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Original Article

In Vitro Effect of α-Tocopherol, Ascorbic Acid and Lycopene on Low Density Lipoprotein Glycation

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Abstract

Nonenzymatic glycation of low density lipoprotein (LDL) is a reaction of glucose and other reducing sugars with apolipoprotein B_{100} (apo-B_{100}) lysine residues. In diabetes, this reaction is greatly accelerated and is important in the pathogenesis of diabetic complications. The objective of this study was to investigate *in vitro* effects of α-tocopherol, ascorbic acid and lycopene on LDL glycation.

LDL was isolated from EDTA-plasma by ultracentrifugation using a single step discontinuous gradient. LDL and glucose were incubated without and with different concentrations of lycopene, ascorbic acid and α-tocopherol. LDL glycation were estimated by sodium periodate assay.

Based on this study results, α-tocopherol, ascorbic acid and lycopene decrease LDL glycation in a dose dependent manner. The electrophoretic mobility of glycated LDL decreased in presence these nutrients.

These effects may be due to antioxidant properties of these nutrients and may have a role in ameliorating atherosclerotic risk of patients with diabetes mellitus.

Keywords: Low density lipoprotein (LDL); Glycation; α-Tocopherol; Ascorbic acid; Lycopene.

Introduction

Nonenzymatic glycation of low density lipoprotein (LDL) naturally occurs in all individuals due to condensation of reducing sugars with apolipoprotein-B_{100} (apo-B_{100}) moiety of LDL particles. LDL glycation is increased in diabetic patients because of their elevated plasma glucose concentrations (1). During glycation, glucose reacts with lysine residues of target proteins (apo-B_{100}) to form a labile Schiff's base. This product may further react into a more stable fructosamine that is characteristic of glycated proteins (2). Chemical modification of lysine residues of apo-B_{100} by glycation is known to decrease the recognition of LDL by its receptor. This cause an increase in relative circulation time of LDL, which may increase particle oxidation, formation of advanced glycation end products (AGEs) and the activation of alternative uptake mechanisms by non-LDL receptor mediated pathways (3). It is to be noted that no AGEs is solely derived from glucose but account for its reactive carbonyl species (RCS) such as 3-deoxyglucosone, glyoxal and methylglyoxal that are critical intermediates during glycation of proteins by glucose and they have identified as important precursors of AGEs *in vivo* (4, 5). Methylglyoxal can readily bind

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to amino groups, thereby modifying biological molecules to form covalently cross-linked aggregates (6). Because these processes are considered proatherogenic, it has been proposed that the nonenzymatic glycation of LDL could contribute to the increased susceptibility of diabetic patients to atherosclerosis and coronary heart disease (2). In consideration of the significance of glycooxidative stress to diabetic pathology, a supplement of antioxidants in response to the inhibition of protein (apo-B_{100}) modification should be a theoretical strategy for preventing diabetic complications (7). This hypothesis has been supported by the clinical results indicating the development of type 2 diabetes may be reduced by the intake of antioxidants in diets (8). Fruits and vegetables are important dietary sources of α-tocopherol, ascorbic acid and lycopene (9). These nutrients are of current interest in research due to their important biological and pharmacological properties attributed to their antioxidant properties (10). Nevertheless, the literature data concerning the effect of these nutrients in preventing glycooxidative modification of LDL are limited. Given the link mentioned above, we hypothesized those antioxidant nutrients (α-tocopherol, ascorbic acid and lycopene) might possess significant antiglycoxidation activities as well. In this study, the effects of α-tocopherol, ascorbic acid and lycopene on LDL glycation were investigated. This study will underline the importance of above naturally occurring nutrients in prevention of hyperglycemia mediated protein (apo-B_{100}) modification.

Experimental

Materials

α-tocopherol, ascorbic acid and lycopene were purchased from Sigma (St. Louis, Mo, U.S.A.) and used without further purification. Ethylene diamidine tetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), potassium bromide, bovine serum albumin (BSA), agarose, glucose, fructose and sodium periodate were obtained from Merck (Darmstadt, Germany). Sodium borohydride was obtained from Riedel-deHaen (Germany). Solutions were freshly prepared with double deionized water.

Methods

LDL isolation

Blood from normolipidemic overnight fasting volunteers (n=25, age 30±5 yr, men, non smokers, non diabetics, not taking any drug since at least 2 weeks before) was collected into syringes containing EDTA (1 mg EDTA/ml blood). LDL was isolated by ultracentrifugation using a single step discontinuous gradient according to the method of Gieseg et al. (11). Briefly, anticoagulated (EDTA) plasma was obtained by centrifugation (3000×g, 10 min). 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 underlaid with dense plasma solution. The dense plasma solution was prepared by dissolving 0.632 g of solid potassium bromide in 2 ml of EDTA-plasma. The centrifuge tubes were ultracentrifuged at 400000 × g for 2 hours in a Damon B-60 ultracentrfuge. LDL fraction as the orange band at the middle of the tubes was carefully withdrawn. EDTA and potassium bromide were separated from LDL by dialysis tubing (10 mm flat width) with a molecular weight cutoff 12 to 14 KDa at 4 °C for 24 h against phosphate buffered saline (PBS), pH 7.4. The LDL protein content was determined by Bradford method (12), using bovine serum albumin as standard. The cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol were determined using the Pars Azmoon 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 (13).

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

LDL glycation

Glycation of LDL was performed by incubation of LDL (0.2 mg protein/ml) at different times (1-11 days) and different concentrations of glucose (0-200 mM) in PBS containing 1 mM EDTA (pH 7.4) at 37 °C. The degree of LDL glycation was measured by periodate method (14). According to this method, 500 μl LDL (0.2 mg protein/ml) was

incubated for 1 h at room temperature with 100 µl of 200 mM sodium borohydride dissolved in ice-cold 0.01 mM NaOH, using 100 µl of 0.01 mM NaOH as control. The sodium borohydride solution was prepared and used freshly. The reaction was stopped by adding 100 µl of 0.2 mM HCl, after which 100 µl of 0.1 mM sodium periodate was added to each sample. After 30 min at room temperature, the samples were chilled in ice bucket for 10 min and then 200 µl each of ice-cold 0.7 mM NaOH and zinc sulfate (15%) were added, with mixing. The samples were centrifuged at 13000 ×g for 10 min and the supernatant was removed, the samples were centrifuged again. Then 600 µl of the final supernatant was mixed with 300 µl color reagent. The color reagent was made by adding 46 µl of acetylacetone to 5 ml of 6.6 mM ammonium acetate. Samples were left at 37 °C for 1 h and absorbance of each sample at 450 nm was then measured.

Fructose solutions (0-0.9 mM) were used to construct a calibration curve for the periodate assay. Both fructose and amadori products (a stable sugar adduct that is formed by the rearrangement of the labile Schiff base formed in the initial stages of glycation) exist predominantly in a ring structure, and after periodate oxidation, both produce 1 mole of formaldehyde per mole of sugar moiety (14). Thus, in this paper degree of glycation of LDL expressed as mmol of formaldehyde released per mg of LDL protein.

Effect of α-tocopherol, ascorbic acid and lycopene on LDL glycation

The effects of three nutrients, i.e. α-tocopherol, ascorbic acid and lycopene on the glycation of LDL were examined by incubation of LDL (0.2 mg protein/ml) with glucose (120 mM) and 0 to 150 µM α-tocopherol, ascorbic acid and lycopene in PBS, pH 7.4 at 37 °C for 7 days. The ascorbic acid was dissolved in PBS, pH 7.4, whereas α-tocopherol and lycopene were dissolved in 10% dimethyl sulfoxide (DMSO) in PBS, pH 7.4. Degree of LDL glycation was determined by sodium periodate assay (14). The electrophoretic mobility of native LDL, glycated LDL in the absence and/or presence of 150 µM α-tocopherol, ascorbic acid and lycopene was compared on 5% polyacrylamide gel. Gels were stained with comassi blue (15).

Statistical analysis

Results are expressed as mean±SD. Degree of LDL glycation in the absence (as control) and presence of α-tocopherol, ascorbic acid and lycopene were compared using ANOVA test. Values of $p \leq 0.05$ were considered statistically significant.

Results and Discussion

Isolation of LDL was assessed by measurement of lipid concentration (Figure 1A). As shown in this Figure, cholesterol and LDL-cholesterol amounts were increased in LDL fraction approximately by 65% and 76%, respectively. This measurement confirms isolation of LDL from plasma. This isolation was also confirmed by agarose gel electrophoresis (Figure 1B). This Figure shows the separated fractions of LDL (Lane 2) and VLDL (Lane 3) compared to plasma (Lane 1).

Glycated LDL was prepared by incubation of LDL with glucose as *in vitro*. Glucose was used as glycating agent, which is commonly adopted in many Millard reaction studies. In nonenzymatic glycation of proteins, also known as Millard reaction, reducing sugars covalently attach to free amino groups and ultimately form advanced glycation end products (AGEs) (16). Free amino groups of apolipoprotein B_{100} (apo-B_{100}) of LDL could serve as a target for glycating agents. Glucose mainly adducts with the ε-amino group of lysine residues in apo-B_{100} (17). In the present study, the best glucose concentration for glucose incorporation with LDL was investigated by incubation of a range of glucose concentrations (0-180 mM) with LDL (0.2 mg protein/ml) in PBS containing 1 mM EDTA, pH 7.4 at 37 °C for 7 days under aseptic condition (Figure 2A). The best incubation time for glucose incorporation with LDL was also investigated by incubating 0.2 mg protein/ml of LDL with 120 mM glucose for 1 to 11 days at 37 °C in PBS and 1 mM EDTA, pH 7.4, under aseptic condition (Figure 2B). As shown in Figure 2 (A and B), LDL glycation was increased in the presence of 20-120 mM of glucose

concentrations at 1 to 7 days of incubation time. However, in higher glucose concentrations (140-180 mM) and longer incubation time (8-11 days) this phenomenon was decreased. Our results were shown that optimum glucose concentration and incubation time for LDL glycation were 120 mM and 7 days, respectively.

A series of experiments were carried out to examine the influence of α-tocopherol, ascorbic acid and lycopene on LDL glycation process. α-tocopherol (0-150 μM) was incubated with LDL (0.2 mg protein/ml) and glucose (120 mM) at 37 °C for 7 days under aseptic condition. The extent of LDL glycation in the absence (as control) and/or presence of α-tocopherol were estimated from mole of formaldehyde released per mg of LDL protein as shown in Figure 3A. Glycation of LDL was decreased in the presence of various α-tocopherol concentrations (25-150 μM). As shown in Figure 3A, this reduction was dependent on α-tocopherol concentration. Same procedure was repeated to investigate the effects of ascorbic acid and lycopene on LDL glycation (Figure 3A). The results showed the nutrients significantly reduced LDL glycation in a dose dependent manner (n=3, ANOVA test, $p<0.05$). According to this study, 150 μM concentration of α-tocopherol, ascorbic acid and lycopene were able to reduce LDL glycation approximately 54%, 54.5% and 63%, respectively (Figure 3B). In glycation process, glucose reacts with an amine group to form a labile Schiff base that rearranges to amadori product. The Schiff base is prone to oxidation and free radical generation, which leads to the formation of reactive carbonyl species such as glyoxal (18). Given the link mentioned above between glycation and oxidation, we suggested that antioxidant nutrients might possess antiglycooxidative activities. α-tocopherol, as a potent antioxidant has been previously shown by Ceriello to be an anti-glycating agent *in vitro* and *in vivo* when administered as a supplement to diabetics (19). Ascorbic acid form ionic bonds with biological molecules such as proteins. The carbonyl group of ascorbic acid may also compete with glucose for proteins (20). Lycopene is a carotenoid without provitamin A activity that occur almost exclusively in tomatoes and tomato products. It is a 40 carbon atom and open chain polyisopronoid with 11 conjugated double bonds. Because of this unique structure, it is one of the most potent singlet oxygen quenchers suggested to possibly have stronger antioxidant properties compared to other major plasma carotenoids (21). Many reports have shown that typical antioxidant nutrients such as vitamin B_1, B_6, C, niacinamide, carnosine and sodium selenite inhibit the *in vivo* and *in vitro* AGEs formation (22). The present study also showed that lycopene, ascorbic acid and α-tocopherol decrease LDL glycation approximately 63%, 54.5% and 54%, respectively. It was found that when LDL was subjected to sugar-mediated modification, the addition of above three nutrients decrease the amount of formaldehyde

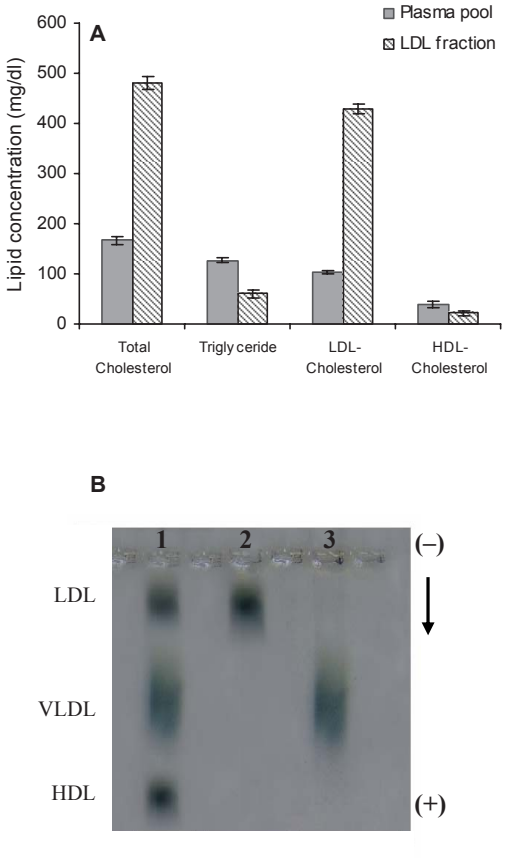

Figure 1. (A) The comparison of lipid concentrations in plasma pool and LDL fraction.
(B) Electrophoresis analysis of plasma (Lane 1), LDL fraction (Lane 2) and VLDL fraction (Lane 3) on 0.8% agarose gel.
Values have represented as the mean±SD of triplicated determinations.LDL= Low density lipoprotein, VLDL= Very low density lipoprotein,HDL= High density lipoprotein

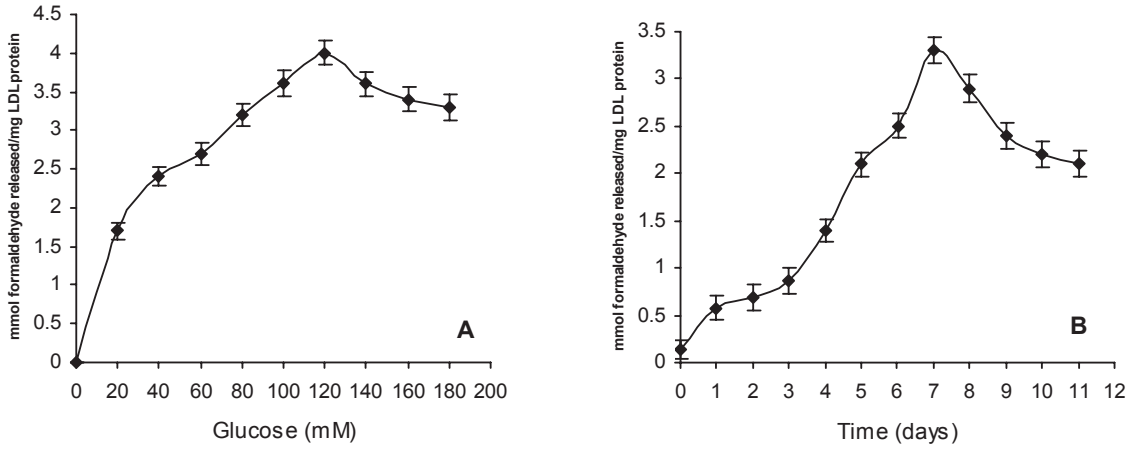

Figure 2. The effect of glucose concentration (A) and incubation time (B) on glycation of LDL.
Values have represented as the means±SD of triplicate determinations.

(mmol) released per mg of LDL protein, as dose dependently. Lycopene was the most effective compound on LDL glycation in our study.

Finally, we investigated electerophoretic mobility of LDL treated with 150 μM α-tocopherol, ascorbic acid and lycopene on polyacrylamid gel (Figure 4A). This Figure shows that glycation, has increased anodic migration of LDL compared to native LDL. This probably resulted from the increased negative charge caused by the modification of lysine amino groups. This result is in agreement with the finding of Witztum et al. (23). Compared samples rate of flow (RF) on gel electrophoresis has also shown in Figure 4B. Lycopene, ascorbic acid and α-tocopherol decreased RF of glycated LDL in comparison with native LDL approximately 23%, 18% and 18%, respectively (Figure 4B).

These results reiterate that lycopene, ascorbic acid and α-tocopherol play an important role in the prevention of LDL glycation by glucose. The mechanism by which these nutrients suppress LDL glycation is still unknown. Generation inhibition and/or scavenging of free

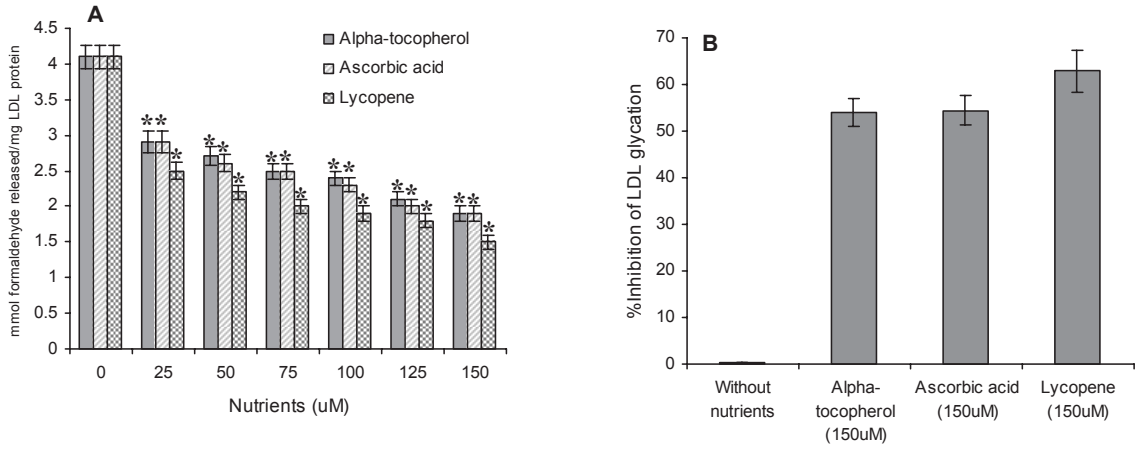

Figure 3. (A) The effect of 25μM to 150μM concentrations of α-tocopherol, ascorbic acid and lycopene on glycation of LDL (0.2mg protein/ml) by glucose (120mM).
(B) The comparison of inhibition percent of LDL glycation in absence and presence of 150μM concentration of α-tocopherol, ascorbic acid and lycopene.
Values have represented as the means±SD of triplicate determinations.
**p<0.05*

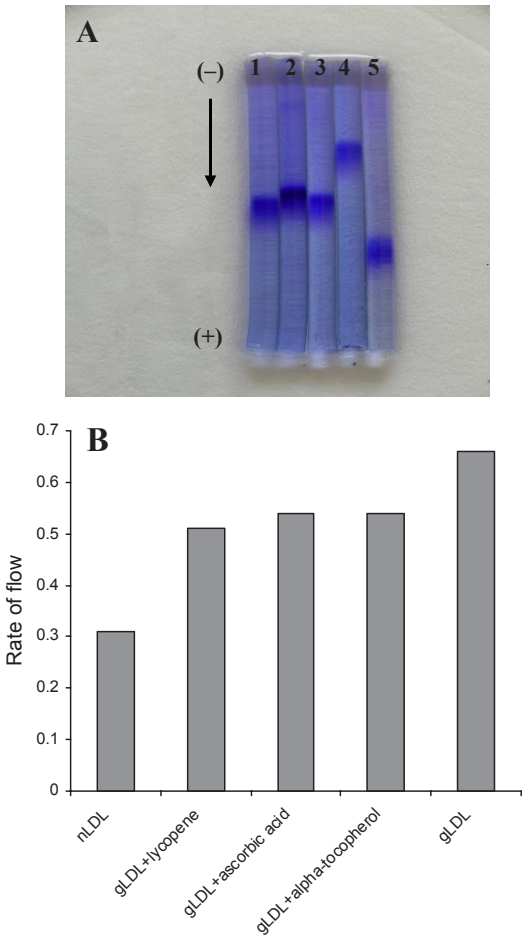

Figure 4. (A) Electrophoresis analysis of native LDL (Lane 4) and glycated LDL in absence (Lane 5) and presence 150μM concentration of α-tocopherol (Lane 1), lycopene (Lane 2) and ascorbic acid (Lane 3) on 5% polyacrylamide gel. (B) The comparison of rate of flow of native LDL (nLDL) with glycated LDL (gLDL) in absence and presence of lycopene, ascorbic acid and α-tocopherol (150μM).

radicals resulted from glycation process and subsequent inhibition of protein modification is one of the probable mechanisms of anti-glycation effect of these nutrients (8). Yim et al. indicated that glycation of proteins generates some active centers for catalyzing one-electron oxidation-reduction reactions, which mimic the characteristics of the metal catalyzed oxidation system (24). In addition, glycated proteins accumulated *in vivo* may provide stable active sites for catalyzing the formation of free radicals (24). Results from Jiang et al. also demonstrated that reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion are generated during glycation process (25). Chien et al. showed that ascorbic acid prevents oxidative modification of LDL primarily by scavenging free radicals and other reactive oxygen species in the aqueous milieu (26). Farvid et al. indicated that α-tocopherol can act as a chain breaking antioxidant by scavenging highly reactive lipid peroxyl and alkoxyl radicals (27). Kiho et al. also showed, lycopen with its unique structure (11 conjugated double bonds and no cyclic groups) can quench singlet oxygen and subsequently inhibit the formation of AGEs (16). Thus, we could suggest that antiglycation activity of α-tocopherol, ascorbic acid and lycopene possibly correlate with their radicals scavenging abilities.

In conclusion, these nutrients, especially lycopene, have inhibitory effects on LDL glycation. All of the studied nutrients naturally occur in the body. But ascorbic acid is known to be lowered in the tissues of subjects with diabetes (28). This fact and the results of the present study point to the necessity of a healthy diet in diabetes and to the possibility of inexpensive and relatively non-toxic therapies for the prevention and treatment of diabetic complications. Because glycated LDL and atherogenesis are correlated *in vivo* (28), inhibition of glycation of LDL by these nutrients may also form the basis of future antiatherogenic strategies in both diabetic and non-diabetic individuals. However, the detailed mechanisms deserve further investigation.

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