Free Rad. Res., Vol. 32, pp. 439–449
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The Role of Antioxidants in the Long-Term Glycation of Low Density Lipoprotein and its Cu^{2+}-Catalyzed Oxidation

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Accepted by Prof. H. Sies

(Received 19 May 1999; In revised form 27 September 1999)

In the present study we investigated the influence of antioxidants such as EDTA, α-tocopherol, troglitazone and acetylsalicylic acid on the long-term-glycation of LDL and its copper ion-catalyzed oxidation. We observed that (a) all antioxidants inhibited AGE-formation, while Amadori product formation was only diminished by extreme concentrations of acetylsalicylic acid, (b) glycated LDL was more susceptible to copper-catalyzed oxidation than unglycated LDL, and (c) the oxidation of native LDL was more dramatically inhibited by the antioxidants than that of glycated LDL. The observed differences may be a consequence of the significantly higher endogenous content in hydroperoxides of glycated LDL as compared to native LDL. Therapeutic implications of these findings regarding vitamin E, which is supposed to slow atherogenesis and the development of microvascular complications in diabetes, are obvious: Vitamin E-monotherapy, while blocking oxidative and AGE-modification of LDL, is unable to inhibit its AP-formation. As a consequence, tocopherol is susceptible to increased consumption by AP-associated radical production in hyperglycemic patients, which could be checked in part by the tocopherol-protecting agent troglitazone and/or by acetylsalicylic acid.

Keywords: LDL-oxidation, LDL-glycation, advanced glycation end products, α-tocopherol, troglitazone, acetylsalicylic acid

Abbreviations: AGE, advanced glycation end product; AP, Amadori product; ASA, acetylsalicylic acid; EDTA, ethylenediaminetetraacetic acid; FA, fructosamine; LDL, low density lipoprotein; g-LDL, glycated LDL; n-LDL, "native" LDL; MDA, malondialdehyde; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; T, troglitazone; TOC, α-tocopherol; TBARS, thiobarbituric acid reactive substances

INTRODUCTION

Oxidative modifications of low density lipoprotein (LDL) are believed to play an important role in atherogenesis and to contribute significantly to the long-term vascular complications in diabetes and aging. Unlike native LDL, oxidatively modified LDL can recruit circulating monocytes, and then, through uptake via the scavenger–receptor

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pathway, transform them into foam cells that characterize early atherosclerotic lesions.[1] Together with lymphocytes and intimal smooth muscle cells the foam cells form fatty streaks within the arterial intima and complex proliferative lesions develop progressively causing eventually arterial insufficiency and occlusion.[1,2]

The mechanism of initiation of LDL-oxidation *in vivo* is largely a matter of speculation. Under diabetic conditions, the Maillard reaction facilitates the production of reactive oxygen species (ROS) and the activity of antioxidant enzymes such as superoxide dismutase is decreased, resulting in a remarkable increase in oxidative stress.[3] Based on the known chemistry of Schiff base and Amadori products (AP) formed during the early phase of nonenzymatic glycation, it is hypothesized that such early glycation products on cells or matrix proteins in the arterial wall could themselves generate free radicals capable of oxidizing lipids. Indeed, free radicals generated by glycated protein increase peroxidation of membranes of linoleic/arachidonic acid vesicles *in vitro* nearly two-fold over control, suggesting that glycation of proteins in diabetes may accelerate vascular wall lipid oxidative modification.[4]

LDL-glycation was first described by Schleicher *et al.*[5] These authors demonstrated that patients with diabetes mellitus undergo increased *in vivo* glycation of apolipoprotein B, a finding that gave rise to the hypothesis that lipoprotein glycation contributes to the accelerated atherosclerosis characteristic of diabetes mellitus. Diabetic patients exhibit high circulating levels of advanced glycation end products (AGEs).[6] Protein-linked AGEs cross-link connective tissue collagen[7] and act as recognition signals for AGE receptor systems present on different cell types.[6,8,9] In general, AGEs are thought to contribute to the development of pathologies associated with both diabetes and aging, such as cataract, retinopathy, nephropathy and vascular disease.[10]

Endothelial dysfunction or activation elicited by oxidatively modified LDL has been implicated in the pathogenesis of atherosclerosis, characterized by intimal thickening and lipid deposition in the arteries. In diabetics, endothelial cell activation may also be induced by AGE-modification of connective tissue molecules such as fibronectin.[11] LDL from diabetic individuals showed significantly greater oxidative modification than the LDL from nondiabetic individuals[12] and was also more significantly glycated.[13,14]

In vivo, the polyunsaturated fatty acids (PUFA) in LDL are protected against free radical attack and oxidation by a number of lipophilic antioxidants. On a molar base, by far the major antioxidant is α-tocopherol (TOC). The antioxidant content of LDL varies, similarly to the PUFA, significantly between individuals.[15] While the effects of various "biological" chain-breaking antioxidants such as TOC, β-carotene or ascorbate on the oxidation of LDL have been extensively investigated,[15,16] similar studies on the potential modulation of LDL-glycation by these antioxidants are rare and controversial.[17,18] In contrast to metal-catalyzed oxidation, LDL-glycation by glucose up to AGE-formation is a long-term process taking several weeks. The antioxidative potential of TOC is, however, preserved for a much shorter time period. It is unclear whether this comparatively short time interval of TOC-activity is sufficient to cause a significant inhibition of advanced glycation reactions. We therefore included the physiologically relevant TOC in our investigations on the long-term glycation of LDL and its modification by antioxidants.

Among the water-soluble antioxidants, ascorbate, the most effective water-soluble chain-breaking antioxidant in human plasma, has been shown to inhibit copper-catalyzed and cell-mediated LDL-oxidation[19,20] and to compete with glucose for binding protein, thereby decreasing glycosylation.[21] Aminoguanidine, a nucleophilic hydrazine compound, is a pharmacological inhibitor of AGE-formation.[22,23] However, its inhibitory effect on LDL-oxidation[24,25] is controversial.

The aim of the present study was to compare the modulation of LDL-glycation by several antioxidants, some of which (troglitazone, acetylsalicylic acid, TOC) are currently evaluated as potential drugs in the treatment of diabetes mellitus or atherosclerosis. The inhibitory effects on the *in vitro* oxidation of native LDL (n-LDL) as compared to glycated LDL (g-LDL) was also determined.

MATERIALS AND METHODS

Materials

Troglitazone (T) was obtained from Dr. Toshihiko Hashimoto (Sankyo, Tokyo, Japan). T was added to the LDL-solutions as a stock solution in DMSO (5.0 mg/ml). α-Tocopherol (TOC, vitamin E; also used as stock solution in DMSO), acetylsalicylic acid (ASA) and bovine pancreatic ribonuclease (RNase) were obtained from Sigma (St. Louis, USA).

Preparation and Characterization of LDL and its Modifications

Human LDL (d 1.019–1.063 g/ml) was isolated from EDTA-treated plasma by density gradient centrifugation.[24] The supernatant was collected and dialyzed extensively at 4°C against PBS containing 1 mM EDTA. The isolated LDL was kept at 4°C for no more than 16 h before all further experiments or analysis.

For oxidation, EDTA-stabilized LDL samples were dialyzed overnight against PBS at 4°C, diluted to 0.25 mg/ml LDL-protein, and then incubated under air with 5.0 μM $CuSO_4$ for different time intervals at 37°C. Formation of conjugated dienes was monitored by continuously recording OD at 234 nm. The baseline absorbance value at 234 nm was set equal to zero. Copper-induced LDL-oxidation resulted in a typical sigmoidal curve consisting of an initial lag-phase, a propagation phase and a decomposition phase. Lag-time was calculated from the intercept of the time-scale axis with the tangent of the slope of the propagation phase. Thiobarbituric acid reactive substances (TBARS) were determined as described below. Oxidation was stopped by addition of 20 μl of 0.1 M EDTA per ml LDL-solution, followed by dialysis against PBS containing 1 mM EDTA at 4°C for 24 h. Lipid peroxides were quantitated by a commercial test ("LPO-CC", Kamiya Biomedical Company, Tokyo, Japan). In the presence of hemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols) and a chromogen is oxidatively cleaved to form methylene blue in an equimolar reaction. Methylene blue is measured at 675 nm.

For glycation, LDL was incubated under air with 0.5 M glucose in phosphate-buffered saline (pH 7.4) in the presence or absence of antioxidants at 37°C in the dark. LDL-glycation was monitored (a) by measuring the fluorescence at 370 nm/440 nm, (b) by AGE-ELISA, (c) by separation of glycated (g-LDL) from nonglycated LDL via affinity chromatography, and (d) by a fructosamine (FA) assay. Monitoring of AGE-formation in LDL via AGE-specific fluorescence is sometimes hampered by the overlapping of fluorescence spectra of oxidized vs. AGE-modified LDL. Separation of the respective fluorescence maxima is not always possible, especially if oxidation is accompanied by strong glycation ("glycoxidation"). Percent glycation of LDL was determined by boronate affinity chromatography using *m*-aminophenyl boronate columns (Pierce Chemical Company, Rockford, IL, USA), as described by Li *et al.*[17] With this technic, we achieved an intra- and inter-assay CV of 4.5% and 7.1%, respectively. This is clearly superior to the performance obtained with the simple and robust FA-test.

Fructosamine Assay

APs were determined with the Roche fructosamine test (Hoffmann-La-Roche, Vienna). Unimate 5 FRUC is an *in vitro* diagnostic reagent based on the ability of ketoamines to reduce nitroblue tetrazolium in alkaline medium. The rate of

formazan formation is directly related to the FA-concentration and is measured photometrically at 550 nm. We obtained an intra-assay CV of 6.7% and an inter-assay CV of 9.8% for this assay.

AGE-ELISA

AGE-RNase was prepared by incubating 25 mg/ml RNase in 0.2 M phosphate buffer, pH 7.4, containing 0.02% sodium azide and 0.5 M glucose-6-phosphate, for six weeks at 37°C. The solution was filtered through a 0.2 μm filter at the start of the incubation period. At the end of the incubation, the unbound carbohydrate was removed by extensive dialysis against PBS. White female New Zealand rabbits were immunized with AGE-RNase (10 or 25 mg/ml), which was emulsified with an equal volume of complete Freund's adjuvant (Sigma). About 250 μl of immunogen were injected subcutaneously every two weeks at two different sites on the back of the animals. A total of six injections were given, all booster injections being performed with the immunogen in incomplete adjuvants. High titers of antibodies developed after ten weeks. Nunc Maxisorp plates (Nunc, Denmark) were coated with 50 μg/ml AGE-Apolipoprotein B (AGE-Apo B) in 0.2 M bicarbonate buffer, pH 9.0, for 3 days at 4°C. This was obtained by incubating Apo-B (Sigma) with 0.5 M glucose for six weeks at 37°C. AGE-LDL samples were preincubated at dilutions ranging from 1 : 5 to 1 : 100 with anti-AGE-RNase antibody (1 : 1000 in PBS containing 1.0% BSA) for 1 h at 37°C and 16 h at 4°C. The antibody/antigen mixtures were then incubated with the solid phase AGE-Apo B for 1 h at 37°C and 16 h at 4°C. Binding of free anti-AGE antibody was quantitated with peroxidase-labeled goat antirabbit Ig antibody (Amersham, UK) and 2,2′-Azino-di-[3-ethyl-benzthiazoline sulfonate(6)], ABTS (Boehringer-Mannheim, Germany), as coloring substrate. As standard, AGE-Apo B was used. One unit of AGE-activity was defined as the amount of antibody-reactive material equivalent to 1 μg of AGE-Apo B.

TBARS-Assay

Lipid peroxidation was determined by quantitation of TBARS with a microtiterplate-modification of the method devised by Buege and Aust.[26] To 120 μl oxidized LDL 70 μl PBS and 50 μl trichloroacetic acid (50%) were added, followed by 75 μl thiobarbituric acid (1.3%). The reaction mixture was incubated for 40 min at 60°C. The tubes were placed on ice for 5 min and the samples were then centrifuged at 2000*g* for 10 min. The supernatants were transferred to microtiter plates and the fluorescence (510/553 nm) was measured in an Anthos microplate reader. TBARS-concentration was calculated using a malondialdehyde (MDA) standard curve and expressed as nM MDA-equivalent per mg LDL protein.

Electrophoretic Mobility of LDL

Native and modified LDL was characterized by agarose gel electrophoresis at pH 8.6 in 60 mM barbital buffer (60 V, 30 mA, 1 h) using the Lipidophor system chamber (Baxter-Immuno AG, Vienna). After fixation, the separated bands were stained using Amido Black 10B (Sigma) as a 0.25% solution in a mixture of 10 vol% acetic acid, 45% ethanol and 45% water.

Statistical Analysis

Statistical differences were tested using Student's *t*-test. A p value of 0.05 or less was considered statistically significant. Data are presented as mean ± SD.

RESULTS

Influence of Antioxidants on Amadori Product- and AGE-Formation

We observed no significantly altered formation of AP during glycation in the presence of 1.0 mM

EDTA as compared to glycation without EDTA (Figure 1). Early glycation product formation was significant after three days and maximal after 25–32 days, and then subsided again. This time curve compares well with results obtained with glycated bovine serum albumin (data not shown). Similar results were obtained when separating g-LDL from nonglycated LDL by affinity chromatography (Table I). Two other radical scavengers and antioxidants tested showed analogous results, i.e. lack of a significant inhibition of AP-formation: TOC and T (Figure 1, Table II). ASA at extreme concentrations (1–11 mM), which could not be tested for TOC and T as a consequence of their limited solubility, significantly inhibited formation of early glycation products. Below this concentration range, however, no inhibitory effects were noted (Table II).

All antioxidants demonstrated a moderate to strong inhibition of AGE-formation, both as measured by fluorescence and by AGE-specific ELISA (Table III). All fluorescence data in Table III are from emission spectra showing a maximum at 440 nm characteristic for AGE-specific fluorescence.

TABLE I Effect of EDTA on LDL-glycation as measured via affinity chromatography[a]

Day	Percent glycation		
	+EDTA	−EDTA	p
0	1.8 ± 0.3		
3	5.7 ± 0.6	6.3 ± 0.9	n.s.
7	20.8 ± 2.0	21.3 ± 3.1	n.s.
12	35.2 ± 4.8	38.7 ± 3.1	n.s.
25	79.8 ± 7.9	81.4 ± 5.0	n.s.
32	78.5 ± 8.1	88.7 ± 6.6	n.s.
39	75.1 ± 4.3	83.2 ± 4.6	n.s.

[a]LDL (2.0 mg/ml) was incubated with 0.5 M glucose in the presence or absence of 1.0 mM EDTA. Glycation was not significantly different in presence or absence of the chelating agent ($n = 4$ experiments).

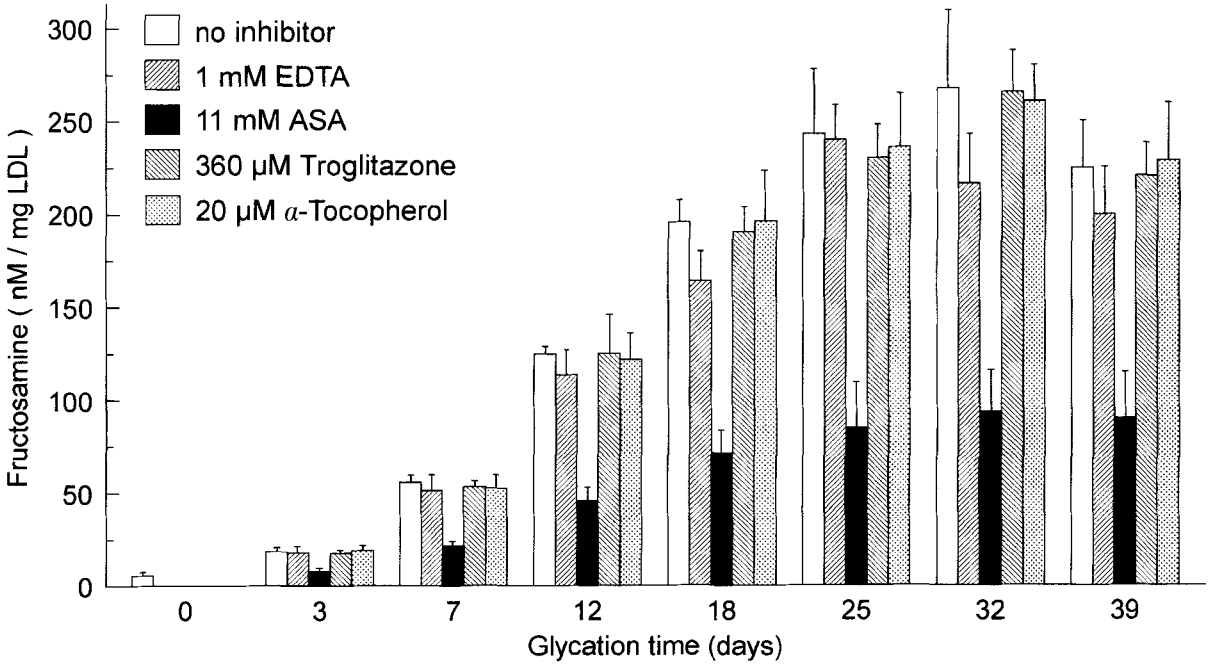

FIGURE 1 Time course of Amadori product formation during glycation of LDL. LDL (2.0 mg/ml) was glycated with 0.5 M glucose in the presence of different antioxidants. Data are mean values of four experiments. Inhibition significant only in case of ASA ($p < 0.01$ for all time points).

TABLE II Inhibition of glycation of LDL by ASA, TOC and T[a]

Concentration (mM)	Percent glycation of LDL		
	ASA	TOC	T
0	21.3 ± 3.1		
0.02	20.7 ± 2.5	22.6 ± 3.0	20.6 ± 1.4
0.05	21.0 ± 3.4	20.7 ± 1.0	19.1 ± 2.7
0.1	19.9 ± 1.7	21.1 ± 2.4	22.4 ± 4.2
0.5	19.5 ± 2.4	19.8 ± 3.2	20.3 ± 1.2
1.1	15.8 ± 1.1[b]	n.d.	n.d.
11.0	9.2 ± 0.8[b]	n.d.	n.d.

[a]LDL was glycated for 7 days in the presence of different amounts of inhibitors ($n = 4$). Measurement of glycation was performed via affinity chromatography. For TOC and T concentrations above 500 μM could not be tested due to the limited solubility of these substances. [b]$p < 0.01$ as compared to an incubation without inhibitor.

TABLE III Inhibition of AGE-modification of LDL by antioxidants[a]

Inhibitor	Concentration	RFU	AGE-units
—	—	5635 ± 370	0.36 ± 0.03
EDTA	1.0 mM	960 ± 270[b]	0.10 ± 0.01[b]
α-Tocopherol	20 μM	2930 ± 420[b]	0.21 ± 0.03[b]
	50 μM	2311 ± 570[b]	n.d.
	500 μM	1987 ± 124[b]	n.d.
ASA	23 μM	5781 ± 370	0.40 ± 0.07
	55 μM	5410 ± 156	0.35 ± 0.01
	550 μM	2282 ± 133[b]	0.27 ± 0.02[b]
Troglitazone	20 μM	5367 ± 498	0.35 ± 0.04
	50 μM	4105 ± 233[c]	0.31 ± 0.09
	500 μM	3442 ± 430[b]	0.23 ± 0.04[b]

[a]LDL (2.0 mg/ml) was glycated for 6 weeks with 0.5 M glucose in the presence of different antioxidants. AGE-formation was verified via measurements of relative fluorescence and via AGE-ELISA using AGE-apolipoprotein B as standard. Fluorescence data are expressed in relative fluorescence units, RFU. Data are mean values ± SD of four assays. Controls without incubation neither show AGE-specific fluorescence nor reactivity in the AGE-ELISA. [b]$p < 0.01$ as compared to an incubation without an inhibitor. [c]$p < 0.05$ as compared to an incubation without an inhibitor.

TABLE IV Inhibition of Cu-mediated LDL-oxidation by ASA: influence of LDL-glycation[a]

Concentration of ASA (mM)	nM MDA/mg LDL protein		p
	n-LDL	g-LDL	
0.0	48.9 ± 1.8	74.0 ± 7.3	
0.055	46.4 ± 3.8	72.4 ± 6.9	n.s.
0.33	44.3 ± 2.4	65.3 ± 1.3	n.s.
0.55	37.4 ± 2.0	61.6 ± 1.3	n.s.
1.1	29.5 ± 0.8	51.1 ± 1.9	< 0.05
11.0	4.8 ± 3.9	26.3 ± 2.7	< 0.01

[a]LDL or glycated LDL was oxidized with 5.0 μM Cu^{++} in the presence of increasing amounts of ASA. TBARS were measured after 4 h. Inhibition data are mean values ± SD of four experiments. p values < 0.05 indicate that the degree of inhibition of oxidation is significantly higher for n-LDL than g-LDL.

Influence of Antioxidants on Copper-Catalyzed Oxidation of LDL and Glycated LDL

LDL (2.0 mg/ml) was glycated in the presence of 1.0 mM EDTA until a plateau value in FA was attained (4 weeks). After extensive dialysis to remove glucose and EDTA, oxidation with 5.0 μM Cu^{++} was performed at a concentration of 0.25 mg/ml LDL protein and TBARS were determined after 4 h, resulting in 49.7 ± 1.8 nM MDA equivalents per mg LDL protein for n-LDL and 74.0 ± 7.3 nM MDA for g-LDL (mean of four experiments). At 4 h, maximal oxidation of n-LDL and g-LDL was normally obtained in the absence of antioxidants. Glycation itself resulted only in moderate oxidation of LDL: 4.1 ± 1.1 nM MDA/mg LDL protein with 0.5 M glucose in the presence of 1.0 mM EDTA; n-LDL generally contained less than 1 nM MDA/mg LDL protein. The content of preformed hydroperoxides was significantly higher in g-LDL (17 ± 15 nM per mg g-LDL as compared to 3.7 ± 3.4 nM per mg n-LDL; $n = 5$ different paired preparations of g-LDL and n-LDL; $p < 0.01$).

ASA markedly inhibited Cu^{++}-catalyzed oxidation of LDL only at high concentrations, i.e. above therapeutically relevant final concentrations: 0.2–2.0 mg/ml corresponding to 1.1–11.0 mM (Table IV, Figure 2). In this concentration range inhibition was significantly reduced for g-LDL after 4 h oxidation as compared to n-LDL (Table IV, Figure 2). The same phenomenon of a reduced inhibition of copper-induced oxidation of g-LDL as compared to n-LDL was observed when using TOC or T as inhibitors (Figure 2).

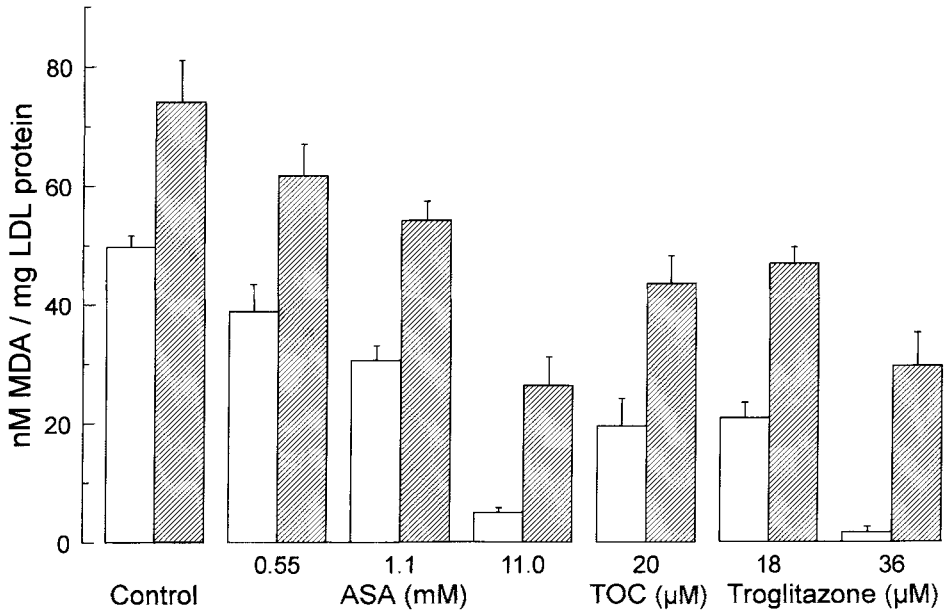

FIGURE 2 Influence of antioxidants on copper-catalyzed oxidation of native LDL (void bars) compared to glycated LDL (hatched bars). LDL (0.25 mg/ml LDL-protein) was oxidized with 5 μM Cu^{++} for 4 h at 37°C. TBARS – data represent the mean values ± SD of four experiments. TOC and T engender a significant reduction in MDA-incorporation ($p < 0.01$), while the antioxidative effects of ASA are significant only above 0.55 mM ($p < 0.05$ at 1.1 mM; $p < 0.01$ at 11 mM).

Below 10 μM, T showed no inhibitory effects either towards n-LDL or g-LDL.

Monitoring of diene formation at 234 nm revealed that g-LDL in contrast to n-LDL showed a severely reduced lag-time (Table V), which was prolonged by addition of TOC or T (Table V). TOC (20 μM) roughly doubled the lag-time of n-LDL. Monitoring of the lag-time of LDL-oxidation via diene formation in the presence of inhibitory concentrations of ASA (1.1–11.0 mM) is not feasible, as ASA has an absorbance at 234 nm of > 3.0 in this concentration range.

To make sure that the antioxidative aspirin effect is not an artifact of LDL-acetylation, we determined relative electrophoretic mobility (REM) of an LDL sample incubated for 4 h at 37°C with and without 11.0 mM ASA. REM values amounted in both cases to 1.10. The REM value of g-LDL after 6 weeks of glycation was 1.26 vs. 1.15 in presence of ASA, whereas copper-oxidized LDL or g-LDL showed after 4 h oxidation a REM of 1.78 vs. 1.91.

TABLE V Lag-times of copper-induced oxidation of native or glycated LDL as modified by antioxidants troglitazone and α-tocopherol[a]

Inhibitor	Concentration (μM)	Lag-time (min)	
		n-LDL	g-LDL
none	—	45.7 ± 3.5	12.3 ± 1.2
α-tocopherol	20	87.4 ± 4 1	51.6 ± 3.2
Troglitazone	360	105 ± 7.9	79.2 ± 4.9

[a]Copper-mediated LDL-oxidation was monitored at 234 nm in the presence or absence of antioxidants. Data are the mean ± SD of four experiments. g-LDL shows significantly reduced lag-times in the presence or absence of antioxidants ($p < 0.01$).

DISCUSSION

Bucala *et al.*[27] investigated the possibility that glucose-mediated advanced glycation, a major pathway for post-translational modification of tissue proteins, may trigger oxidative LDL-modification *in vivo*. While there are several reports that *in vitro* or *in vivo* glycation of LDL

enhances its oxidizability,[12–14,28,29] Babiy *et al.*[30] contend that g-LDL is more resistant to oxidation than n-LDL. The same authors found, however, that LDL from type II diabetic patients was more easily oxidized than control LDL. This finding was confirmed by Bowie *et al.*,[31] who observed a similar correlation between LDL-glycation and LDL-oxidation as Bucala.[27] Finally, according to Tsai *et al.*, the LDL from type I diabetic patients with poor metabolic control showed a higher susceptibility to oxidation than that of healthy individuals.[32] As shown by our results, g-LDL contains significantly more hydroperoxides than n-LDL. This can affect oxidizability induced by Cu^{2+}.[33]

We performed LDL-glycation in the presence or absence of antioxidants and followed the time course of AP- and AGE-formation over several weeks ("phase I"). To obtain clear cut, i.e. immunologically reactive AGE-formation, we had to use both 0.5 M glucose concentration and a six week incubation period. With 25–50 mM glucose, as used by other authors, we only achieved Amadori product generation, which was already noted after 3 days incubation. The long-term glycated LDL modifications still retained the characteristic electrophoretic mobility of slightly oxidized LDL. In a second step, inhibitory effects of the antioxidants on the copper-catalyzed oxidation of g-LDL vs. n-LDL were compared ("phase II"). We think that this rationale is an appropriate model for approximating the *in vivo* situation of diabetic patients with systemic LDL-glycation, covalent binding of AGE-LDL to atherosclerotic lesions and local LDL-oxidation. *In vivo*, however, a glycation interval of 4–6 weeks for LDL is not realistic in the light of the shorter half-life of LDL. Furthermore, the highest plasma concentrations of glucose encountered in diabetics are 30–50 mM, not 0.5 M.

Zyzak *et al.*[34] report that antioxidative conditions retard the decomposition to tetroses, pentoses and 3-deoxyglucosone both of a model Amadori compound and glycated rat tail type I collagen. Since these sugars or residual sugar fragments contribute to the formation of AGEs,[35] it is logical that this process is slowed down by antioxidants. It is indeed a general finding that antioxidants such as metal chelators significantly retard AGE-formation, while glycation itself is only weakly inhibited.[36] Fu *et al.* term this phenomenon "uncoupling" of glycation from oxidative AGE-formation. We observed this uncoupling phenomenon for EDTA, TOC and T, not ASA. The reason for ASA's unique behavior probably resides in its capacity to acetylate the free ε-amino groups of proteins such as human serum albumin,[37] enabling this drug to inhibit nonenzymatic early glycation via the blockade of these groups,[38] i.e. via inhibition of the initiating Schiff's base formation.

One might expect that TOC is totally consumed during long-term glycation, i.e. over a time period of 4–6 weeks. In Cu^{++}-catalyzed oxidation of LDL, depending on the Cu^{++} : LDL concentration ratio, as well as during oxidation via macrophages, "physiological" concentrations of TOC (approx. 10 nM/mg LDL protein) indeed are consumed within 0.5–4 h.[39,40] In cell-free aerated control incubations TOC-consumption is less rapid (24 h).[24] Since our glycation of LDL was performed in the absence of Cu^{++} and cells at an at least five times higher TOC-concentration and TOC : LDL ratio (50 nM/mg LDL protein), as well as without additional aeration, we can assume that TOC's antioxidant and chain-breaking activity lasted significantly longer to inhibit radical generation by early glycation products. Like TOC, T inhibits AGE-formation while having no effect on early glycation. T stabilizes LDL-bound TOC and acts itself as ROS-scavenger. The T concentration of 100–500 μM at which a significant inhibition of AGE-formation was noted is, however, far beyond the concentration range reached *in vivo* (4 μM).

The enhanced susceptibility to copper-catalyzed oxidation of g-LDL ("phase II" experiments) as compared to n-LDL can be explained by the additional oxidative stress exerted by LDL-bound AP and, to a minor extent, AGE-moieties. In the

presence of transition-metal catalysts AP would be expected to amplify their increased rate of oxygen radical production even further via Fenton reaction chemistry. In addition, metal-catalyzed decomposition of lipid hydroperoxides and aminoacid hydroperoxides, formed during glycation under aerobic conditions in apo B,[14] might contribute to the induction of oxidative stress. Thus, one reason for an apparently increased ease of oxidation of g-LDL resides is the fact that g-LDL contains significantly more hydroperoxides than n-LDL. Their function is to reoxidize Cu^{+} under formation of alkoxyl radicals. These are "primordial" radicals[33] like tocopheroxyl and lipid peroxyl radicals. Their formation rates govern the total rate of initiation, which is essential for the whole oxidation process.[33] Thus, our results do not establish that glycation *per se* renders LDL more susceptible to Cu^{++}-induced oxidation. Increased peroxidation of g-LDL could, however, be a consequence of the glycation procedure.

ASA should be of special importance as inhibitor of LDL oxidation via g-LDL, as – in contrast to EDTA, aminoguanidine, T and TOC – ASA efficiently blocks both AP- *and* AGE-formation, although only in a concentration range that exceeds by far the therapeutically achieved plasma concentrations of 5–20 μM. This supratherapeutical concentration range of 1–11 mM could not be studied for TOC and T, because the solubility of these drugs was not high enough even when applied as DMSO stock solutions. A recent paper shows, however, that even low-dose aspirin can indeed reduce glycoxidative damage via pentosidine formation in type II diabetic patients.[42]

Three mechanisms should be basically considered in the antagonism of copper-mediated LDL oxidation: 1. metal complexation–inactivation involving abrogation of metal-catalyzed decomposition of lipid or protein hydroperoxides[41] (e.g. by EDTA), 2. scavenging of radical species such as peroxyl, alkoxyl or lipid radicals, 3. stabilization of the lipid moiety of LDL, conceivably via chemical interactions between drug hydrophobic groups and polyunsaturated residues of LDL–phospholipids. Items 2 and 3 best explain the effects of TOC and T, the antioxidant activity of which can be attributed to the similarity of its molecular structure to that of TOC. T has a scavenging effect on ROS and tends to be in the radical form.[43] Like TOC, it can be incorporated into LDL, and reacts rapidly with radicals to give chromanoxyl radicals characterized by the same ESR-spectrum as TOC.[44] T incorporated exogenously into LDL inhibits the oxidation of LDL by aqueous or lipophilic peroxyl radicals and suppresses the formation of lipid hydroperoxides.[45] It is important to note as in case of ASA that significant antioxidative effects of T were only observed at concentrations exceeding by far the plasma concentration of 4 μM transiently attained after therapeutic dosage (400–800 mg/day).

When comparing the efficiency of inhibition, i.e. retardation, of the copper-mediated oxidation of LDL vs. g-LDL by antioxidants we generally observed that copper-induced oxidation of g-LDL was less efficiently inhibited than that of n-LDL. One explanation for this finding is the severely reduced lag-time of g-LDL as compared to n-LDL, indicating inactivation of LDL-bound TOC and other endogenous antioxidants on LDL during phase I glycation. Another explanation could be again the different endogenous content of hydroperoxides in n-LDL vs. g-LDL at the beginning of the oxidation.

Therapeutic implications of these findings with respect to the antioxidant vitamin E, which is supposed to slow atherogenesis and the development of microvascular complications in diabetes, are obvious: TOC-monotherapy, while blocking oxidative and AGE-modification of LDL, is unable to inhibit AP-formation. As a consequence, TOC is susceptible to increased consumption by AP-associated ROS-production in patients with circulating or tissue-bound glycated proteins, i.e. in hyperglycemic patients. This enhanced TOC-consumption could be checked

in part by the TOC-protecting agent T and/or by ASA which itself reduces AGE-formation *in vivo* at a dose of 100 mg/day.[42] Such a combined pharmacologic treatment, together with dietary measures aiming at optimizing glycemic control, could most effectively inhibit the deleterious consequences of lipoprotein glycation.

Acknowledgments

This study was supported by a grant from the Jubiläumsfonds der Österreichischen Nationalbank (no. 7584).

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