Evaluation of the Resistance of Blood Plasma to Oxidative Stress by Oxidizability of Proteins and Lipids during Metal-Catalyzed Oxidation

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 9, pp. 268-272, September, 2006
Original article submitted February 14, 2006

A new approach for the evaluation of oxidizability of proteins and lipids in the same sample of blood plasma was proposed. We tested a method for evaluation of metal-catalyzed oxidation of fibrinogen by the formation of bityrosine cross-links during oxidation detected by the increase in fluorescence at 415 nm. A correlation was revealed between parameters of oxidizability estimated by this marker and carbonyl derivatives (dinitrophenylhydrazine method). Oxidizability of total proteins from whole plasma was compared with oxidizability of plasma lipids (marker malonic dialdehyde). Study of these parameters in patients with coronary heart disease showed that the proposed experimental approach allows us to divide the sample into several subgroups differing in the resistance to oxidative stress. These data can be used for diagnostic and prognostic purposes.

Key Words: *Cu^{2+}-catalyzed oxidation of lipids and proteins; malonic dialdehyde; blood plasma; oxidative stress*

Pathological conditions induced by oxidative stress are characterized by the appearance of modified intra- and extracellular macromolecules. They are formed during free radical oxidation and serve as a biomarker of oxidative stress. Oxidative stress is a pathogenetic factor for various diseases. Identification and measurements of these biomarkers are important to evaluate the course of the disease. Peculiarities of lipid peroxidation were studied in details. The intensity of this process is estimated by the content of oxidized lipids in blood plasma or serum (*e.g.*, malonic dialdehyde, MDA) [2].

The content of modified components in blood plasma depends on their stability and absorption by vascular cells and other cells. In light of this, valuable information can be obtained in the analysis of potential vulnerability of plasma lipids to Cu^{2+}-catalyzed oxidation *in vitro* (oxidizability or oxidative resistance). Under these conditions, oxidation occurs at high dilution, depends little on the influence of water-soluble plasma antioxidants, and is mainly affected by fat-soluble compounds [1].

Plasma proteins are another target for oxidative stress. Oxidation products of these compounds are revealed during various diseases [3]. Oxidation products of amino groups from the lateral chain of proteins containing carbonyl groups are the major markers that can be identified by the interaction with dinitrophenylhydrazine (DNPH) [4]. Bityrosine derivatives formed during oxidation of tyrosine residues also serve as the marker of protein oxidation [5,6].

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0007-4888/06/1423-0299

Oxidized lipids and proteins are assayed in various studies. It should be emphasized that the method to study oxidizability was developed only for lipids.

Here we developed a new method to study oxidizability of proteins and lipids in the same sample of oxidized plasma. Parameters of oxidative resistance were compared in the detection of several biomarkers, including MDA (for lipids) and carbonyl or bityrosine derivatives (for proteins).

MATERIALS AND METHODS

The solution of fibrinogen (3 mg/ml) in phosphate buffered saline (0.15 M NaCl and 5 mM NaH_2PO_4, pH 7.4) was incubated with $FeSO_4 \times 7H_2O$ in various concentrations (10^{-4}, 3×10^{-4}, and 5×10^{-4} M) at 37°C over different time intervals. Desferrioxamine mesylate 100 mM (10 μl per 1 ml fibrinogen solution, final concentration 1 mM) was added for binding of Fe^{2+} and formation of chelate complexes. Oxidized protein was subjected to single dialysis in phosphate buffered saline (pH 7.4) for 15 h.

Standard buffered saline (4.9 ml, pH 7.2-7.4) and aqueous solution of $CuSO_4$ were added to 100 μl plasma (final concentration 20 μM). The mixture was incubated at 37°C for various time intervals.

Fluorescence was estimated by recording of the emission spectrum at 340-500 nm (excitation wavelength 325 nm). Fluorescence was expressed in relative units of fluorescence and calculated as follows: $I_{415}-(I_{350}+I_{500})/2$, where I_{415}, I_{350}, and I_{500} are emission values at 415, 350, and 500 nm, respectively.

Carbonyl products of protein oxidation were assayed in the reaction with 2,4-DNPH in 2 nM HCl [7]. Aliquot samples (0.2 ml) were taken from each tube at fixed time intervals. HCl (1 ml, 2 nM) was added to control samples. 2,4-DNPH (1 ml, 0.1 M in 2 nM HCl was added to experimental samples. The samples were incubated in darkness at room temperature for 1 h, mixed with 1 ml 25% trichloroacetic acid, and centrifuged at 3000 rpm for 15 min. The pellet was washed 2 times with a mixture of ethanol and ethyl acetate (1:1). The precipitate was dried and dissolved in 2 ml 8 M urea in a boiling water bath. Optical density was recorded on a Beckman spectrophotometer at 370 nm. The concentration of carbonyl products was calculated as follows: $C\ (nmol/ml)=(Abs_{370\ nm}\times10^6/E_{370\ nm})\times X$, where C is the concentration of carbonyl groups; $Abs_{370\ nm}$ is optical density of the sample at 370 nm; $E_{370\ nm}$ is the molar extinction coefficient (22,000 $M^{-1}cm^{-1}$); and X is the factor of dilution. Protein concentration was measured by the biuret micromethod.

MDA concentration was determined in the reaction with thiobarbituric acid (TBA). TBA-reactive substances were detected by absorption at $\lambda_{max}=532$ nm. The molar extinction coefficient for products of the reaction between TBA and aldehyde groups was 1.56×10^5 $M^{-1}cm^{-1}$.

RESULTS

In series I we tested the method to study metal-catalyzed oxidation of plasma protein fibrinogen. This method suggested the detection of bityrosine cross-links (oxidation products). Fibrinogen was chosen because it is characterized by the highest oxidizability among plasma proteins [8]. The formation of bityrosine cross-links was detected by the increase in fluorescence at 415 nm (excitation wavelength 325 nm).

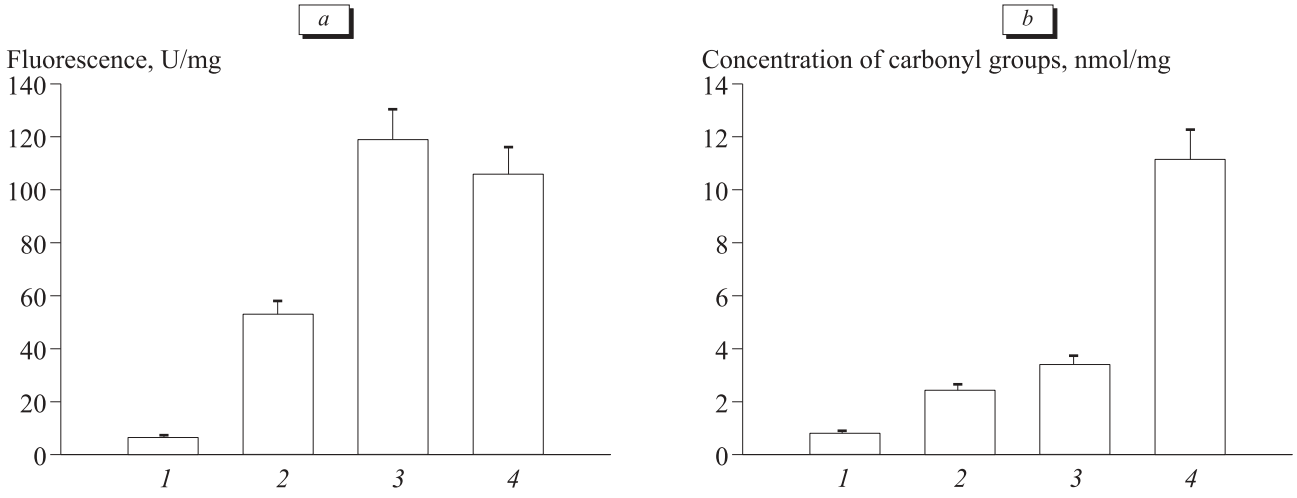

Fig. 1. Evaluation of fibrinogen oxidation by the fluorescence (*a*) and DNPH method (*b*). Nonoxidized fibrinogen (control, *1*); fibrinogen oxidized by Fe^{2+}-H_2O_2 in concentrations of 10^{-4} (*2*), 3×10^{-4} (*3*), and 5×10^{-4} M (*4*).

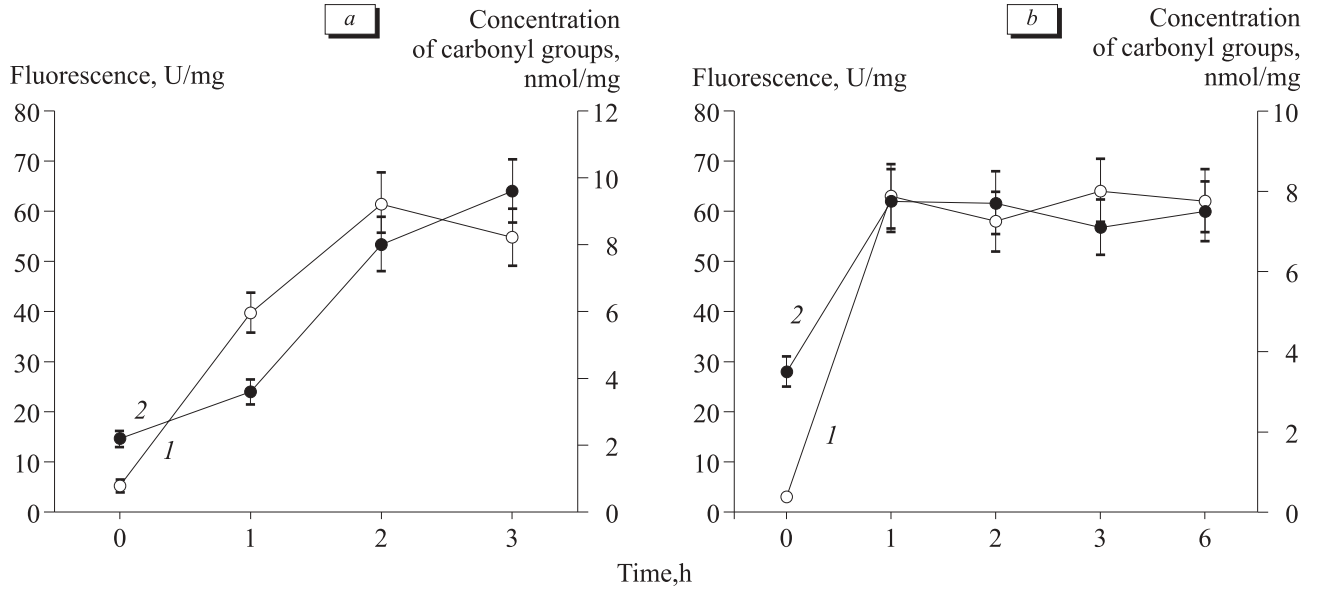

Fig. 2. Dependence of fibrinogen oxidation on the time of incubation with oxidizing agent under conditions of moderate (*a*) and thorough agitation of reagents (*b*). Detection by the fluorescent (*1*) and DNPH methods (*2*).

We studied the dependence of the number of bityrosine cross-links on the concentration of the oxidizing agent. Fluorescence initially increased, but then slightly decreased with increasing the concentration of the oxidation mixture from 10^{-4} to 3×10^{-4} M (Fig. 1, *a*). The content of carbonyl products in samples of oxidized fibrinogen was estimated using DNPH. The content of carbonyl groups also increased with increasing the concentration of the oxidizing agent (Fig. 1, *b*). The study by the bityrosine method showed that the maximum value is achieved at the oxidizing agent concentration of 3×10^{-4} M. In experiments by the carbonyl method, this value was observed under more strict conditions.

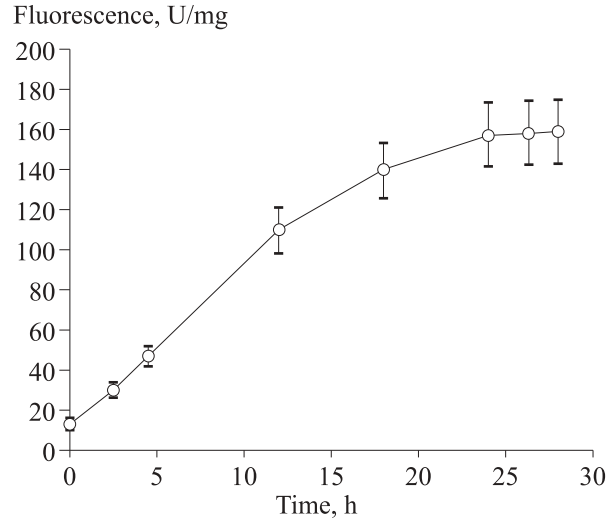

Fig. 3. Accumulation of bityrosine cross-links in plasma proteins.

In series II we evaluated the time-dependence of accumulation of oxidation products during fibrinogen incubation with the oxidizing agent. Detection was performed by the fluorescence or DNPH method (Fig. 2). The rate of oxidation depended on agitation of components in the reaction mixture. Under conditions of moderate agitation the content of oxidation products increased during the first 2 h of incubation. The slight decrease in this parameter during the follow-up period of study coincided with the formation of bityrosine cross-links (Fig. 2, *a*). The content of oxidation products increased after 3-h incubation with DNPH (Fig. 2, *b*). After thorough agitation of reagents the rate of oxidation significantly increased, and saturation was achieved over 1-h incubation. Further experiments were performed under conditions of moderate agitation.

In series III we studied total protein oxidizability in the whole plasma and compared the results with the data on lipid oxidizability evaluated by MDA content. Copper sulfate was used as an oxidizing agent similarly to previous experiments where lipid oxidizability was determined by the content of oxidation product MDA [3]. The plasma was oxidized after 50-fold dilution with phosphate buffered saline in the presence of 20 μM copper sulfate. Similarly to lipid oxidation [3], the kinetic curve for oxidation with the formation of bityrosine cross-links reached a plateau (Fig. 3). In further experiments, the plasma was oxidized for 20-24 h under these conditions. Table 1 shows oxidizability of plasma proteins estimated by the DNPH and fluorescence methods. Lipid oxidizability in 15 patients

TABLE 1. Cu^{2+}-Catalyzed Oxidation of Plasma Proteins and Lipids

Sample	Concentration of bityrosine derivatives, rel. U fluorescence/ml plasma		Concentration of carbonyl groups, nmol/ml plasma		Protein concentration, mg/ml plasma		Concentration of bityrosine derivatives, rel. units/mg protein		Concentration of carbonyl groups, nmol/mg protein		MDA concentration, nmol/ml plasma	
	before therapy	after therapy	before therapy	after therapy	before therapy	after therapy	before therapy	after therapy	before therapy	after therapy	before therapy	after therapy
1	8314	6904	910.0	780.0	90.0	75.0	92.4	92.1	10.1	10.4	66.0	77.3
2	8781	8178	925.0	980.0	80.0	72.5	109.8	112.8	11.6	13.5	69.8	119.3
3	7844	6535	765.0	655.0	77.5	72.5	101.2	90.1	9.9	9	57.8	71.3
4	11753	5939	1110.0	580.0	90.0	75.0	130.6	79.2	12.3	7.7	150.8	57.8
5	8854	7158	780.0	835.0	82.0	80.0	108.0	89.5	9.5	10.4	90.8	86.3
6	6493	5909	765.0	800.0	100.0	77.5	64.9	76.2	7.7	10.3	63.8	99.8
7	10326	10930	1072.5	1020.0	80.0	67.5	129.1	161.9	13.4	15.1	150.0	86.0
8	10728	6704	1145.0	835.0	87.5	80.0	122.6	83.8	13.1	10.4	171.8	96.0
9	8721	8866	945.0	1090.0	80.0	75.0	109.0	118.2	11.8	14.5	80.3	129.0
10	10472	11027	1072.5	1035.0	77.5	72.5	135.1	152.1	13.8	14.3	90.0	95.3
11	8061	7870	410.0	280.0	67.5	62.5	119.0	126.0	6.1	4.5	117.8	120.0
12	10192	11567	365.0	345.0	60.0	67.5	170.0	171.0	6.1	5.1	106.5	115.5
13	12027	9278	455.0	225.0	62.5	57.5	192.0	161.0	7.3	3.9	100.5	50.3
14	14338	10755	435.0	272.5	57.5	57.5	249.0	187.0	7.6	4.7	93.0	76.5
15	8096	11825	272.5	372.5	57.5	62.5	141.0	189.0	4.7	6.0	61.5	132.8

Note. Relative error in measurements of lipid and protein oxidizability is 7-10 and 5-8%, respectively.

with cardiovascular diseases was determined before and after therapy (Table 1). This experimental approach allowed us to divide the sample into several subgroups differing in the resistance of plasma components to oxidative stress. Oxidizability of proteins and lipids in plasma samples from patients 4, 8, 13 and 14 decreased after the course of therapy. These changes were accompanied by an increase in oxidative resistance (from patients 9, 10, 12, 15). In some samples, the course of therapy had opposite effects on the ability of plasma proteins and lipids to undergo peroxidation.

We conclude that data on oxidative resistance of plasma proteins and lipids can be used in combination with the results of clinical and laboratory examination to make the diagnosis and predict the course of the disease.

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