Chemiluminescence Study of the Contents of the Products of Cu^{2+}-Induced Lipid Peroxidation in Different Fractions of Human Blood Lipoproteins

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It is demonstrated that the content of the primary products of lipid peroxidation reaches the maximum after about 1-h incubation with Cu^{2+} and then declines. At a Cu^{2+} concentration of about 10-15 μM, the content of lipid peroxidation products is maximal; it does not rise with a further increase in the Cu^{2+} concentration. Comparison of the kinetics of lipid peroxidation in different lipoprotein fractions shows that low density lipoproteins are much more strongly oxidized than high density lipoproteins. A strong positive correlation between the amplitude of the chemiluminescence burst and the diene conjugate content is established in 79 independent measurements.

Key Words: *lipoproteins; chemiluminescence; copper ions; lipid peroxidation*

Recently it has been found that oxidative modification of lipoproteins (LP) is an important factor in the pathogenesis of atherosclerosis [8,13,14]. The mechanisms of activation of lipid peroxidation (LPO) leading to alterations in LP structure are unclear. However, there is a considerable body of evidence indicating that oxidation of LP impairs cholesterol transport between low density and high density lipoproteins (LDL and HDL), on the one hand, and blood cells and vascular endothelium [11] on the other. Determination of the degree of LP oxidation can be useful for the identification of atherosclerosis risk groups and evaluation of the disease severity.

It has been demonstrated *in vitro* that LP oxidation leads to the accumulation of primary (hydroperoxides) and secondary (aldehydes, specifically, malonic dialdehyde, ketones, etc.) LPO products [4,6]. Generally, the level of 2-thiobarbituric acid-reactive substances has been used as a parameter characterizing the degree of LP oxidation. This colorimetric method is sensitive enough to malonic dialdehyde but it is not specific, since many other compounds react with 2-thiobarbituric acid with the formation of the same chromophore [5]. In addition, malonic dialdehyde is a minor LPO product whose content is reportedly not more than 2% of the total concentration of unsaturated fatty acids which are the LP components, and alterations in the biological properties of oxidized LP are probably not associated with the accumulation of malonic dialdehyde [6].

Presumably, lipid hydroperoxides, the primary products of free radical reactions involving unsaturated fatty acids, are the most important factors for oxidative modification of LP. In addition, in the presence of transition metals lipid hydroperoxides induce LPO branching [2,16]. Spectrophotometric determination of diene conjugates formed upon free-radical oxidation of polyunsaturated fatty acids is a method commonly used for analysis of the hydroperoxide content [12]. Determination of hydroperoxides by high-performance liquid chromatography with a chemiluminescence analysis in the

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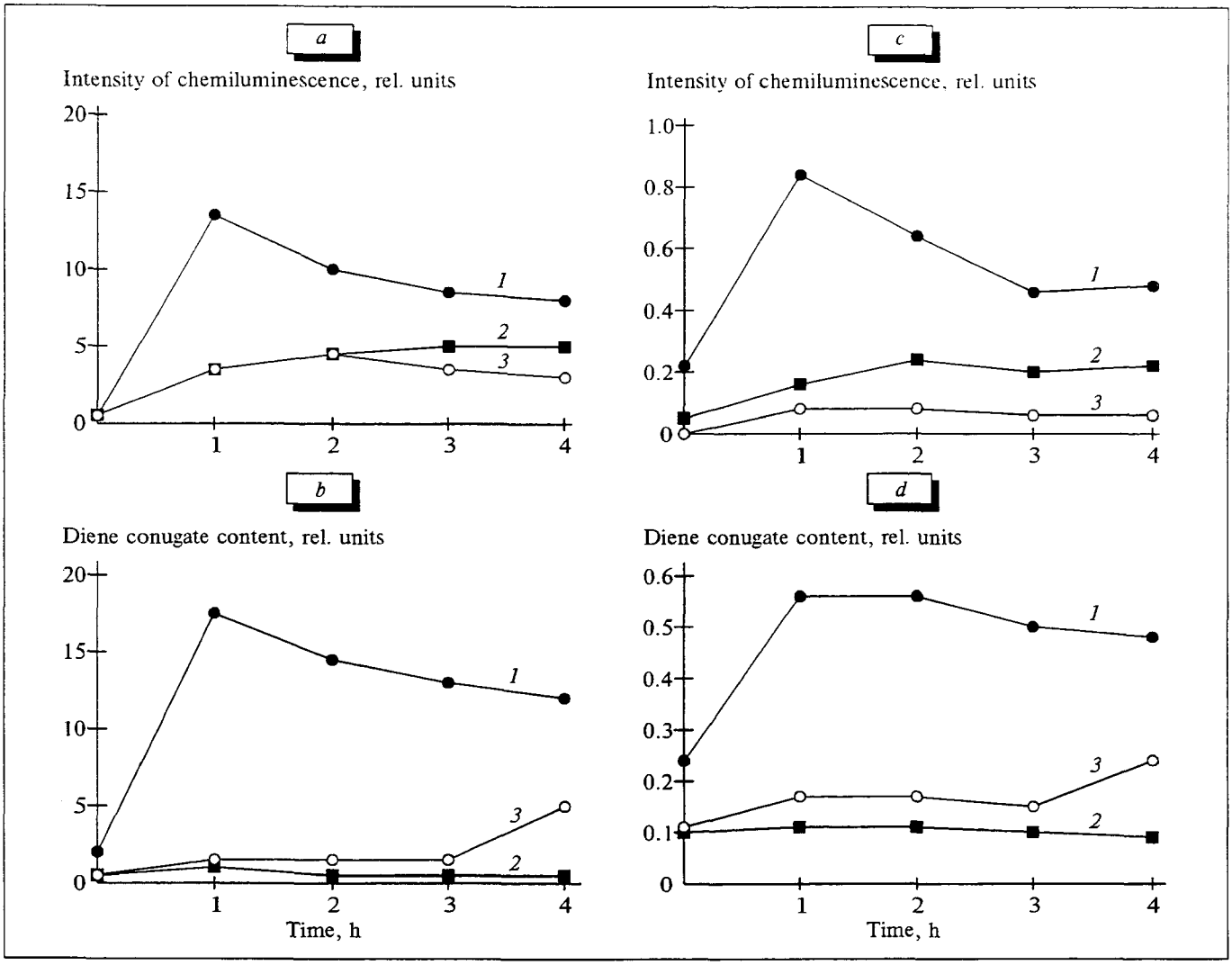

Fig. 1. Kinetics of LP oxidation at 37°C in the presence of 10 μM Cu^{2+}. *1*) LDL; *2*) HDL_2; *3*) HDL_3. Amplitudes of chemiluminescence (*a*: donor 1, *b*: donor 2) were measured after the addition of 1 mM Fe^{2+} in aliquots containing 0.08 mg protein/ml in 0.1 M NaCl, 0.02 M Tris–HCl, pH 7.4. The concentration of diene conjugates (*c*: donor 1; *d*: donor 2) is expressed as the difference between light absorbance at 231 and 300 nm.

presence of peroxidase and luminol derivatives is the most sensitive method [7]. However, this method is not widely applied in clinical laboratories, since it requires complicated equipment. Recently, we showed that the reaction of lipid peroxides in LDL with Fe^{2+} is accompanied by a chemiluminescence burst, the intensity of which under certain conditions depends on the peroxide content in human plasma LDL [3].

Our objective was to study the Cu^{2+}-induced LPO in different LP fractions isolated from human plasma with the use of the chemiluminescence reaction of lipid peroxides with Fe^{2+}.

MATERIALS AND METHODS

LDL and HDL fractions (HDL_2 and HDL_3) were isolated from sera of healthy donors by ultracentrifugation in a NaBr density gradient [9]. The LP were dialyzed for 18 h against 500 volumes of the standard buffer (10 mM Tris-HCl, 100 mM NaCl, pH 7.4). The protein concentration was determined by the microbiuret method [15]. Oxidation was carried out for 4 h at 37°C in a dry thermostat in the presence of varied concentrations of Cu^{2+}. The amplitude of chemiluminescence bursts was measured in an LKB-1251 chemiluminometer as described elsewhere [3] after the addition of Fe^{2+} (1 mM) to LP samples containing 0.08 mg protein/ml in the standard buffer. The samples were thoroughly mixed and thermostated at 25°C. The diene conjugate content was determined from the absorbance spectra of heptane extracts and expressed as the difference between the absorbances at 231 and 300 nm [12]. The absorbance spectra were measured in a Beckman DU-7 spectrophotometer.

Tris-buffer from Merck (Germany) and Russian-manufactured reagents of chemically pure grade were used for preparation of the solutions. Fe^{2+} solutions were prepared from $Fe_2SO_4 \times 7H_2O$ in 0.01 N HCl, Cu^{2+} solutions from $Cu_2SO_4 \times 5H_2O$.

RESULTS

Figure 1, *a-d* shows the kinetics of accumulation of lipid hydroperoxides (primary LPO products) measured by the chemiluminescence (Fig. 1, *a, b*) and diene conjugate (Fig. *1, c, d*) methods in three LP fractions (LDL, HDL_2, and HDL_3) isolated from sera of two donors. Oxidation of different LP fractions with a protein concentration of 0.8 mg/ml was performed in the presence of 10 μM Cu^{2+}. It can be seen from the figure that the kinetics of LDL, HDL_2, and HDL_3 oxidation differed both in the initial and maximal contents of primary LPO products and in the rate of their increase and decrease. The level of LPO products in LDL was considerably higher than in HDL_2 and HDL_3. In addition, the primary LPO products were rapidly accumulated (compared with HDL_2 and HDL_3) at the first stage of the process (during 1 h); with time the rate of accumulation slowed down, and subsequently the amount of primary products declined.

Changes in the Cu^{2+} concentration in the sample had a noticeable effect on the kinetics of LPO product accumulation. This is illustrated by Fig. 2, *a-d* showing the dependence of accumulation of LPO products on the Cu^{2+} concentration. It can be seen from the figure that small concentrations of Cu^{2+} induce the maximum (10 μM) accumulation of primary LPO products. The LPO product content decreased with an increase in the Cu^{2+} concentration. Figure 2 shows that the degree

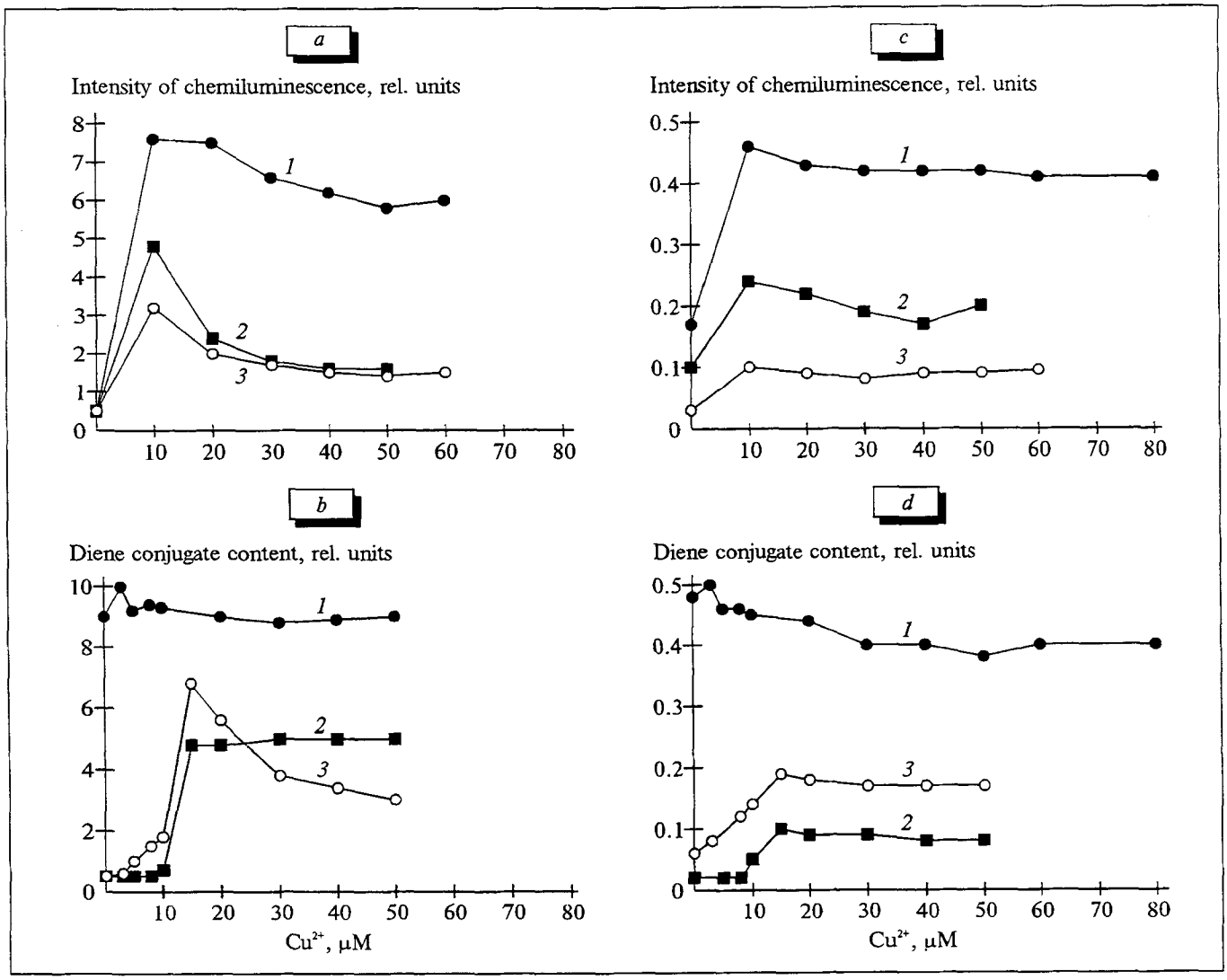

Fig. 2. LP oxidation as a function of the Cu^{2+} concentration after 4 h of incubation. The conditions of measurements and symbols are the same as in the legend to Fig. 1.

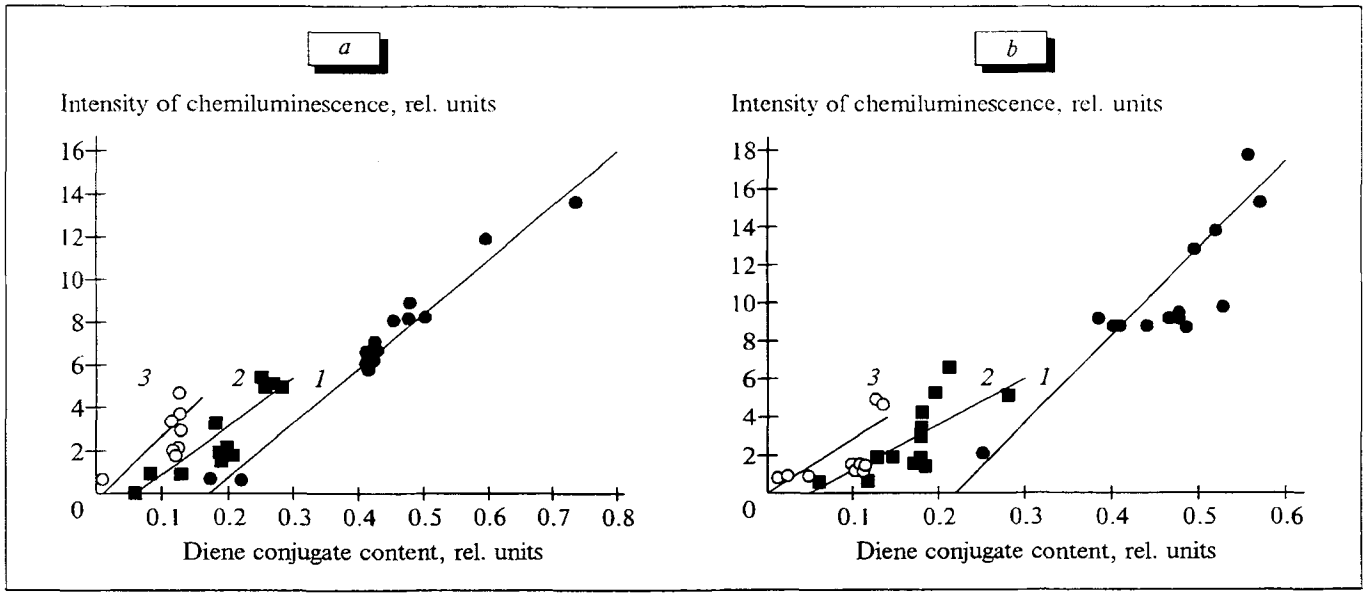

Fig. 3. Correlation between the amplitude of the chemiluminescence burst and the diene conjugate content (*a*: donor 1; *b*: donor 2). *1*) LDL; *2*) HDL_2; *3*) HDL_3. All values were obtained on the basis of the data presented in Fig. 1.

of LDL oxidation is appreciably higher than that of HDL over the entire range of Cu^{2+} concentrations. The difference between the oxidation of HDL_2 and HDL_3 was less pronounced. From the data on the Cu^{2+}-induced oxidation of the different LP fractions it can be concluded that under the same conditions LDL oxidation is more intense compared with that of HDL_2 and HDL_3. This is probably associated with the differences in the fatty acid composition of the different LP fractions.

TABLE 1. Coefficients of Linear Regression and Correlation between the Amplitude of the Fe^{2+}-Induced Chemiluminescence Burst and the Content of Diene Conjugates in LP Resulting from Cu^{2+}-Induced LPO

	$a \pm S_a$	$b \pm S_b$	r_{xy}
	Donor 1		
LDL ($n=13$)	24.8±1.3*	−4.1±0.6*	0.98
HDL_2 ($n=11$)	24.1±5.3*	−1.5±1.0*	0.84
HDL_3 ($n=12$)	31.4±8.0*	−0.4±0.7*	0.78
	Donor 2		
LDL ($n=13$)	42.2±7.7*	−9.0±3.6*	0.86
HDL2 ($n=15$)	31.0±10.3*	−0.4±0.9*	0.64
HDL3 ($n=14$)	28.1±1.2*	−1.7±1.3**	0.71
Total ($n=78$)	20.0±1.1*	−0.1±0.3**	0.89

Note. *a* and *b* are the coefficients in the linear regression equation $y=a_x \pm b$, where *y* is the amplitude of the chemiluminescence burst, *x* is the absorbance of diene conjugates in an LP sample; S_a and S_b are the standard errors of the corresponding coefficients, *n*: number of observations, r_{xy}: empirical correlation coefficient with correction for a small sample; significance level: one asterisk indicates $\alpha<0.01\%$ and two asterisks indicate $\alpha<1\%$.

An increase in the Cu^{2+} concentration starting from 10-15 μM induces no increase in the oxidation product content in any of the LP fractions and can even reduce it. Judging from the kinetics of LP oxidation, the decrease in the concentration of primary LPO products results from their degradation during just a 4-h incubation with Cu^{2+}. Such a dependence of LP oxidation on the Cu^{2+} concentration, one which is similar for all fractions, can be explained by the fact that Cu^{2+} binds to LP, and there is a saturating Cu^{2+} concentration on the surface of an LP particle. In this case, an excess of this concentration of copper ions should not lead to additional accumulation of LPO products. The more intense oxidation of LDL compared with HDL_2 and HDL_3 and their competition for Cu^{2+} binding may be the cause of the antioxidant activity of HDL upon their oxidation together with LDL observed *in vitro* [10].

Analysis of the LPO product contents measured by the two methods (absorbance of diene conjugates and amplitude of the Fe^{2+}-induced chemiluminescence burst) showed their good qualitative match (Figs. 1 and 2). We performed correlation analysis [1] between these indexes using the kinetic curves and the concentration dependences for all the LP fractions. For all the results obtained in experiments with sera of two donors a linear correlation was established between the amplitude of the burst of the Fe^{2+}-induced chemiluminescence and the diene conjugate content (r=0.89, Table 1).

We also performed regression analysis [1] both for the aggregate of results and for LP fractions

individually for the different donors. The results of this analysis are shown in Fig. 3, *a, b*. It can be seen that the results of chemiluminescence and diene conjugate measurements are well resolved for the different LP fractions of the same donor and for the same LP fractions of different donors. The regression coefficients and the reliability levels for the fractions are summarized in Table 1. Despite the high coefficient of correlation between the absorbance of diene conjugates and the amplitude of the Fe^{2+}-induced chemiluminescence burst for the entire set of results, regression analysis revealed a significant difference in the regression coefficient (more than 2-fold) for different LP fractions and for different donors.

A strong positive correlation (r=0.89) was established between the contents of primary LPO products measured by the light absorbance of diene conjugates and the amplitude of the Fe^{2+}-induced chemiluminescence. However, regression analysis showed that the linear regression coefficients for the different LP fractions and for the same LP fractions of different donors varies considerably. For example, in the case of LDL the regression coefficients varied from 24.8 to 42.16 for the two donors. Individual changes in the regression coefficient are probably associated with a different quantum efficiency of chemiluminescence and result from individual differences in the LP composition of the different fractions obtained from different donors.

It should be noted that the convenience and simplicity of chemiluminescence analysis in comparison with other methods of determining primary LPO products are obvious advantages. However, when using this method, one should remember the possibility of variations in the quantum efficiency of chemiluminescence from sample to sample. The cause of these variations is so far unclear.

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