL to oxidative modification in a cell free system.

Experimental

Materials

α -tocopherol, ethylene diamine tetraacetic (EDTA), copper Sulfate and sephadex G-25 were obtained from sigma (St. Louis, Mo, U.S.A). Dimethyl sulfoxide, potassium bromide, bovine serum albumin, agarose and 2-thiobarbituric acid were purchased from Merck (Damstadt, Germany). All reagents were freshly prepared and copper solutions were diluted from a freshly prepared 10 mM stock solution.

Methods

Isolation of LDL

EDTA-plasma (1 mg EDTA/ml blood) was prepared from blood (40 ml) of healthy male donor (30 year old and fasting for 14 h). LDL (density 1.019-1.063g/ml) was isolated from the EDTA-plasma by ultracentrifugation using a single step discontinuous gradient (16-17). 4.5 ml of 1 mg/ml EDTA solution (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 tubes were then ultra centrifuged at 60000 rpm for 2 h in a Damon B-60 ultracentrifuge. LDL band was removed through the side of the centrifuge tube with a syringe needle.

For removing 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 h against the phosphate buffer saline (PBS), pH7.4, consisting of NaCl (140mM), Na₂HPO₄ (8.1mM), NaH₂PO₄ (1.9m M) and EDTA (100 μ M). Again LDL was dialyzed at 4°C for 12 h against the phosphate buffer as described above, but without EDTA (18). LDL solution was assayed for protein and lipids contents. The protein content was measured by the method of Lowry *et al.* (19), using bovine serum albumin as standard. The cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol concentrations were determined using the Pars Azmon Kit. 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 (20-21).

LDL was sterilized by filtration (0.45 μ m Millipore filter). Then stored in darkness under nitrogen gas at 4°C and was used within 4 weeks.

Assessment of LDL oxidation and lipid peroxidation

For the preparation of oxidized LDL, LDL was incubated with CuSO₄ (22). Briefly, first 50 μ l LDL (50 μ g protein/ml) was added to eight pre-acid washed test tubes. Then 200 μ l CuSO₄ with different copper concentrations (0 to 50

μM) was added to the series of tubes and the volumes were adjusted up to 1 ml with PBS, pH=7.4, without EDTA. The solutions were then mixed vigorously by vortexing and left for 3 h at 37°C. Lipid peroxidation of LDL was assessed by measuring thiobarbituric reactive substances (TBARS) (23-24). In this method, samples were incubated with 0.5 ml of 20% trichloroacetic acid and then 1 ml of 0.67% aqueous solution of thiobarbituric acid was added. After heating at 100°C for 20 minutes, the reaction mixtures were centrifuged at 4000 rpm for 5 minutes. The absorbance of red pigment in the supernatant fractions was estimated at $\lambda_{\text{max}}=532$ nm. The concentration of TBARS was calculated using the extinction coefficient of 165000 mol/cm and expressed as nmol of malondialdehyde (MDA) equivalents per mg LDL protein. Calibration curves were drawn using a freshly diluted 1, 1, 3, 3-tetraethoxypropane.

Effect of α -tocopherol on oxidation of LDL

To examine the effect of α -tocopherol 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 α -tocopherol (10 μM) and 200 μl dimethyl sulfoxide (DMSO) of 10% (vol/vol) were added to the series of tubes. The α -tocopherol was dissolved in 10% DMSO in PBS, pH7.4. The solutions were incubated for 1 h at 37°C. Finally, 200 μl CuSO_4 with concentrations of different copper (0 to 50 μM) was added to the tubes and the volumes were adjusted up to 1ml with PBS. The solutions incubated for 3 h at 37°C. Same procedure was repeated for concentrations of 50 and 100 μM of α -tocopherol.

In second process, 50 μl LDL (50 μg protein/ml) was added to four pre-acid washed test tubes. Then 200 μl α -tocopherol with concentrations of 0, 10, 50 and 100 μM and 200 μl DMSO were added to the series of tubes. The solutions were incubated for 1 h at 37°C. Then 200 μl CuSO_4 with concentration copper of 10 μM was added to the tubes and the volumes were adjusted up to 1 ml with PBS, pH=7.4. The solutions were mixed vigorously by vortexing and left for 3 h at 37°C. Finally, lipid peroxidation of LDL in samples was assessed as described previously.

Gel filtration of oxidized LDL

After oxidation of LDL (1.5 mg protein/ml) with 50 μM CuSO_4 with or without subsequent dialysis against PBS at pH=7.4 consisting 140 mM NaCl, 8.1 mM Na_2HPO_4 and 1.9 mM NaH_2PO_4 for 24 h at 4°C, one 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 then eluted with the same buffer (24). Fractions (each of 2 ml volumes) were collected and assayed for protein and copper content. Protein content was measured by the method of Lowry *et al.* (19) and the copper concentration was determined by atomic absorption spectrophotometry using a Varian Model 220 (25).

The same method was repeated in the presence of α -tocopherol (1mM). At first LDL (1.5 mg protein/ml) was incubated with 1 mM α -tocopherol for 1 h at 37°C and then 50 μM CuSO_4 was added and again it was incubated for 3 h at 37°C. 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 and Discussion

The isolation of LDL from plasma using ultracentrifugation was assessed by measuring lipid concentrations (Table 1). Also, this isolation was confirmed by agarose gel electrophoresis (Figure 1). Incubation of freshly isolated human LDL (50 μg protein/ml) with various concentrations of copper (0-50 μM) at 37°C for 3 h led to oxidation of LDL. This oxidation was dependent on copper concentration (Figure 2A). The copper catalyzed oxidation of LDL was determined in absence or presence of 10, 50 and 100 μM α -tocopherol (Figure 2A). As shown in Figure 2A, the addition of α -tocopherol significantly reduced ($p<0.05$) TBARS formation in medium. This reduction was dependent on concentration of α -tocopherol (Figure 2B).

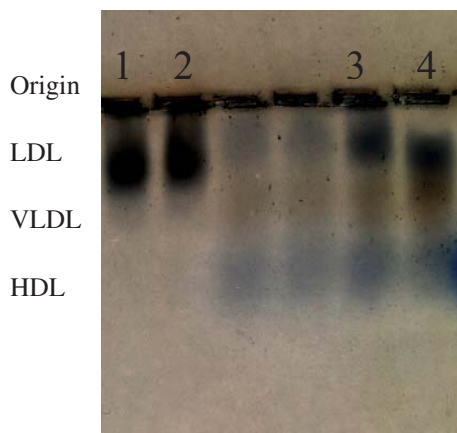


Figure 1. Electrophoresis analysis of LDL fraction (1, 2) and plasma (3, 4) on 0.8% agarose gel. LDL=Low density lipoprotein, VLDL=Very low density lipoprotein, HDL=High density lipoprotein

α -tocopherol is quantitatively, the most abundant lipophilic antioxidant in LDL (26). This vitamin (α -tocopherol) is known to be a highly efficient lipid soluble antioxidant protecting the unsaturated lipids in a variety of biological and model systems including cell membranes, LDL and liposome (27). Previous reports suggested that α -tocopherol associated with LDL and act as chain breaking antioxidant (28) that suppress lipid peroxidation by reducing chain carrying lipid peroxyl radical (LOO) to LOOH or by forming covalent adducts with these radicals (29). In this study, we determined the susceptibility of LDL to copper induced oxidation with or without α -tocopherol. Presence of α -tocopherol with concentrations of 10, 50 and 100 μ M was accompanied by reduced susceptibility of LDL to oxidative modification approximately by 2, 13 and 21 percent, respectively. This result supports the suggestion that α -tocopherol plays an important role in the prevention of LDL oxidation mediated by copper ions in vitro.

Following incubation of LDL (1.5 mg protein/ml) with 50 μ M copper in the absence and presence of α -tocopherol (1 mM), 1 ml sample was diluted with 1ml 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 lipoprotein (LDL) while second was seen in aqueous phase. These observations suggest that the copper ions exist not only in a free form but

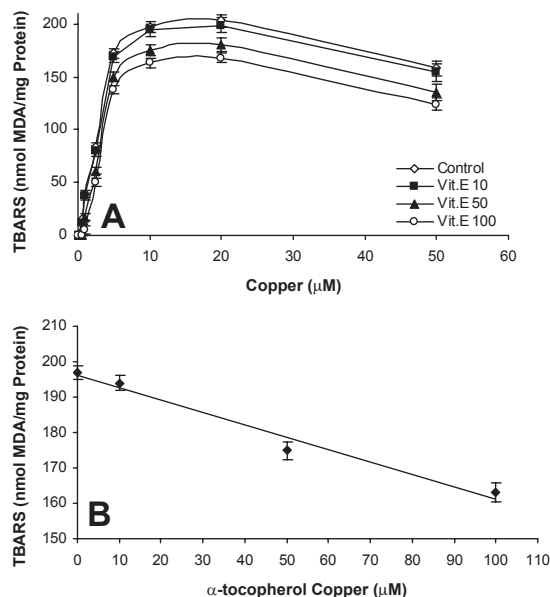


Figure 2 . (A) Effect of various concentrations of copper (0-50 μ M) on oxidation of LDL (50 μ g protein/ml) in absence (\diamond) and presence of 10 μ M (\blacksquare), 50 μ M (\blacktriangle) and 100 μ M (\circ) α -tocopherol. **(B)** Effect of various concentrations of α -tocopherol (0-100 μ M) on oxidation of LDL (50 μ g protein/ml) by copper (10 μ M). Values represent the means \pm SD of triplicate determinations.

also bound to the LDL particle.

Following incubation of LDL with copper, in the absence and presence of α -tocopherol, the samples were dialyzed at 4 $^{\circ}$ C for 24 h 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 α -tocopherol before incubation of LDL with copper (CuSO_4) increased the concentration of free copper in aqueous phase (Figure 3B). As shown in Figure 3B and Figure 4, in the presence of α -tocopherol

Table 1. A comparison 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

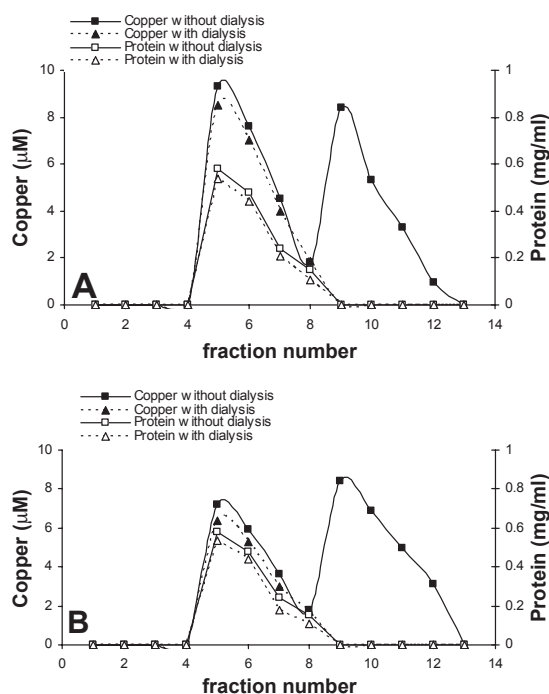


Figure 3. Elution pattern of copper in absence (A) and presence (B) of α -tocopherol. LDL (1.5mg protein/ml) was incubated in PBS, pH7.4 with $50\mu\text{M}$ CuSO_4 for 3 hat 37°C (■) and subsequently dialyzed against PBS, pH7.4 for 24 at 4°C (▲). Samples were then diluted with PBS, pH7.4 (1:1, v/v) and 2ml of aliquot was applied to sephadex G-25 column (600×9mm) and eluted with PBS. Fractions of 2ml were collected and assayed for copper (■, ▲) and protein (□, △). The flow rate was 1ml/min.

binding of copper ions to LDL was decreased. In this study, we also showed that copper could bind to LDL during incubation. Measurement of the copper content in both the lipoprotein and aqueous phases demonstrated that most of the copper is bound to LDL. The addition of α -tocopherol to the LDL and CuSO_4 containing mixtures beforehand, decreased the formation of LDL-copper complex approximately by 30 percent; therefore it will increase the amount of free copper in the aqueous phase. Previous investigations have shown that antioxidants such as vitamin C, quercetin and myricetin could inhibit lipid peroxidation by LDL bound copper (18, 21, 30). Kuzuya et al. reported that copper binds to LDL and that copper binding is a prerequisite for LDL oxidation (24). The vitamin C decreased copper binding to LDL and thus prevented copper induced oxidative damage. This effect of vitamin C is justified since it could reduce Cu^{+2} to Cu^{+1} (21). Roland *et al.* have shown that quercetin and myricetin inhibited

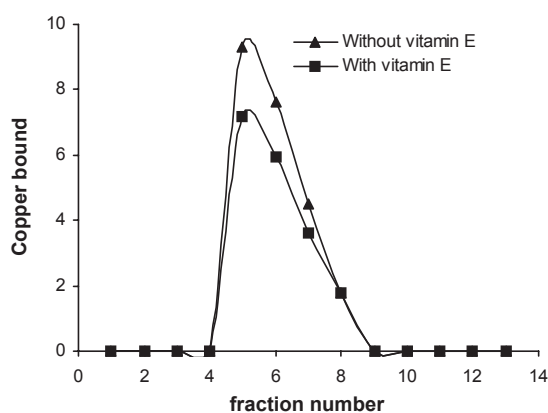


Figure 4. The effect of α -tocopherol (1mM) on binding of copper to LDL.

copper binding to LDL (18). These two flavonols possess a carbonyl group at the 4 position and hydroxyl groups at the 3 and 5 positions. Each of these hydroxyl groups could act in concert with the carbonyl oxygen to chelate transition metal ions (18). Our study also showed that α -tocopherol can prevent binding of copper to LDL. This vitamin possesses a hydroxyl group, therefore we suggest α -tocopherol could chelate copper ions and with this mechanism it may inhibit the copper binding to LDL.

It can be concluded that α -tocopherol decreases the susceptibility of LDL to copper ions induced oxidation by inhibition of copper binding to LDL. Therefore, this mechanism may be a reason for α -tocopherol effect in preventing atherosclerosis.

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