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Effect of gangliosides on the copper-induced oxidation of human low-density lipoproteins

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Abstract

The role of gangliosides in the copper-induced oxidative modification of human low-density lipoprotein (LDL) was studied focusing on the early stage of LDL oxidation in which the concentration of conjugated dienes increases only weakly. The changes in the protein and lipid component were followed using fluorescence spectroscopy. The results indicate that binding of gangliosides to LDL causes slower destruction of tryptophan fluorescence and suppresses cross-linking between the reactive groups of the protein and the products of lipid peroxidation. The protective role of gangliosides could be assigned to their interference with the lipid–protein interaction in the LDL particle, which might be important for the maintenance of the native plasma antioxidant status in vivo.

Keywords: LDL; Gangliosides; Oxidation; Fluorescence

1. Introduction

Gangliosides are sialic acid containing glycosphingolipids present in all mammalian tissues so far examined [1]. They are particularly abundant in neuronal cells and the research in this field is extremely intensive [2,3]. The distribution and the role of gangliosides in the plasma membranes and intracellular membranes has been studied in various systems [4–6]. In particular, the role of gangliosides in modulation of transmembrane signaling via induced structural and dynamic changes in the lipid bilayer has been emphasized [7].

The exogenous insertion of gangliosides from the culture media into the cell membrane has been reported to have various repairing and protective effects [8,9]. In this context, the effect of gangliosides on the pathological modification of human plasma low-density lipoproteins (LDLs) is interesting since the majority of gangliosides in the blood of healthy humans is predominantly associated with LDL [10,11]. To the best of our knowl-

Abbreviations: LDL, low-density lipoprotein; PBS, phosphate-buffered saline; Apo B, apolipoprotein B; Trp, tryptophan

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edge, the research involving oxidation of LDL in the presence of gangliosides has not been performed. The oxidatively modified LDL is assumed to play an important role in atherogenesis contributing to the formation of lipid-loaded foam cells [12,13]. It has been reported that the average concentration of gangliosides in human plasma (10–20 μM) increases five-fold in atherosclerotic patients [14]. This observation is not understood with respect to the above-mentioned protective effect of gangliosides. In addition, the detailed biological roles of gangliosides circulating with lipoproteins remain to be elucidated. This is partially hampered by the extremely complex structure of the LDL particle which consists of different lipids in a non-covalent interaction with apolipoprotein B (apo B), the largest monomeric protein composed of 4536 amino acids [15]. Since the three-dimensional structure of the native LDL particle is still not known at the atomic resolution [16,17], different experimental approaches have been tried to probe the topology of LDL interactions [18,19]. Some earlier studies have been performed considering the interaction of LDL with gangliosides [14,20,21]. It has been reported that binding of gangliosides changes the surface organization of LDL and interferes with the normal clearance of LDL from the circulation stimulating binding of LDL to the scavenger receptor of macrophages.

The aim of this study was to investigate the possible influence of gangliosides in a model of LDL oxidation in vitro. The common procedure with copper ions was used to initiate the LDL oxidation [22], which proceeds via a free radical chain mechanism including initiation (lag phase), propagation and termination reactions [23]. The chemical reactions governing the process affect both the lipid and the protein component of lipoproteins. The research was focused on the early stage of oxidation, i.e. the lag phase, since the concept of lag time has been widely used to test the efficiency/ability of various substances to inhibit the oxidation of LDL [24,25]. LDL samples were enriched with different concentrations of gangliosides and copper-induced LDL oxidation was followed using spectrophotometric and fluorescence measurements.

2. Material and methods

2.1. LDL isolation and oxidation

Reagents were from Merck (Darmstadt, Germany) and Kemika (Zagreb, Croatia), except for gangliosides type III from bovine brain which were from Sigma–Aldrich (Steinheim, Germany). Using thin layer chromatography [26] it was estimated that the gangliosides used in this study were a mixture of GM_1 (30%), GD_{1a} (50%), GD_{1b} (10%) and GT_{1b} (10%).

Human plasma was obtained from the blood bank as a pooled sample from two normolipidemic donors. LDL was isolated by density gradient ultracentrifugation [27,28] using the fixed angle rotor Ti70 on a Beckman preparative ultracentrifuge. To avoid the oxidation of lipoproteins during the isolation procedure, EDTA (1 g/l) was present in all steps of the LDL isolation and all buffers were flushed with argon. The purity of the LDL samples was checked by electrophoresis using a Radiophor electrophoresis system with lipidophor agar medium. The protein concentration was measured by the method of Lowry [29] using bovine serum albumin as a standard. In this study, the LDL concentration is expressed as its protein content. All the experiments were repeated with LDL isolated from three independent blood bank plasma samples. The results presented here, refer to one chosen LDL preparation out of these three which all gave consistent results.

Prior to oxidation, LDL was dialyzed overnight against a 200-fold volume of EDTA-free phosphate-buffered saline (PBS, 0.01 M, pH 7.4) flushed with argon. The oxidation of LDL (100 μg LDL protein/ml) was induced at 37 °C by 5 μM $CuSO_4$ in PBS in the presence/absence of gangliosides and monitored in 15-min intervals. The gangliosides were dissolved in PBS and added to the LDL sample immediately before the start of the oxidation process. The maximal final concentration of gangliosides in the experiments was 80 μM. The capability of gangliosides to modify copper-mediated LDL oxidation was evaluated using a Varian Cary 50 UV-visible spectrophotometer. The increase in the absorbance at 234 nm was monitored as an indication for the formation

of conjugated dienes. A kinetic study of the time course of the lag phase in the LDL oxidation process, an indicator of the resistance to oxidation, was performed according to a previously established protocol [23,28].

2.2. Steady state fluorescence measurements

For fluorescence measurements the LDL samples were entrapped in a particular oxidation state by adding EDTA to a final concentration of 1 g/l.

Intrinsic LDL fluorescence spectra were measured in a Varian microcuvette (400 μl of the sample volume) using a Varian Cary Eclipse fluorescence spectrophotometer at 37 °C if not otherwise stated, with a sampling data interval of 0.5 nm. The bandwidths of excitation and emission monochromators were 5 nm and 2.5 nm, respectively. The LDL concentration was 0.2 μM. Steady state fluorescence spectra were recorded with excitation at 282 nm and 355 nm. The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the Trp residues in apo B [30]. Quenching of the intrinsic fluorescence was studied at 332 nm (excitation at 282 nm) with potassium iodide (KI) prepared as a 5 M stock solution in H_2O containing 0.1 mM sodium thiosulfate [14]. Trp exposure to the aqueous surrounding at the start of LDL oxidation was estimated using a modified Stern Volmer equation and assuming two distinct Trp populations differing with respect to potassium iodide accessibility [31].

Steady state anisotropy measurements were performed at 300 nm excitation and 340 nm emission detection, with the bandwidth of excitation and emission monochromators of 10 nm. Samples were excited with vertically polarized light and vertically (I_{vv}) and horizontally (I_{vh}) polarized fluorescence intensities were measured using L-geometry optical path. The anisotropy (r) was calculated using the relationship:

$$r=(I_{vv}-GI_{vh})/(I_{vv}+2GI_{vh})$$

The correction factor (G) is given by $G=I_{hv}/I_{hh}$ and is determined by measuring the vertically (I_{hv}) and horizontally (I_{hh}) polarized fluorescence intensities using the horizontally polarized exciting light.

3. Results

Spectrophotometric analysis of copper-induced LDL oxidation based on the conjugated dienes formation due to the oxidation of unsaturated lipids is presented in Fig. 1a. The results indicate that LDL samples enriched with gangliosides exhibit a prolonged lag phase (135 min) as compared to samples without gangliosides (100 min).

The results of fluorescence measurements indicated that the spectral band is centered at approximately 332 nm, and assigned to the Trp residues in apo B, decreased in intensity already during the lag phase of the induced LDL oxidation (Fig. 1b). It can clearly be seen that the process is slowed down with increasing amount of gangliosides added to the LDL samples.

The analysis of fluorescence quenching by KI at the start of the LDL oxidation is presented in Table 1. The approach is based on the ability to separate the accessible and inaccessible fraction of Trp in apo B with respect to their interaction with KI. The data indicate that, in LDL samples loaded with gangliosides, a significantly smaller percentage of Trp residues (approx. a half) is exposed to aqueous iodide, as compared to the LDL samples without gangliosides. Measurements performed at two different temperatures provided mutually consistent results indicating higher exposure of Trp at higher temperature for both LDL samples. However, this simple approach cannot be applied in the analysis of fluorescence quenching during LDL oxidation. The inability to model fluorescence quenching curves assuming only two distinct Trp populations in oxidized LDL is indicative of the imposed change of LDL structural properties. Tryptophyl side chains, residing in different hydrophobic parts of apo B, start to become exposed to aqueous surrounding already during the early stage of LDL oxidation, thus, exhibiting complex fluorescence quenching behavior (Fig. 2a). It can be noticed that approaching the end of the lag phase, LDL samples not loaded with gangliosides exhibit increased quenching of the native fluorescence. On the other hand, LDL enriched with ganglio-

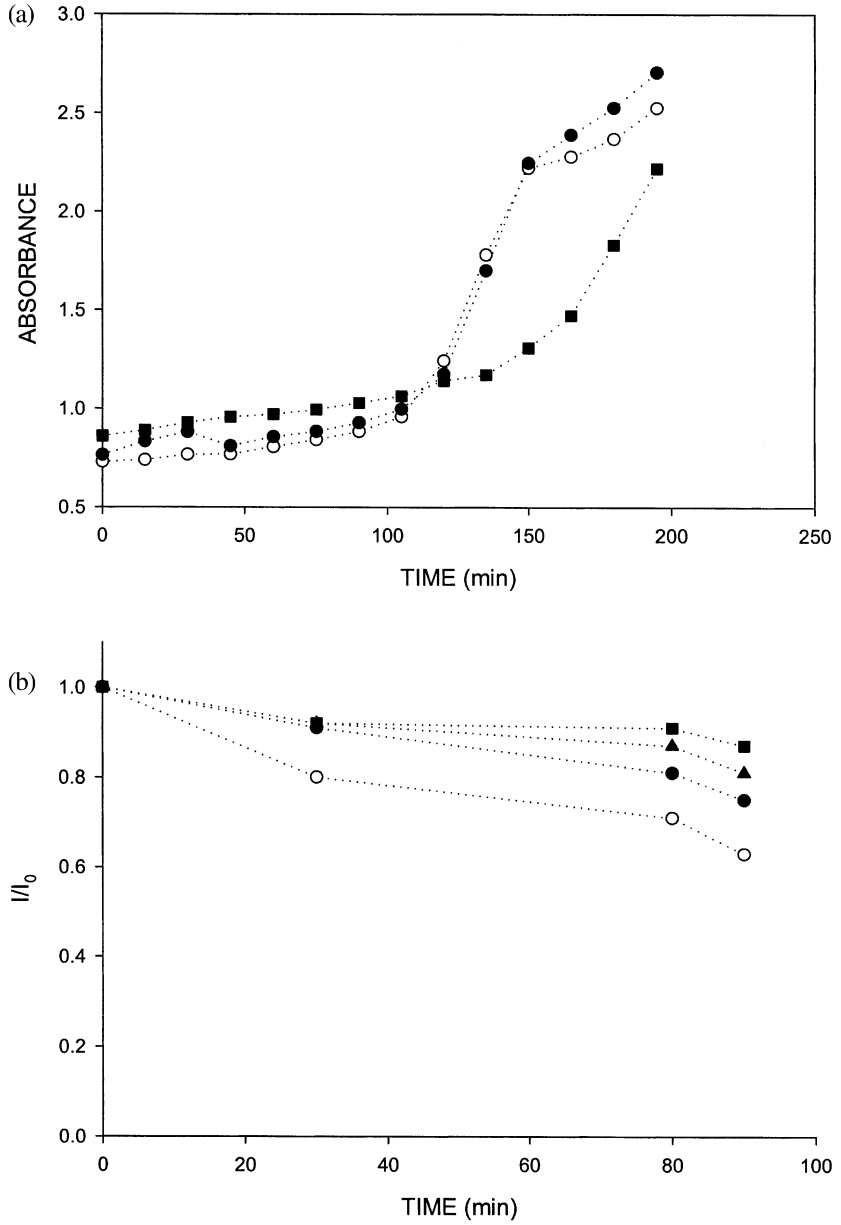

Fig. 1. The effect of gangliosides on the kinetics of copper-induced LDL oxidation. (a) The formation of conjugated dienes measured spectrophotometrically at 234 nm. The following symbols are used: (○) LDL, (●) LDL with 10 μM gangliosides, and (■) LDL with 80 μM gangliosides. (b) Decrease of fluorescence intensity at 332 nm (excitation at 282 nm) during the lag phase of copper-induced LDL oxidation. The fluorescence intensity (I) is normalized to the intensity (I_0) at the start of the oxidation process. The following symbols are used: (○) LDL, (●) LDL with 10 μM gangliosides, (▲) LDL with 30 μM gangliosides, and (■) LDL with 80 μM gangliosides.

Table 1
Trp exposure to aqueous surrounding at the start of LDL oxidation estimated from the analysis of fluorescence quenching by KI

T (°C)	% of Trp exposed to aqueous surrounding out of total Trp in LDL	
	LDL	LDL + 80 μM gangliosides
25	18 ± 2	11 ± 3
37	32 ± 6	16 ± 1

sides, apart from having less Trp exposed to aqueous quencher, show hardly any increase in fluorescence quenching during the lag phase of the LDL oxidation process (Fig. 2b).

The involvement of lipids in the process of copper-mediated LDL oxidation can be traced through the appearance of the emission spectra at wavelengths higher than 430 nm (excitation at 355 nm), as presented in Fig. 3. The formation of various fluorophores during the oxidation of lipids is clearly suppressed in the presence of gangliosides.

The results of steady state fluorescence anisotropy measurements of different LDL samples are summarized in Table 2. During the copper-induced oxidation of LDL particles not loaded with gangliosides, the overall trend of data indicates a continuous increase in anisotropy. On the other hand, LDL samples enriched with gangliosides show somewhat higher initial anisotropy which does not change during the lag phase. In the propagation phase of LDL oxidation, the anisotropy starts to increase but at a slower rate than in LDL samples not loaded with gangliosides.

4. Discussion

The influence of exogenous gangliosides on the early phase of LDL oxidation was investigated. The results of previous research [32] indicated that the lag phase was prolonged depending on the concentration of gangliosides (10–80 μM) in the LDL samples. Here, LDL samples enriched with 80 μM gangliosides exhibited a 35 min longer lag phase as compared to LDL samples not loaded with gangliosides.

Fluorescence spectroscopy proved to be a very sensitive approach for detecting changes in the LDL particle during the early stage of the induced oxidation. Since the fluorescence peak at approximately 332 nm is assigned to Trp residues [30], the observed decrease in its fluorescence intensity can be interpreted as protein destruction, which already starts in the lag phase. The significantly slower decay of fluorescence intensity in the presence of gangliosides implies their protective role at the level of apo B, at least in the beginning of LDL oxidation.

The role of gangliosides during the early stage of LDL oxidative damage was corroborated by experiments on fluorescence quenching with aqueous iodide. LDL particles not enriched with gangliosides exhibit higher exposure of Trp to polar environment, thus experiencing a lower hydrophobic barrier. They lose their structural integrity much faster during the oxidation process and consequently their Trp residues become even more exposed towards the aqueous quencher. Therefore, these LDL particles already exhibit a profound change in structural organization during the lag phase of the oxidation process. On the other hand, in LDL samples loaded with gangliosides, there are fewer contacts between Trp and the aqueous solvent. This suggests that gangliosides bind to LDL in the vicinity of native chromophores, providing sterical shielding from the polar quencher, and also presumably from oxidation damage. This interpretation is consistent with reports stating that loading of LDL particles with other types of gangliosides inhibited binding of LDL to a variety of cells [33].

Regarding the oxidative damage of the lipid component in LDL a similar protective role of gangliosides can be anticipated. Namely, cross-linking between aldehydes or hydroperoxides and the ε-NH_2 groups of the lysyl residues of apo B, indicated by fluorescence emission above approximately 430 nm [27,34–36], was clearly suppressed in the presence of gangliosides. These observations could be interpreted assuming that gangliosides are adsorbed/anchored at the lipoprotein surface, thus interfering with direct interaction between apo B and the products of lipid peroxidation. This conclusion is in accord with the

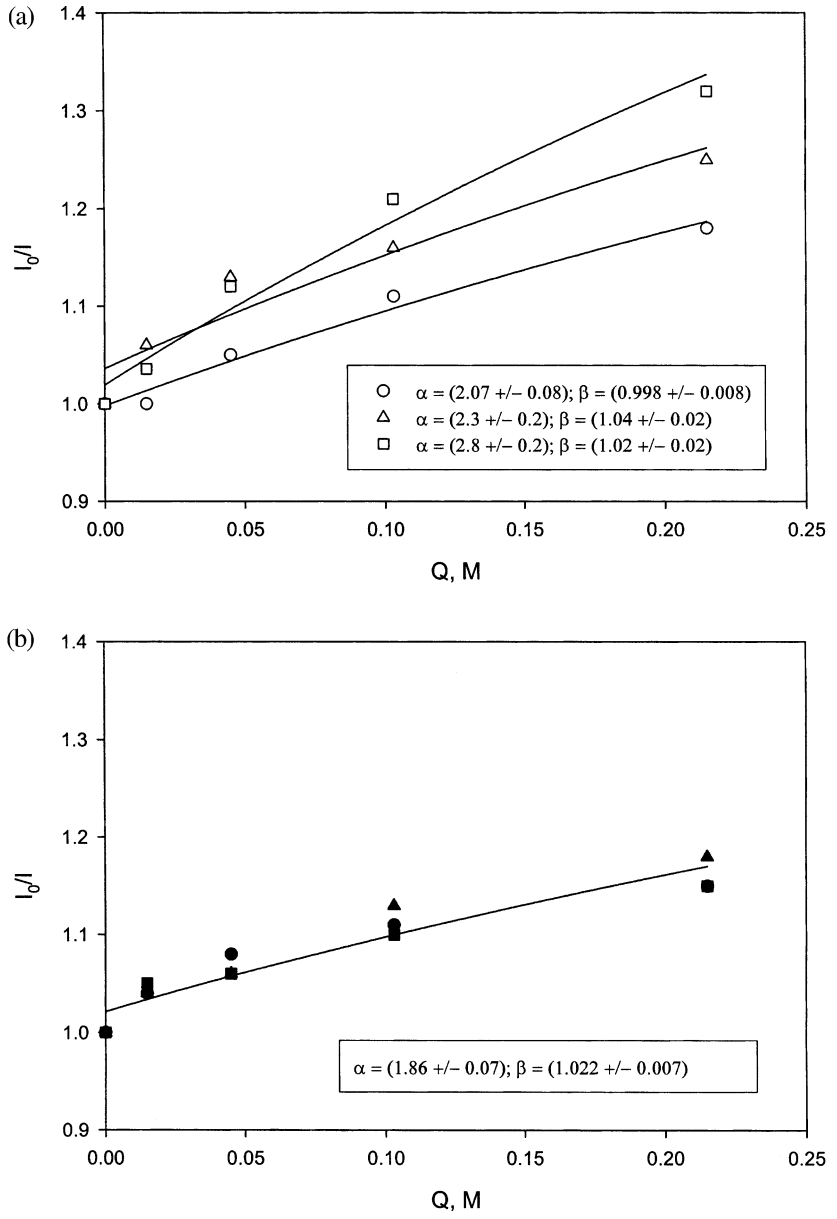

Fig. 2. Quenching of Trp fluorescence at 332 nm (excitation at 282 nm) with KI in the absence (a) and presence of 80 μM gangliosides (b) during the early stage of LDL oxidation. Fluorescence intensities (I) are normalized with respect to the intensity in the absence of quencher (I_0). The experimental data are depicted with symbols which refer to different oxidation states of LDL depending on the duration of oxidation (time = 0 min, ○, ●; time = 90 min, △, ▲; time = 120 min, □, ■). Full lines denote an arbitrarily chosen theoretical description of the experiment according to $I_0/I = (\alpha^* Q + \beta)/(Q + 1)$ which was applied to help to evaluate fluorescence quenching curves. The concentration of potassium iodide is denoted with Q and values of the free parameters derived in the fitting procedure, α and β, are indicated in the legend to the Figure. All three experimental data sets for LDL enriched with gangliosides could be described with the same α and β parameters (Fig. 2b).

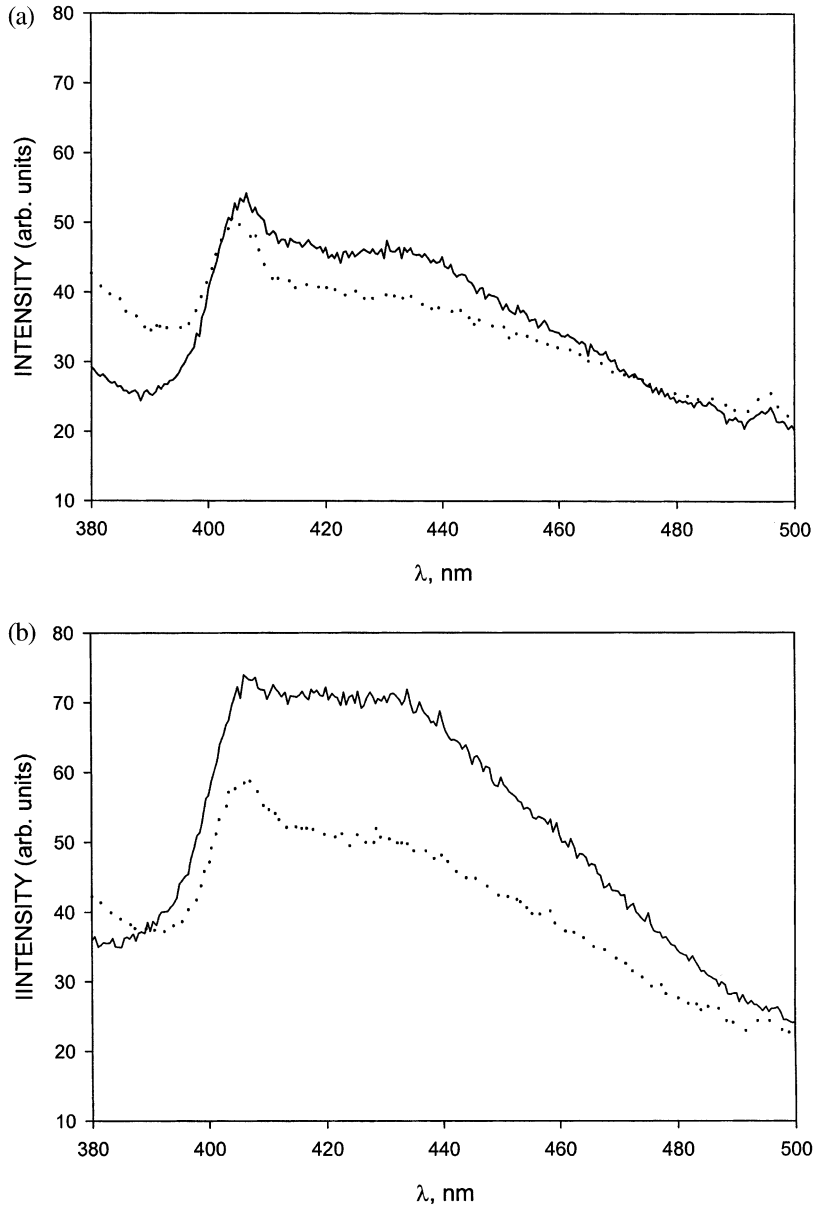

Fig. 3. Fluorescence spectra (excitation at 355 nm) of various lipid fluorophores detected at 90 min (a) and 120 min (b) of the copper-induced LDL oxidation. Dotted lines denote LDL samples loaded with 80 μM gangliosides and full lines LDL samples without them. The peaks approximately at 410 nm are due to Raman scattered light.

reported binding of exogenously added gangliosides to LDL, which is described as predominantly non-specific, mediated by weak hydrophobic and electrostatic interactions at the surface of the lipoprotein particle [37]. Thus, during copper-induced LDL oxidation, gangliosides may provide a steric barrier between reactants and thus postpone the ultimate damage of the particle.

Table 2
Fluorescence steady state anisotropy values during the time course of the copper-induced LDL oxidation

Time (min)	Steady state anisotropy	
	LDL	LDL + 80 μM gangliosides
0	0.249 ± 0.002	0.283 ± 0.003
90	0.265 ± 0.003	0.287 ± 0.003
120	0.286 ± 0.004	0.286 ± 0.003
180	0.355 ± 0.003	0.321 ± 0.003

The results of fluorescence anisotropy measurements indicated changes in protein dynamics during LDL oxidation. In the LDL samples not enriched with gangliosides, Trp anisotropy increased with the time of oxidation. This result could be assigned to an oxidatively modified environment, which progressively restricts the mobility of the native fluorophores. A similar observation has been reported for VLDL labeled with an extrinsic fluorophore [38]. In the presence of gangliosides the initial LDL anisotropy was somewhat higher, an effect which could be related to the reported LDL-ganglioside complexes being even 2–3 times larger than the native LDL particles [14]. Following this reasoning, it can be anticipated that binding of gangliosides to the LDL particle modulates its surface monolayer properties by restricting the mobility of certain parts of the protein. During the early stage of LDL oxidation, the corresponding fluorescence anisotropy is conserved implying a longer preservation time for initial protein dynamics in LDL in the presence of gangliosides.

5. Conclusion

In this study, the effect of gangliosides in the model of LDL oxidation in vitro has been investigated. The experimental results based on fluorescence spectroscopy indicate a protective role of gangliosides during the early stage of copper-induced LDL oxidation. This observation might be explained assuming binding of gangliosides to the LDL particle, thus preventing direct contact between the reactive groups of the protein with the products of oxidation. This phenomenon might be important for the maintenance of the native plasma antioxidant status in vivo, but has to be further investigated in other relevant models of LDL oxidation.

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