Autofluorescence of Low-Density Lipoproteins Modified as a Result of Autooxidation

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Autooxidation of low-density lipoproteins during incubation at 37°C was accompanied by accumulation of LPO products, decrease in UV autofluorescence (F_{UV}), and increase in autofluorescence in the visible band (F_{VIS}). The degree of low-density lipoprotein modification was estimated by calculating the F_{VIS}/F_{UV} ratio. A positive correlation was revealed between this ratio and concentration of thiobarbituric acid-reactive LPO products (r=0.76, p<0.001). Autooxidation of low-density lipoproteins increased availability of tryptophanyls for fluorescence quenchers and inductive resonance energy transfer from tryptophanyls to adducts formed in the reaction of apoprotein and LPO products. These changes probably play a role in the decrease in F_{UV}.

Key Words: *low-density lipoproteins; lipid peroxidation; autofluorescence*

Lipid peroxidation (LPO) results in modification of low-density lipoproteins (LDL) with the formation of highly atherogenic particles [7]. The method of fluorescence spectroscopy allows detection of even minor physicochemical changes in lipoprotein particles. LPO modifies both intrinsic LDL autofluorescence [3-5] and fluorescence (FL) of probes incorporated into LDL particles [6,8]. UV autofluorescence region is mainly determined by tryptophan residues in apoprotein molecule. The intensity of autofluorescence decreases in oxidized LDL [3-5]. FL in the visible region (F_{VIS}) is associated with the presence of structures formed during the interaction of aldehydes (LPO products) with free amino groups. F_{VIS} increases during LPO in LDL [5].

Here we studied the dependence of changes in autofluorescence on LPO intensity in autoxidized LDL and the mechanisms underlying the decrease in UV autofluorescence (UV-FL).

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MATERIALS AND METHODS

LDL were isolated from blood plasma of healthy donors by ultracentrifugation with 0.01% EDTA, which prevents LPO [6]. LDL were dialyzed against a 500-fold volume of 10 mM Tris-HCl buffer (pH 7.4) containing 0.28 M sucrose for 15 h. LDL concentration was estimated by protein content (Lowry method).

LDL were autoxidized by incubation at 37°C. The intensity of LPO was evaluated by measuring the amount of thiobarbituric acid-reactive substances (TBA-reactive substances) [2]. It was expressed as an equivalent amount of malonic dialdehyde (MDA). The molar extinction coefficient for reaction products of TBA and aldehyde groups was 156,000 $M^{-1}cm^{-1}$.

LDL concentration during FL measurement was 0.04 mg protein/ml. The sample contained 2 mM EDTA. The measurements were performed on a Hitachi F-3000 spectrofluorometer using a rectangular quartz cuvette (1 cm optical path, 5 nm slit for excitation and FL measurement). F_{UV} was mea-

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sured at 340 nm (excitation wavelength 286 nm). F_{VIS} was measured at 430 nm (excitation wavelength 360 nm). Anthracene (Sigma) and 4-(*p*-dimethylaminostyryl)-1-dodecylpyridinium *p*-toluenesulfonate (DSP-12; Institute of Organic Synthesis, Latvian Academy of Sciences) were used as FL probes.

RESULTS

The decrease in F_{UV} was detected soon after the start of autooxidation. After 3- and 24-h incubation F_{UV} decreased to 67 and 22% from the initial value (nonoxidized LDL), respectively (Table 1). F_{VIS} underwent other changes. FL at 430 nm was low under basal conditions and remained unchanged over the first 2 h of incubation, but after 3 and 24 h F_{VIS} increased by 17 and 190%, respectively. The inhibition of LPO with EDTA prevented changes in F_{UV} and F_{VIS}, which attests to a relationship between changes in intrinsic FL and LDL modification during LPO.

The decrease in F_{UV} during LDL oxidation can be associated with oxidation of tryptophanyls, increase in their availability for FL quenchers due to changes in microenvironment, and inductive resonance energy transfer from tryptophanyls to LPO adducts [5]. It is currently accepted that the decrease in F_{UV} for oxidized LDL reflects the degree of tryptophanyl oxidation [3,4]. However, other factors cannot be excluded.

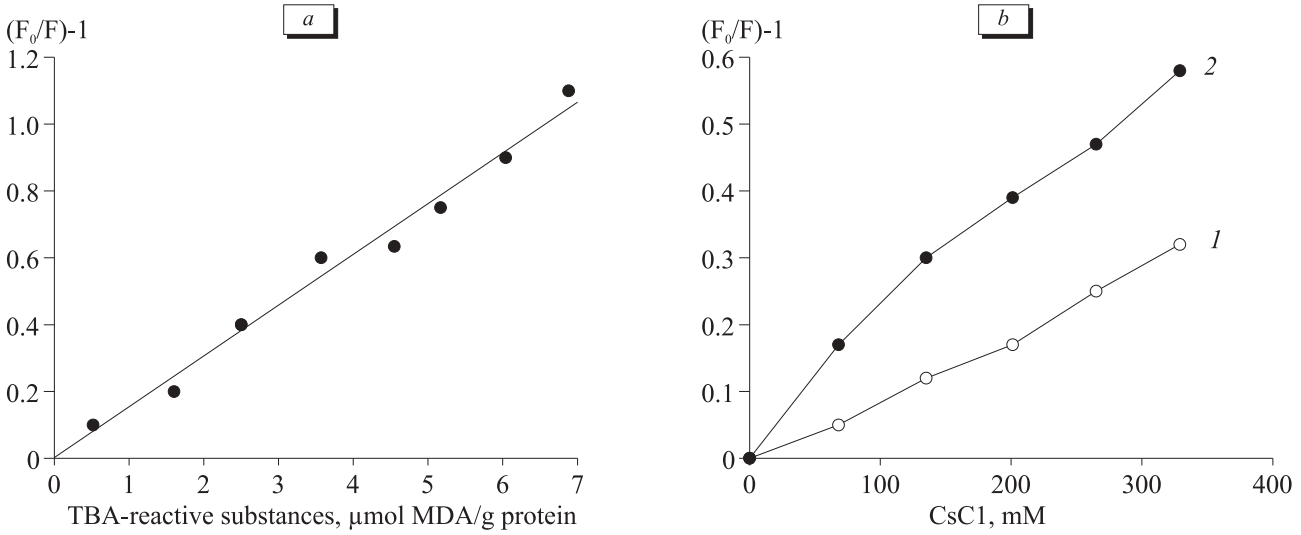

Fig. 1. Dependence of variations in autofluorescence LDL UV-FL on the intensity of LPO (*a*) and quenching with cesium ions (*b*). Here and in Fig. 2: F_0 and F, FL before and after autooxidation, respectively. Native LDL (1 μmol MDA/g protein, *1*); oxidized LDL (6.88 μmol MDA/g protein).

TABLE 1. Autofluorescence of LDL in the UV Region during Incubation with or without EDTA (*M*±*m*, *n*=9)

Time of incubation, h		TBA-reactive substances, μmol MDA/g protein	F_{UV}, %	F_{VIS}, %
Without EDTA	0	1.10±0.07	100	100
	1	1.70±0.08	93.0±1.9	102.0±3.8
	2	2.50±0.08	82.0±2.1	104.0±3.2
	3	3.1±0.1	67.0±2.3	117.0±4.5
	6	4.20±0.12	44.0±2.1	121.0±5.1
	24	12.00±0.58	22.0±2.3	290.0±4.8
With 0.01% EDTA	0	1.00±0.07	100	100
	1	1.10±0.08	100.0±1.9	100.0±3.8
	2	0.90±0.07	99.0±1.8	98.0±4.2
	3	1.10±0.08	98.0±1.9	100.0±3.9
	6	1.00±0.08	98.0±1.9	102.0±4.0
	24	1.10±0.09	96.0±2.1	104.0±3.9

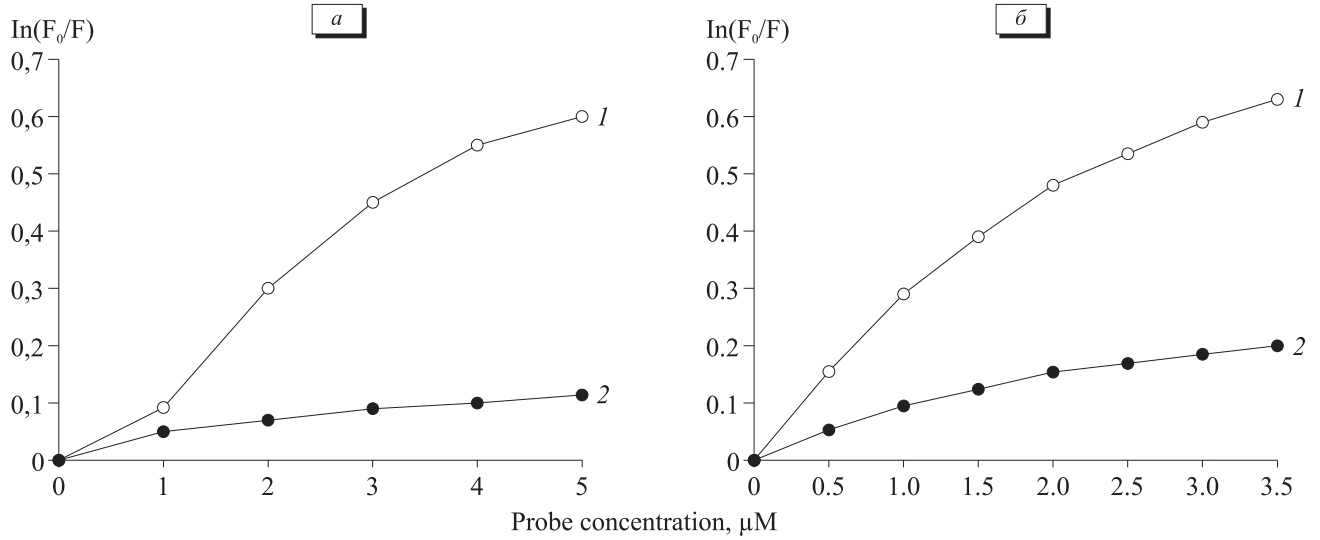

Fig. 2. Quenching of autofluorescence LDL UV-FL due to energy transfer to FL probes anthracene (*a*) and DSP-12 (*b*).

The absorption spectrum for substances formed during interaction of LPO products with protein overlaps with the spectrum of LDL UV-FL and therefore Forster energy transfer can occur between chromophores. To test this hypothesis, we studied the dependence of F_{UV} on the concentration of TBA-reactive substances (Fig. 1, *a*). The dependence was typical of energy transfer. Hence, energy transfer to chromophores resulting from LPO can contribute to the decrease in UV-FL.

Study of dynamic quenching of LDL UV-FL with water-soluble Cs^+ showed that the efficiency of quenching in oxidized LDL is higher than in native particles (Fig. 1, *b*).

Availability of tryptophanyls for water-soluble quenchers of FL can be modified by structural changes in apoprotein molecule. Study of energy transfer from tryptophanyls residues to FL probe pyrene showed that nearly all tryptophan residues in native LDL are situated near the particle surface and have similar microenvironment. They are embedded into the lipid phase at a distance of 10-20 Å from the lipid—water boundary. Long-term storage is probably accompanied by LPO and results in apoprotein "sliding" from the lipoprotein surface and the formation of protein projections. In our experiments, 2 probes served as energy acceptors to study the structure of apoprotein during LDL autooxidation. DSP-12 is localized on the surface of LDL. Anthracene is distributed over the volume of the lipid nucleus. The efficiency of energy transfer from tryptophanyls to probes in oxidized LDL was higher than in native particles (Fig. 2), which provides support for apoprotein sliding from the surface of LDL during autooxidation. It can be hypothesized that changes in the microenvironment of tryptophan residues increase their availability for water-soluble molecules, including FL quenchers.

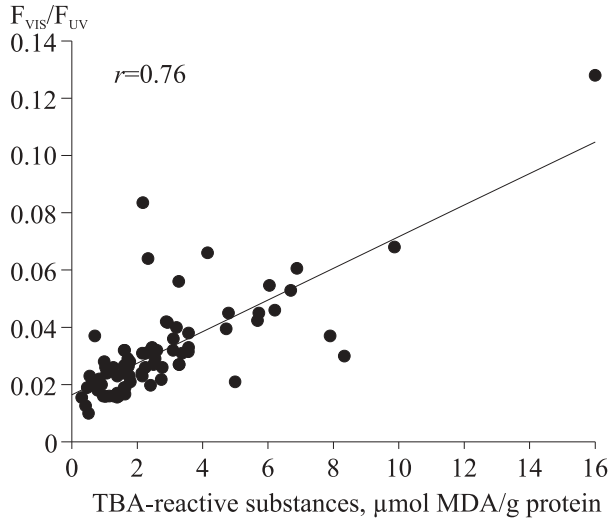

Fig. 3. Dependence of the F_{VIS}/F_{UV} ratio for LDL FL (*n*=76) on the intensity of LPO.

The F_{VIS}/F_{UV} ratio was calculated to estimate the degree of LDL modification. This approach does not require standardization of FL measurement. The relationship between F_{VIS}/F_{UV} and LPO was estimated. LDL were isolated from the blood of healthy donors (n=24) and incubated at 37°C for various periods. The study was performed with 76 LDL samples differing by the degree of modification. A positive correlation was found between F_{VIS}/F_{UV} and concentration of TBA-reactive substances (r=0.76, p<0.001; Fig. 3). The mean value of F_{VIS}/F_{UV} for LDL after 3-h autooxidation was 0.036±0.003, which is significantly lower compared to native LDL (0.019± 0.001). The conventional intergroup boundary was drawn at 0.027. In only 1 of 24 native samples

F_{VIS}/F_{UV} that exceeded this value and in 1 sample of modified LDL F_{VIS}/F_{UV} was below 0.027. The difference between F_{VIS}/F_{UV} for native and oxidized LDL increased under conditions of strong oxidation (24-h incubation of LDL at 37°C). Hence, study of variations in LDL autofluorescence by calculating the F_{VIS}/F_{UV} ratio allows us to distinguish oxidized and native LDL.

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