Effects of Glucose and α-Tocopherol on Low-Density Lipoprotein Oxidation and Glycation

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ABSTRACT: Glycation of blood proteins is considered to be a major contributor to hyperglycemic complications in diabetes mellitus patients. In this study, we demonstrate the efficacy of α-tocopherol in reducing low-density lipoprotein (LDL) oxidation and glycation *in vitro*. Native LDL isolated from healthy subjects was exposed to various concentrations of glucose and malondialdehyde (MDA) with or without α-tocopherol enrichment for 7 days in sealed vacuum ampoules. The degree of glycation, copper-induced lag time, content of thiobarbituric acid–reactive substances (TBARS), and α-tocopherol levels in LDL were then assessed. LDL lag time was significantly reduced with high levels of glucose and MDA. α-Tocopherol enrichment dramatically inhibited the oxidation of LDL in the lag-time assay. However, the length of incubation time was inversely related to the LDL lag time. Longer incubation time resulted in shorter LDL lag time, with or without α-tocopherol enrichment. The level of TBARS associated with LDL oxidation was highest in native, MDA-supplemented, and high-glucose samples. The α-tocopherol levels were inversely related to glucose levels and incubation times. In conclusion, high-glucose concentrations heightened the oxidative susceptibility of LDL. α-Tocopherol enrichment reduced this trend and prevented LDL from undergoing architectural modification.

KEYWORDS: α-tocopherol; glucose; LDL; oxidation; glycation

INTRODUCTION

The primary therapeutic goal in treating diabetic patients is to maintain an optimal blood glucose level. Recently, therapies of diabetes have been extended to prevention of complications of diabetes, including macrovascular disorders secondary to accelerated atherogenesis in large vessels,[1–3] as well as microvascular disturbances such as foot ulcers and retinopathy.[4] The rationale for placing a high priority on controlling blood glucose levels in diabetic patients is to prevent aberrant and irreversible sugar–protein alterations. Poorly controlled blood glucose levels with enhanced oxidative capability of glucose modification of proteins have been proposed

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to be instrumental in the development of vascular complications in diabetes. These pathological processes may involve lipid peroxidation and irreversible oxidative modifications of proteins (i.e., collagen and low-density lipoprotein [LDL]).[5,6] However, the precise mechanisms that facilitate these complications of diabetes are poorly understood. High blood-glucose levels favor nonenzymatic reactions, resulting in the generation of low-molecular-weight aldehydes such as methylglyoxal and malondialdehyde.[7,8] Proteins' interaction with reducing sugars (i.e., glucose) and lipids in nonenzymatic manners may also generate Amadori products, leading to the formation of advanced glycation end products (AGEs). Amadori products as well as reactive oxygen species (ROS), such as superoxide and hydrogen peroxide,[9–11] are likely to be involved in glycation-induced enhancement of the oxidative modifications of LDL. Nonenzymatic modification of proteins can contribute to the dysfunction of functional proteins such as lipoprotein, collagen, and membrane components.[12,13]

In vitro and *in vivo* studies have shown that hyperglycemia leads to increased oxidative stress and endothelial dysfunction. In addition, many studies suggest that patients with diabetes appear to have a decreased antioxidant capability reflected by lower levels of vitamin E, glutathione, and reduced activities of antioxidant enzymes, such as catalase, superoxide dismutase, or glutathione peroxidase. α-Tocopherol is considered to be a strong antioxidative reagent that may be salutary to slow down progression of diseases that involve oxidative stresses. However, the role of α-tocopherol in preventing LDL oxidation and glycation has not been fully explored. In this study, we sought to demonstrate the efficacy of α-tocopherol in delaying or reducing LDL oxidation caused by high glucose levels under heightened oxidative states.

MATERIALS AND METHODS

Materials

All solutions were prepared with deionized distilled water (Milli Q system; Millipore-Waters, Billerica, MA) prior to use. Glucose, α-tocopherol, EDTA, $CuCl_2$, and malondialdehyde (MDA) were purchased from Sigma (St. Louis, MO). The analytical columns (Poly-Prep Chromatography Column; Bio-Rad, Hercules, CA) for LDL glycation were obtained from Pierce Chemical Company (Rockford, IL). All other chemicals were of analytical grade and all solvents were of high-performance liquid chromatography (HPLC) grade from TEDIA (Fairfield, OH) with the highest purity and laboratory standards.

LDL Preparation

Native LDL

Ten healthy subjects were recruited from clinics at the Municipal Wan-Fang Hospital, Taipei, Taiwan. Blood (30 mL) was drawn and centrifuged to collect native LDL. LDL (density, 1.019–1.063 g/mL) was prepared by density gradient ultracentrifugation as described previously.[14] After isolation, LDL was dialyzed with an appropriate volume (corresponding to 1000-fold dilution of the LDL fraction) of phosphate-buffered saline (PBS; 8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of Na_2HPO_4, and 0.21 g/L of NaH_2PO_4, pH 7.4) for 4 h at 4°C twice. Dialyzed LDL fractions were pooled and dialyzed with 2000-fold volume of PBS for 16 h at 4°C. The redialyzed LDL fractions (native LDL) were pooled for glycation and oxidation assays. The protein content was analyzed using a commercial kit from Bio-Rad.

Vitamin E–Enriched LDL

Partial plasma was fortified with a predetermined volume (10 μL of dimethyl sulfoxide [DMSO]/mL of serum) of a prepared vitamin E solution (250 μmol α-tocopherol acetate/L of DMSO) and incubated at 37°C under shaking, in a germ- and oxygen-free atmosphere for 4 h, as described elsewhere.[15] Preparation of the vitamin E–enriched plasma followed the previously described procedures of LDL preparations.[16] The final LDL fraction was named vitamin E–enriched LDL.

Assays of LDL Glycation and Oxidation

Native and vitamin E–enriched LDL fractions were incubated at 37°C with different levels of glucose and MDA with shaking, in germ- and oxygen-free sealed ampoules for 7 days. Samples were assayed at different time intervals (on days 0, 1, 3, and 7). Assays to determine the degree of glycation, copper-induced lag time, content of thiobarbituric acid–reactive substances (TBARS), and α-tocopherol levels were conducted as reported previously.[17]

Incubated samples were separated into glycated (fraction A) and nonglycated (fraction B) LDL via *m*-aminophenyl boronate affinity chromatography. The partially modified step-by-step procedures followed those described elsewhere.[18] In brief, fractions were measured at 414 nm, and absorbance (optical density [OD]) was calculated to derive the degree of LDL glycation. The degree of LDL glycation (%) was expressed as [OD of fraction A × 2/(OD of fraction A × 2 + OD of fraction B × 5.9)] × 100%.

To measure the oxidative stress of LDL, we determined the level of MDA, a product of lipid peroxidation that reacts with TBARS, based on the MDA–TBARS complex at 532 nm with a spectrophotometer (Hitachi U2000; Hitachi, Tokyo, Japan); the detailed preparation procedures for measuring the MDA–TBA complex are described elsewhere.[17] Quantities of MDA presented in the results were adjusted based on the LDL protein content.

In vitro oxidizability of LDL was evaluated by a spectrophotometric technique described by Esterbauer *et al.*[15] Glucose-treated LDL samples were adjusted to a protein concentration of 200 μg/mL of PBS in the presence of 5 mM of Cu^{2+}. The oxidation kinetics was monitored by the change in absorbance at 234 nm with a Hitachi spectrophotometer (Hitachi U2000) at room temperature for 180 min. The oxidative resistance of LDL was assessed in terms of the period when no oxidation occurred (lag phase) and was calculated as the intercept of extrapolations of the parts of the curve representing the lag and propagation phases. The propagation rate was measured from the slope of the absorbance curve during the propagation phase based on the levels of conjugated dienes.

Vitamin E levels in serum were measured by HPLC (Hitachi L-7100 with an L-7420 UV/VIS detector) with a reversed-phase C_{18} column (Vydac 201TP54 C_{18}; 4.6 × 250 mm; 5 µm) with a 100% methanol mobile phase, 1 mL/min flow rate, and detection at 292 nm.[19]

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences, version 9.0 (SPSS, Chicago, IL). Results are expressed as mean ± standard deviation. The difference between the means of samples incubated under different conditions was analyzed statistically using analysis of variance and Fisher's test. Statistical calculations were conducted using the Student's t test for paired observations. A P value less than .05 was considered statistically significant.

RESULTS

The glycation level of LDL increased significantly with 25 and 50 mM glucose on day 7 ($P < 0.05$); with the addition of MDA, the glycation level was significantly elevated with 25 mM and 50 mM glucose on day 3 ($P < 0.05$) and day 7 ($P < 0.01$). The lag time dramatically decreased with high glucose concentrations (25 and 50 mM) and longer incubation times (days 3 and 7) (TABLES 1 and 2). The TBARS formation associated with LDL oxidation was highest in native, MDA-supplemented, and high-glucose level samples. α-Tocopherol levels were inversely correlated with the glucose level (25 mM, $P < 0.01$; 50 mM, $P < 0.001$) and incubation time (day 3, $P < 0.01$; day 7, $P < 0.001$) (TABLE 2). α-Tocopherol enrichment dramatically inhibited the oxidation of LDL as reflected by lag-time prolongation (25 mM, $P < 0.01$; 50 mM, $P < 0.001$). However, the incubation time was inversely related to the LDL lag time, with longer incubation time resulting in shorter LDL lag time with or without α-tocopherol enrichment.

DISCUSSION

In this study, the volunteers were healthy and were not known to be suffering from hyperlipidemia, hyperglycemia, or other metabolic diseases. The normal biochemical characteristics of these blood samples were ascertained based on fasting blood sugar, hemoglobin A_{1c} (HbA_{1c}), total cholesterol, LDL, HDL, and total triglyceride levels that were within reference ranges. Among key blood proteins, LDL appeared to be more susceptible to glucose modification than Hb in streptozotocin-induced hyperglycemic rats in a glucose concentration–dependent response.[20] This finding suggests that the extent of LDL glycation reflects the oxidation status better than HbA_{1c} in a short-term study. In our study, subjects were healthy, with stable HbA_{1c} levels (3.78 ± 0.79%) that were within the reference range; the pooled LDL representing native LDL may have excluded many confounding factors such as difference in glycation level and cholesterol content among subjects. Variation among subjects could have affected the subsequent *in vitro* glycation studies.

TABLE 1. Glycation levels and vitamin E contents of LDL under various levels of glucose incubation[1,2]

Treatment	Glucose conc. (mM)	Glycation (%)				Vitamin E concentration (nmol/mg protein)			
		Day 0	Day 1	Day 3	Day 7	Day 0	Day 1	Day 3	Day 7
Native LDL	0	3.21 ± 0.29	3.54 ± 0.42	3.93 ± 0.26	3.83 ± 0.25	16.7 ± 0.5^{a}	8.6 ± 0.2^{b}	7.6 ± 0.2^{c}	3.8 ± 0.3^{d}
	5	3.46 ± 0.12	3.60 ± 0.17	4.26 ± 0.48	4.25 ± 0.33	$11.5 \pm 1.0^{a*}$	$7.7 \pm 0.2^{b*}$	$6.9 \pm 0.3^{c*}$	$3.4 \pm 0.2^{d*}$
	25	3.36 ± 0.23^{a}	3.96 ± 0.62^{a}	4.00 ± 0.31^{a}	$5.34 \pm 0.44^{b*}$	$9.3 \pm 0.6^{a*}$	$7.1 \pm 0.3^{b*}$	$6.8 \pm 0.1^{c*}$	$2.8 \pm 0.1^{d*}$
	50	3.91 ± 0.23^{a}	3.98 ± 0.60^{a}	4.68 ± 0.12^{a}	$5.47 \pm 0.34^{b*}$	$8.6 \pm 0.4^{a*}$	$6.6 \pm 0.1^{b*}$	$6.2 \pm 0.1^{b*}$	$2.6 \pm 0.1^{c*}$
Native LDL with MDA	0	3.95 ± 0.19	3.85 ± 0.53	3.98 ± 0.11	3.93 ± 0.26	9.8 ± 0.8^{a}	6.8 ± 0.2^{b}	6.8 ± 0.2^{b}	3.0 ± 0.2^{c}
	5	3.84 ± 0.37	4.10 ± 0.80	4.44 ± 0.25	4.77 ± 0.60	8.7 ± 0.5^{a}	6.5 ± 0.2^{b}	$6.2 \pm 0.2^{b*}$	$2.4 \pm 0.1^{c*}$
	25	3.79 ± 0.69^{a}	4.34 ± 0.14^{a}	$4.97 \pm 0.25^{b*}$	$7.58 \pm 0.27^{c*}$	$7.7 \pm 0.4^{a*}$	6.4 ± 0.2^{b}	$6.3 \pm 0.1^{b*}$	$1.7 \pm 0.2^{c*}$
	50	3.84 ± 0.48^{a}	4.58 ± 0.16^{a}	$5.08 \pm 0.13^{a*}$	$8.90 \pm 0.29^{c*}$	$6.6 \pm 0.3^{a*}$	6.3 ± 0.1^{b}	$3.9 \pm 0.0^{c*}$	$1.7 \pm 0.1^{d*}$
Vit E-enriched LDL	0	3.65 ± 0.22	3.88 ± 0.50	3.80 ± 0.24	3.67 ± 0.19	68.7 ± 0.6^{a}	47.8 ± 0.4^{b}	25.6 ± 1.1^{c}	20.7 ± 0.7^{d}
	5	3.51 ± 0.19	3.87 ± 0.48	4.03 ± 0.13	4.06 ± 0.17	$25.7 \pm 1.4^{a*}$	$20.4 \pm 0.4^{b*}$	$16.9 \pm 1.4^{c*}$	$14.2 \pm 0.3^{c*}$
	25	3.89 ± 0.13	3.89 ± 0.29	4.18 ± 0.67	$4.78 \pm 0.16^{*}$	$22.9 \pm 1.2^{a*}$	$18.4 \pm 0.6^{b*}$	$11.3 \pm 0.2^{c*}$	$10.8 \pm 0.5^{c*}$
	50	3.69 ± 0.26^{a}	3.97 ± 0.36^{a}	4.37 ± 0.50^{a}	$5.13 \pm 0.18^{b*}$	$23.0 \pm 0.8^{a*}$	$18.2 \pm 0.3^{b*}$	$10.1 \pm 0.5^{c*}$	$7.9 \pm 0.4^{d*}$
Vit E-enriched LDL with MDA	0	3.44 ± 0.91	3.87 ± 0.82	4.07 ± 0.30	4.28 ± 0.18	66.1 ± 0.9^{a}	36.1 ± 1.6^{b}	21.3 ± 0.5^{c}	13.1 ± 0.4^{d}
	5	3.61 ± 0.08^{a}	3.85 ± 0.23^{a}	4.12 ± 0.41^{a}	4.31 ± 0.19^{b}	$19.5 \pm 0.4^{a*}$	$11.3 \pm 0.7^{b*}$	$10.0 \pm 0.6^{b*}$	$9.5 \pm 0.2^{c*}$
	25	3.98 ± 0.16^{a}	4.16 ± 0.26^{a}	4.54 ± 0.34^{a}	$5.61 \pm 0.57^{b*}$	$16.5 \pm 0.2^{a*}$	$10.0 \pm 0.2^{b*}$	$9.1 \pm 0.5^{c*}$	$8.9 \pm 0.8^{c*}$
	50	4.15 ± 0.16^{a}	4.31 ± 0.44^{a}	$5.07 \pm 0.28^{b*}$	$6.04 \pm 0.06^{c*}$	$16.0 \pm 0.2^{a*}$	$8.2 \pm 0.4^{b*}$	$8.0 \pm 0.3^{b*}$	$7.1 \pm 0.2^{c*}$

[1]Superscripts in rows with different letters indicate a significant difference from the day 0 value ($P < 0.05$).
[2]Superscripts in columns with an asterisk "*" indicate a significant difference from the glucose-free status ($P < 0.05$).

TABLE 2. Oxidative status of LDL under various levels of glucose incubation[1,2]

Treatments	Glucose conc. (mM)	LDL lagtime (min)				TBARs (nmol MDA/mg protein)			
		Day 0	Day 1	Day 3	Day 7	Day 0	Day 1	Day 3	Day 7
Native LDL	0	41.8 ± 4.1	40.6 ± 3.2	41.6 ± 2.4	37.2 ± 4.0	0.84 ± 0.08^{a}	0.93 ± 0.04^{a}	2.56 ± 0.19^{b}	3.00 ± 0.27^{c}
	5	41.9 ± 2.4^{a}	40.0 ± 3.3^{a}	41.0 ± 3.3^{a}	33.7 ± 2.4^{b}	0.94 ± 0.09^{a}	0.98 ± 0.12^{a}	2.73 ± 0.20^{b}	6.93 ± 0.83^{c*}
	25	40.1 ± 3.2^{a}	36.1 ± 1.6^{a}	38.0 ± 2.4^{a}	31.3 ± 1.8^{b}	1.01 ± 0.18^{a}	1.10 ± 0.19^{a}	2.76 ± 0.22^{b}	7.78 ± 0.88^{c*}
	50	41.3 ± 2.4^{a}	34.6 ± 2.1^{b}	30.0 ± 2.5^{b*}	28.4 ± 2.0^{c*}	1.08 ± 0.16^{a}	1.08 ± 0.19^{a}	3.31 ± 0.21^{b*}	9.11 ± 0.68^{c*}
Native LDL with MDA	0	41.1 ± 1.4^{a}	39.7 ± 2.8^{a}	35.8 ± 3.2^{a}	33.5 ± 3.2^{b}	0.94 ± 0.18^{a}	1.25 ± 0.21^{a}	5.12 ± 0.24^{b}	9.65 ± 0.65^{c}
	5	39.7 ± 4.0^{a}	35.4 ± 2.5^{a}	34.6 ± 3.1^{a}	30.3 ± 2.9^{b}	1.06 ± 0.30^{a}	1.27 ± 0.25^{a}	5.36 ± 0.29^{b}	9.99 ± 0.64^{c}
	25	38.9 ± 1.9^{a}	31.2 ± 1.8^{b*}	27.6 ± 3.1^{b*}	25.7 ± 1.6^{b*}	1.19 ± 0.09^{a}	1.32 ± 0.28^{a}	5.82 ± 0.12^{b}	11.84± 1.35^{c*}
	50	38.5 ± 2.3^{a}	28.0 ± 2.5^{b*}	22.8 ± 1.8^{c*}	21.0 ± 2.2^{c*}	1.18 ± 0.21^{a}	1.42 ± 0.26^{a}	7.44 ± 0.79^{b*}	11.58± 0.61^{c*}
Vit E-enriched LDL	0	58.1 ± 3.9	50.2 ± 2.8	50.2 ± 3.0	49.8 ± 2.7	0.77 ± 0.07^{a}	0.82 ± 0.11^{a}	1.83 ± 0.09^{b}	2.15 ± 0.13^{c}
	5	55.2 ± 3.4^{a}	49.8 ± 2.2^{a}	49.0 ± 1.6^{a}	47.4 ± 3.2^{b}	0.78 ± 0.08^{a}	0.87 ± 0.00^{a}	1.78 ± 0.07^{b}	2.28 ± 0.14^{c}
	25	53.9 ± 3.5^{a}	48.0 ± 2.8^{a}	46.1 ± 2.4^{b*}	41.7 ± 2.2^{c*}	1.00 ± 0.12^{a}	0.89 ± 0.11^{a}	1.93 ± 0.14^{b}	2.47 ± 0.25^{c}
	50	49.8 ± 2.7^{a}	45.7 ± 2.7^{a}	43.3 ± 3.1^{a*}	40.6 ± 5.3^{b*}	1.13 ± 0.17^{a*}	1.19 ± 0.17^{a*}	1.97 ± 0.13^{b}	2.95 ± 0.17^{c*}
Vit E-enriched LDL with MDA	0	57.5 ± 4.3^{a}	51.4 ± 3.6^{a}	49.8 ± 3.0^{a}	44.9 ± 3.6^{b}	0.84 ± 0.08^{a}	1.09 ± 0.14^{a}	1.83 ± 0.29^{b}	2.89 ± 0.38^{c}
	5	53.2 ± 2.5	49.0 ± 3.2	47.7 ± 2.6	45.2 ± 4.2	0.79 ± 0.02^{a}	1.08 ± 0.27^{a}	1.93 ± 0.29^{b}	2.48 ± 0.21^{c}
	25	49.7 ± 3.0	48.7 ± 2.5	44.0 ± 5.1	38.5 ± 1.8^{a}	0.83 ± 0.14^{a}	1.13 ± 0.24^{a}	1.95 ± 0.20^{b}	3.02 ±0.26^{c}
	50	48.8 ± 2.8^{a*}	45.5 ± 2.2^{a}	43.4 ± 3.6^{a}	36.3 ± 2.6^{b*}	1.08 ± 0.06^{a}	1.24 ± 0.19^{a}	2.18 ± 0.05^{b}	3.41 ± 0.30^{c*}

[1]Superscripts in rows with different letters indicate a significant difference from the day 0 value ($P < 0.05$).
[2]Superscripts in columns with an asterisk "*" indicate a significant difference from the glucose-free status ($P < 0.05$).

When proteins react with reducing sugars (i.e., glucose) and lipids in a series of nonenzymatic reactions, they generate Amadori products, ultimately leading to the formation of advanced glycation end products (AGEs). Amadori products as well as ROS, such as superoxide and hydrogen peroxide,[9–11,21] are likely to be involved in glycation-induced enhancement of the oxidization modification of LDL. In our study, we applied 5 (90 mg/dL), 25 (450 mg/dL), and 50 (900 mg/dL) mM glucose to simulate normoglycemic and hyperglycemic status and to evaluate glucose concentration–dependent modifications of LDL. Not surprisingly, formation of glycated LDL increased with increasing glucose level and incubation time. The incubation time and the level of glucose are the two major contributing factors to the glycation of heterogeneous compounds. Much has been shown to suggest that the complications of diabetes are associated with oxidative stress induced by the generation of free radicals.[9,11] Glycated LDL, resembling modified LDL, is highly prone to oxidation. Glycation and oxidation processes are closely linked and occur simultaneously in diabetes, accelerating LDL oxidization. Studies have reported that some stable chronic patients such as those with cardiovascular disease and diabetes not only had a shortened oxidative lag phase during copper-catalyzed oxidation of LDL but also showed elevated TBARS levels in the blood.[23,24] Vitamin E exerts antioxidant actions in chronic diseases associated with heightened oxidative stress.[17] In this study, with or without α-tocopherol enrichment, a significant increase in MDA production by LDL and a decrease in the LDL lag time at various glucose levels and incubation times were observed. The results suggest that higher glucose levels and longer incubation times contributed to the irreversible architectural alterations of LDL. Some clinical studies[25,26] showed that diminished vitamin E levels were accompanied by increased LDL susceptibility to chemical modification in type 2 diabetes mellitus patients with or without macrovascular complications. Free radicals (i.e., superoxide) and their byproduct, MDA, another ROS, may both contribute to the initial step of glycation, chemically modifying fat-enriched albumin to accelerate lipid oxidation.[27] Chemical modification of proteins by ROS results in the formation of more susceptive carbonyl residues and MDA, which contribute to enzyme and lipoprotein dysfunction.

In conclusion, high glucose levels heightened the oxidative susceptibility of LDL. α-Tocopherol enrichment reduced the oxidative susceptibility of LDL and reduced LDL architectural modification.

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