

Glucose Influence on Copper Ion-Dependent Oxidation of Low Density Lipoprotein

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ABSTRACT

Background: It is well established that oxidative modification of low density lipoprotein (LDL) plays a causal role in human atherogenesis and the risk of atherosclerosis is increased in patients with diabetes mellitus. We examined the *in vitro* effect of glucose on native and glycated LDL oxidation using copper ion dependent oxidation system. **Methods:** In this study, LDL was isolated from plasma by ultracentrifugation using a single step discontinuous gradient. Native LDL preparations were glycated by glucose and also were oxidized by copper ions. LDL glycation and oxidation levels were estimated by sodium periodate assay and thiobarbituric acid reactive substances (TBARS), respectively. Then, native LDL was incubated with glucose and copper and LDL oxidation was estimated by TBARS. Finally, oxidation of glycated LDL was studied in presence of copper ions by TBARS and relative electrophoretic mobility on polyacrylamide gel. **Results:** This study showed that glucose considerably decreased the oxidation of native LDL by copper ions. But oxidation of glycated LDL elevated with presence of copper ions. **Conclusion:** The results of this investigation show that LDL glycated *in vitro* is prone to oxidation by copper ions. Thus, promotion of glycated LDL oxidation by glucose is specific for copper ion dependent oxidation and involves increased copper ion reduction. These results provide one mechanism that may enhance LDL oxidation in diabetes and thus contribute to the pathogenesis of atherosclerosis in diabetic patients. *Iran. Biomed. J.* 13 (1): 59-64, 2009

Keywords: Low density lipoprotein (LDL), Glucose, Copper, Glycation, Oxidation

INTRODUCTION

Atherosclerosis is a leading cause of morbidity and mortality in patients suffering from diabetes mellitus [1]. The risk for development of atherosclerosis is increased by approximately three fold in patients with diabetes as a result of a number of processes which are still poorly understood [2]. One hypothesis is that increase modification of low density lipoprotein (LDL) by oxidation and/or glycation may enhance the atherogenic process in individual with diabetes. There is increasing evidence that both LDL and plasma from individuals with diabetes may be more susceptible to oxidation [3, 4]. Although LDL levels are frequently normal in diabetic patients, hyperglycemic or other metabolic alterations secondary to the diabetic state may alter lipoproteins to a form that promotes atherosclerosis. One such

modification may be the non-enzymatic glycation of LDL which has been shown in some studies to be taken up rapidly by macrophages in culture [5]. LDL can be oxidized by all major groups of arterial wall cells, namely macrophages, smooth muscle cells, endothelial cells and lymphocytes. A number of agents, such as transition metal ions, lipoxygenases, proxidases and heme proteins are capable in the absence of cells oxidizing LDL. Of these oxidants, transition metal ions (such as iron and copper) seem to be most likely involved in LDL oxidation [6]. For *in vitro* studies of oxidized LDL, oxidation by copper is frequently used and produces LDL with characteristics similar to LDL oxidized by cells. Copper is more potent than iron its ability to oxidize LDL *in vitro* [7]. Therefore, the present study was designed to determine influence of glucose on the susceptibility of LDL to oxidative modification by copper ions in a cell free system.

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MATERIALS AND METHODS

Chemicals. Copper sulfate, agarose and EDTA were obtained from Sigma Chemical Co. (Saint-Louis, Mo, USA). Potassium bromide, BSA, glucose, fructose and sodium periodate were supplied by Merck (Darmstadt, Germany) and sodium borohydride was purchased from Riedel-deHaen (Germany). All other chemicals were of the highest purity available from Sigma or Merck Companies. Solutions were freshly prepared with deionized water.

Preparation and treatment of LDL. Blood samples were obtained from normolipidemic overnight fasting volunteers ($n = 25$, age 30 ± 5 y, men, non-smoker, non-diabetics, not taking any drug since at least 2 weeks). Blood samples were collected into syringes containing EDTA (1 mg EDTA per ml blood). LDL ($d = 1.0240-1.050$) was isolated from plasma pool by ultracentrifugation using a single step discontinuous gradient according to the method of Gieseg *et al.* [8] as previously described [9]. After isolation from plasma pool, LDL was dialyzed by 10-mm flat width dialysis tubing with cutoff 12 to 14 kDa, for 24 hours at 4°C against pH 7.4, 10 mM PBS to remove EDTA and potassium bromide [9]. The LDL protein content was determined by Bradford method [10] using bovine serum albumin as standard. Total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol were determined by standard enzymatic Kit (Pars Azmoon Co, Iran) according to the manufacturer's instruction. The purified LDL was also examined by electrophoresis carried out at pH 8.6 in 0.05 M barbital buffer on 0.8% agarose gel [11]. Unless specified in the text, LDL preparations were stored in darkness under nitrogen gas at 4°C and used within 4 weeks. We obtained the best glucose concentration and incubation time for LDL glycation, and also the best copper concentration for LDL oxidation, as previously described [9, 12]. Glycation and oxidation of LDL were measured as described below.

LDL glycation measurement. The degree of LDL glycation was measured by periodate method [13]. A volume of 500 μl LDL (0.2 mg protein. ml^{-1}) was incubated with 100 μl of fresh prepared sodium borohydride (200 mM) in ice cold NaOH (0.01 mM). A volume of 100 μl of NaOH (0.01 mM) was used as control. The reaction was stopped by adding 100 μl of HCl (0.2 mM). Then, 100 μl of sodium

peridate (0.1 mM) was added and incubated at room temperature for 30 minutes. The samples were put on ice for 10 minutes and then 200 μl of ice cold NaOH (0.7 mM) and 200 μl of zinc sulfate (15%) were added to each sample. The samples were centrifuged at 13,000 $\times g$ for 10 minutes and then supernatants were removed and centrifuged again. Final supernatant (600 μl) of each sample was mixed with 300 μl color reagent. Color reagent was prepared by adding 46 μl of acetylacetone to 5 ml of ammonium acetate (6.6 mM). The samples were incubated at 37°C for 1 hour and then absorbance of each sample was measured at 450 nm. We used fructose solutions (0-0.9 mM) to prepare a calibration curve for the periodate assay. Both fructose and amadori products (in the initial stages of glycation a labile Schiff base is formed, which rearranges to form a stable sugar adduct known as an amadori product) exist predominantly in a ring structure and after periodate oxidation, both product 1 mol of formaldehyde per mole sugar moiety [13]. Thus, in this study, the degree of glycation of LDL expressed as mmol of formaldehyde released per mg LDL protein.

LDL oxidation measurement. Lipid peroxidation of LDL was assessed by thiobarbituric acid reactive substance (TBARS) [14]. 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 2000 $\times g$ for 5 minutes. The absorbance of red pigment in the supernatant fractions was estimated at 532 nm. The concentration of TBARS was calculated using the extinction coefficient of 165,000 $\text{mol} \cdot \text{cm}^{-1}$ and expressed as μmol of malondialdehyde equivalents per mg LDL protein.

Statistical analysis. Results are expressed as the mean \pm SD. Degree of LDL oxidation by copper ions in absence (as control) and presence of glucose was compared using ANOVA test. $P < 0.05$ was assumed to be statistically significant.

RESULTS

LDL isolation. Isolation on LDL was assessed by measurement of lipid concentration (Table 1). As shown in this Table, total cholesterol and LDL cholesterol amounts were increased in LDL

preparation approximately 65% and 75%, respectively. This measurement can confirm the isolation of LDL from plasma pool. This isolation was also confirmed by agarose gel electrophoresis (Fig. 1). Figure 1 shows the separated fraction (Lane 2) according to LDL band in plasma, as control (Lane 1).

The best glucose concentration and incubation time on LDL glycation process. The best glucose concentration for incorporation with LDL was investigated by incubation of varying concentrations of glucose (0-180 mM) with LDL (0.2 mg protein.ml⁻¹) in PBS containing 1 mM EDTA, pH 7.4, at 37°C for 7 days under sterile conditions. The best incubation time for LDL glycation was also investigated by incubation 0.2 mg protein per 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 sterile condition [9]. The optimum glucose concentration and incubation time were obtained for LDL glycation 120 mM and 7 days, respectively (data are not shown).

The best copper concentration on LDL oxidation process. To examine the best copper concentration on LDL oxidation, LDL (0.2 mg protein.ml⁻¹) was incubated at 37°C for 4 hours with various concentrations of CuSO₄ (0-10 μ M), in 10 mM PBS, pH 7.4, without EDTA [12]. According to this study, 4 μ M copper had highest effect on copper induced LDL oxidation (data are not shown).

Influence of glucose on LDL oxidation process. A series of experiments was performed to examine the influence of glucose on LDL oxidation process by copper ions. In first experiments, LDL (0.2 mg protein.ml⁻¹) was incubated at 37°C for 4 hours with copper (4 μ M) and various concentrations of glucose (0-180 mM), in 10 mM PBS, pH 7.4, without EDTA.

Table 1. Lipid profile of plasma pool and LDL preparation.

Parameters	Plasma pool	LDL preparation
Total cholesterol (mg.dl ⁻¹)	167 \pm 2.0	480 \pm 2.5
Triglyceride (mg.dl ⁻¹)	127 \pm 1.5	60 \pm 0.5
LDL-Cholesterol (mg.dl ⁻¹)	104 \pm 1.3	430 \pm 1.8
HDL-Cholesterol (mg.dl ⁻¹)	38 \pm 0.9	22 \pm 0.4

Values are mean \pm SD of triplicate determination on plasma and separated LDL. LDL, very low density lipoprotein; HDL, high density lipoprotein.

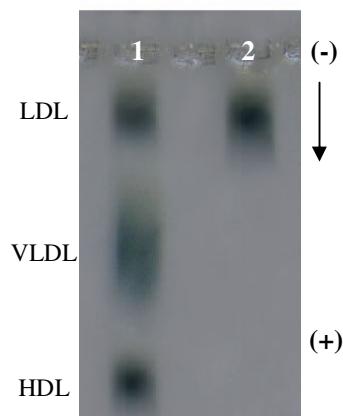


Fig. 1. Electrophoresis analysis of plasma pool (Lane 1) and LDL preparation (Lane 2) on 0.8% agarose gel. LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

LDL oxidation at absence (as control) and/or presence of glucose was monitored by changes in TBARS (Fig. 2A). Oxidation of LDL decreased in presence of 20-180 mM of glucose concentrations. As shown in Figure 2A, this reduction was dependent on glucose concentration. Glucose presence in comparison to the control (without glucose) was shown a significant inhibition of copper induced LDL oxidation by the ANOVA test, $P<0.005$. According to this study, 20-180 mM glucose concentrations are able to reduce LDL oxidation approximately 14% to 93%, respectively (Fig. 2B). In second series of experiments we investigated the effect of glycated LDL on LDL oxidation by copper ions. After incubation of LDL (0.2 mg protein.ml⁻¹) with 120 mM glucose at 37°C for 7 days, in 10 mM PBS, pH 7.4, without EDTA, under sterile conditions, LDL solutions were exposed to with 2-10 μ M copper concentrations. Four hours after copper addition, the extent of LDL oxidation was estimated from TBARS measurements as shown in Figure 3. This experiment was also performed by incubation of native LDL with 2-10 μ M copper concentrations at 37°C for 4 hours, as control (Fig. 3A). The addition of copper to the incubation medium of glycated LDL led to accelerated LDL oxidation. As shown in Figure 3A, oxidation of glycated LDL by copper increased significantly (ANOVA test, $P<0.003$) in a dose dependent manner. The comparison of glycated and native LDL oxidation by 4 μ M copper showed copper ions are able to increase LDL oxidation approximately 56% (Fig. 3B).

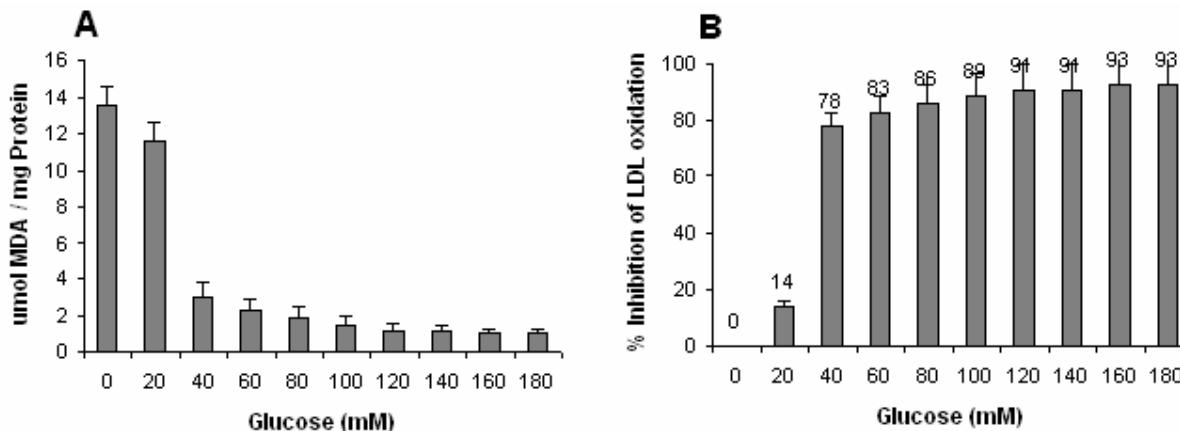


Fig. 2. (A) Effect of glucose on LDL oxidation induced by 4 μ M copper and (B) The comparison of inhibition percent of copper induced LDL oxidation in absence and presence of 20-180 mM glucose. Values are mean \pm SD of at least three similar experiments performed with LDL preparations. Figure 2B was obtained by Figure 2a data.

Relative electrophoretic mobility. In another set of experiments, we studied the electrophoretic mobility and flow rate of native, glycated, oxidized and glycol-oxidized LDL on polyacrylamide gel (Fig. 4). As Figure 4 shows, electrophoretic mobility glycated, oxidized and glycol-oxidized LDL were enhanced compared to native LDL (as control) and also flow rate glycated, oxidized and glycol-oxidized LDL were increased when compared to native LDL approximately 40.5%, 60%, and 69%, respectively. These observations suggest that glycation of LDL can increase LDL oxidation in presence of copper ions.

DISCUSSION

The oxidation of LDL has been extensively studied during the past years and various *in vitro* models have been developed in an attempt to better

understand the *in vivo* situation in relation to the potential role of LDL oxidation in pathological or prepathological situations, particularly in atherosclerosis [15, 16]. Much attention has been devoted to the oxidation of LDL by copper ions which are widely used as a model system [17]. Our main goal was to investigate the effect of glucose on the copper induced oxidation of LDL by model system *in vitro*. The oxidant properties of glucose have been studied, which suggests this sugar has a role in human atherosclerosis and the risk of atherosclerosis is increased in patients with diabetes mellitus [18]. Glucose was used as glycated agent, which is commonly adopted in many Millard reaction studies [19]. Apo-B₁₀₀ LDL, the amine source could serve as a target for glycated agents.

Considering that glucose adducts mainly with ϵ -amino group of lysine residues of which about 90%

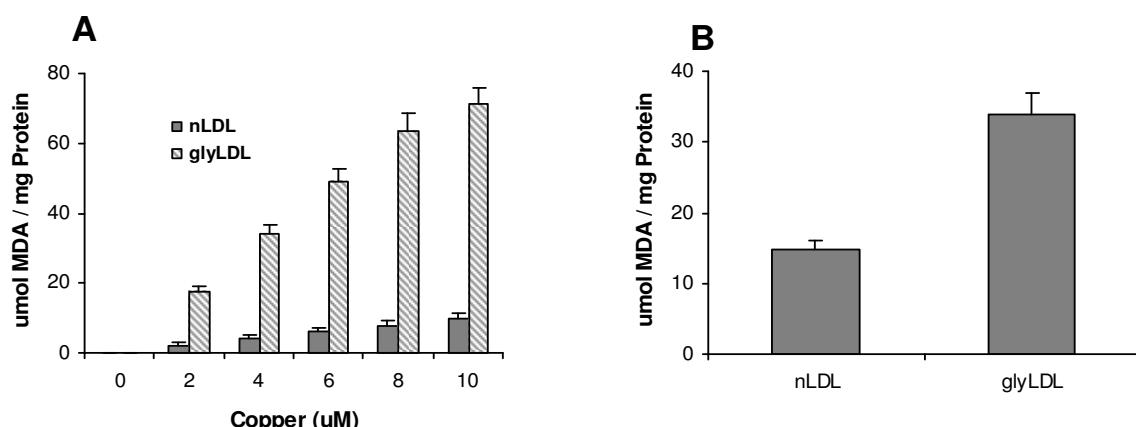


Fig. 3. (A) Effect of copper on native and glycated LDL oxidation and (B) The comparison of the effect of 4 μ M copper on native LDL (nLDL) and glycated LDL (glyLDL) oxidation. Values are the means \pm SD of at least three similar experiments performed with LDL preparations. Figure 3B was obtained by Figure 3A data.

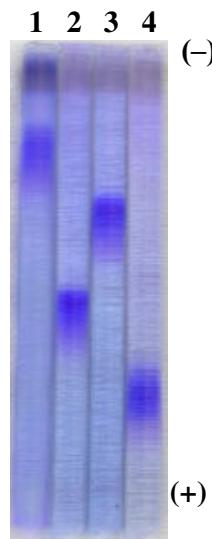
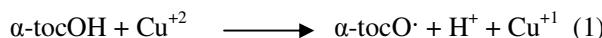


Fig. 4. Analysis of native LDL (Lane 1), oxidized LDL (Lane 2), glycated LDL (Lane 3) and glycooxidized LDL (Lane 4) on 5% polyacrylamide gel by electrophoresis.

are contained in apo-B₁₀₀ [20]. Results of the present study revealed that the addition of synchronous glucose and copper to LDL solutions led to decreased LDL oxidation by copper ions. According to the previous reports, copper (II) bound to LDL is necessary for catalyzing the oxidation of LDL by this metal [12, 21]. There is evidence that Cu⁺² ions may oxidize LDL by extracting an electron and proton from α -tocopherol within LDL and forming α -tocopheroxyl radical (reaction 1) [22].



The α -tocopherol radical may extract a hydrogen atom from a polyunsaturated fatty acid (reaction 2), thereby initiating a free radical cascade of lipid peroxidation.



In the presence model, we hypothesize that glucose can generate Cu⁺¹ by reducing the Cu⁺². The copper (I) is low affinity to LDL [21], thus it decreases binding of this ion to LDL and thereby may lower the rate of reaction 1 and 2. Therefore, glucose by this mechanism may inhibit the susceptibility of LDL oxidation to copper ions. This view is supported by other study that has suggested a mechanism which glucose could serve as an antioxidant by involves its interference with copper binding to LDL [23]. We also showed that the addition of copper ions to glycated LDL led to increased LDL oxidation. It is acknowledged that after binding of copper (II) ions to LDL, Cu⁺²

reductions to Cu⁺¹ is required for triggering lipid peroxidation in LDL [24]. In glycated LDL, glucose forms the Amadori compound, fructoselysine. The Amadori compound is then subject to 1, 2-enediol rearrangement with carbohydrate hydrolysis and formation of the dicarbonyl compound, D-glucosone [25]. Copper (II) ion reduction may result from enediol oxidation and/or direct reduction by the Amadori compound itself, glucosone [25]. Therefore, we suggested that glycated LDL may reduced Cu⁺² to Cu⁺¹ and the Cu⁺¹ reacted faster with lipid hydroperoxides (reaction 3) than did the Cu⁺² (reaction 4) [26].



Our study also showed that glycated, oxidized and glycol-oxidized LDL had increased in mobility on polyacrylamide gel, which probably resulted from the increased negative charge caused by the modification of lysine amino groups. The increased electrophoretic mobility is in agreement with the finding of Witztum *et al.* [27]. The presence of copper ions increased rate of flow glycated LDL compared to control (native LDL). Thus, these results support the suggestion that glucose play an important role after LDL glycation in enhancement of LDL oxidation by copper ions. In conclusion, glucose inhibited LDL oxidation by copper in a simple phosphate buffer. This inhibition may be due to (1) the rapid conversion of Cu⁺² to Cu⁺¹ and thereby (2) a decrease in copper binding to LDL, which may prevent the formation of the pro-oxidant α -tocopheroxyl radical in LDL. The pro-oxidant effect of glucose (after LDL glycation) toward oxidized LDL may be explained by the reduction of Cu⁺² to Cu⁺¹, followed by rapid breakdown of preformed lipid hydroperoxides by Cu⁺¹ ions to form lipid radical, thereby propagation lipid peroxidation. The present study support the hypothesis that hyperglycemia stimulates LDL oxidation accounting, at least in part, for the increased atherosclerosis risk in type I and II diabetes. However, further studies will be needed to determine if this applies *in vivo*.

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