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Indicator Characterizing Carbonyl-Dependent Modification of Erythrocytic Superoxydismutase as a Biochemical Marker of Oxidative Stress in Coronary Heart Disease

Aim	To study the oxidative modification of red blood cell Cu,Zn superoxide dismutase (SOD) in patients with ischemic heart disease (IHD) in vivo and in vitro to substantiate the use of a new oxidative stress marker.
Material and methods	Red blood cell Cu,Zn SOD was measured by depression of nitrotetrazolium blue reduction by the superoxide anion generated in xanthine oxidase xanthine oxidation. Red blood cell Cu,Zn SOD was measured immunochemically. The biochemical study was performed in the control group (patients with low extremity fracture without known history of cardiovascular diseases and hyperlipidemia) and in groups of patients with acute myocardial infarction, stable angina, and decompensated heart failure. For evaluation of oxidative stress intensity in IHD patients, an empirical SOD oxidative modification coefficient (OMC_{SOD}) was proposed, which is a Cu,Zn SOD activity/Cu,Zn SOD content ratio.
Results	The red blood cell Cu,Zn SOD activity was significantly decreased in all IHD groups compared to the control group. Furthermore, OMC_{SOD} was also considerably decreased in IHD patients, which warrants the use of this biochemical index as an oxidative stress marker.
Conclusion	It was shown that the Cu,Zn SOD modification was induced by interaction of the enzyme molecules with a natural dicarbonyl, malonic dialdehyde, and OMC_{SOD} can be used for evaluation of oxidative stress intensity in IHD patients.

Keywords	Red blood cell superoxide dismutase; natural dicarbonyls; oxidative stress; modification coefficient; ischemic heart disease
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A large number of observations indicate that oxidative stress is a significant factor in the origin and pathogenesis of atherosclerosis, diabetes mellitus (DM), and neurodegenerative diseases [1–6]. The oxidative stress in atherosclerosis and coronary artery disease (CAD) involves free-radical oxidation of the polyene acyl groups of the phospholipids in the outer layer of low-density lipoproteins (LDL), which are the primary component in the lipid transport system of blood plasma [1].

Acylhydroperoxidation derivatives of LDL phospholipids undergo continued oxidative destruction, leading to the accumulation of various alpha-oxoaldehydes, such as 4 hydroxinonenal and other nonenals, and the most abundant, malon dialdehyde (MDA) [1]. Aldehyde groups of MDA can react quickly with free protein amino groups, which leads to their structural and functional modification [1]. For example, apoprotein B-100 of the LDL particles can undergo this modification, and such carbonyl-modified LDL particles are recognized and captured by the vessel wall cells via the scavenger receptors, which leads to the accumulation of these atherogenic LDL particles and the pre-atherogenic damage (lipoidosis) of the vessel wall [1].

It is clear that other blood plasma and blood cell proteins can undergo carbonyl modification. Due to small size and high hydrophilicity, a three-carbon MDA molecule is more likely to easily penetrate the pores and channels of various cell membranes. We have already shown that patients with type 2 DM and severe disorders of carbohydrate metabolism (high levels of glycated hemoglobin) had not only increased plasma levels of carbonyl-modified LDL but also a very significant decrease in the activity of erythrocytic superoxide dismutase (Cu,Zn-SOD) [7]. This may be due to the

accumulation in the DM patient's plasma of non-MDA dicarbonyls (glyoxal and methylglyoxal), which are formed in hyperglycemia as a result of the auto-oxidation of glucose and enzymatic oxidation of triosophosphates accumulated during glycolysis.

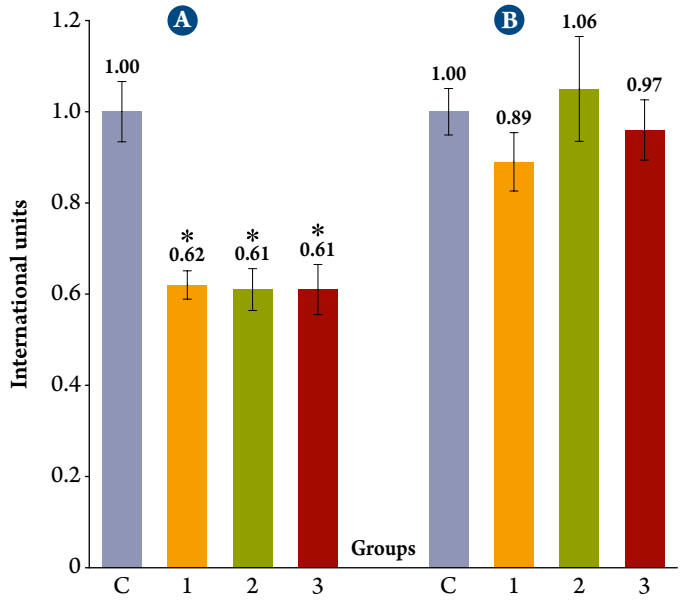
Thus, the objective of this work was to study the possibility of carbonyl modification of erythrocyte Cu,Zn-SOD, which can be accompanied by inhibition of the enzyme's activity and its dysfunction. It should be noted that such work is very relevant due to the lack of scientifically validated markers of oxidative stress, which seem necessary for correcting oxidative stress in patients with CAD and estimating the efficacy of the treatment.

Materials and Methods

The study included 72 male patients aged 50–70 years (mean age 61.3±0.78 years) urgently admitted to V.V. Veresaev City Clinical Hospital. The treatment group included 56 patients with verified CAD (diagnosis was based on coronary angiography findings). All patients were diagnosed with stage III hypertension and dyslipidemia. Forty patients with CAD underwent percutaneous coronary intervention. All patients were divided into three groups: Group 1 (n=32; mean age 60.1±1.0 year) included patients with acute myocardial infarction; Group 2 (n=8; mean age 62.1±1.3 years) comprised patients with functional class II–III stable angina admitted for routine revascularization; and Group 3 (n=16; mean age 63.3±1.0 years) consisted of patients with decompensated chronic heart failure (CHF). The control group comprised 16 male patients (mean age 56.8±1.9 years) from the Trauma Department with lower limb fractures without a known history of cardiovascular diseases and hyperlipidemia who were exposed to moderate risk of cardiovascular complications (Systematic COronary Risk Evaluation [SCORE] 3–4%). All patient groups were comparable in age (see Table 1). In the treatment group, blood samples for biochemical tests were taken at admission to the hospital immediately after cardiological examination and patient's signing of informed consent. In the control group, patients signed the informed consent form for biochemical testing, which was performed 1–2 weeks after the trauma at the stage of callus formation.

The activity of Cu,Zn-SOD was determined in erythrocytes isolated from the venous blood of fasting patients in the presence of anticoagulant and antioxidant EDTA 1 mg/mL. Erythrocytes were lysed in 5 mM buffer of K,Na-phosphate pH 7.4 for 15 minutes in an ice bath. Erythrocyte lysate was centrifuged for 10 minutes at 20 krpm in a Sigma 3-16KL refrigerated general-purpose centrifuge (Japan). Then two volumes

Figure 1. Activity of Cu,Zn-SOD (A) and levels of the enzyme (B) in erythrocytes in patients with CAD (international units; in the control group, values are taken as 1)



Column symbols: C = control; 1, 2, 3 = patients with CAD: 1 =patients with acute myocardial infarction; 2 =patients with stable angina; 3 =patients with decompensated CHF. CAD, coronary artery disease; CHF, chronic heart failure. *, significance versus control, p<0.05.

of chloroform/ethanol (3:5) mixture were added to five volumes of supernatant and shaken in an Elmi Skyline vortex shaker. Hemoglobin sediment was removed by centrifugation. The activity of Cu,Zn-SOD in the supernatant was determined by suppressing the recovery rate of nitrotetrazolium blue by superoxide anion radicals generated during the oxidation of xanthine with xanthine oxidase [8]. The kinetics of the recovery of nitrotetrazolium blue was recorded at 560 nm using a Shimadzu UV-2600 spectrophotometer. One unit of Cu,Zn-SOD activity was defined as the amount of enzyme that inhibited 50% of the recovery of nitrotetrazolium blue during the reaction. SOD activity was expressed in units of activity per gram of hemoglobin (IU/g Hb). The content of Cu,Zn-SOD was determined in the blood using a human Cu/Zn-SOD enzyme-linked immunosorbent assay (ELISA) kit. Based on the data on activity and content of SOD, an empirical Cu,Zn-SOD oxidative modification coefficient (OMC_{SOD}) was calculated, which is the ratio of Cu,Zn-SOD activity (IU/g Hb) to its content (ng/g Hb). The model experiments were used to investigate the effect of intra-erythrocyte Cu,Zn-SOD modification with natural dicarbonyl MDA on the enzyme activity. Erythrocytes isolated from donor blood (apparently healthy males aged 30–40 years) were

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Table 1. Activity of Cu,Zn-SOD and its erythrocyte levels in patients with CAD

Group	Age, years	Activity of Cu,Zn-SOD, IU/g Hb	Content of Cu,Zn-SOD, ng/g Hb	OMC_{SOD}
Control (trauma); n=16	56.8±1.9	880.0±58.0	390.0±20.0	2.30±0.17
Group 1 (acute myocardial infarction; n=32)	60.1±1.0	543.2±27.4*	349.2±13.4	1.63±0.09*
Group 2 (stable angina; n=8)	62.4±1.3	536.1±40.6*	411.6±45.2	1.38±0.14*
Group 3 (decompensated CHF; n=16)	63.3±1.5	533.7±12.1*	377.3±6.4	1.50±0.03*

*, p<0.001 versus the control group. CAD, coronary artery disease; Hb, hemoglobin; CHF, chronic heart failure; SOD, superoxide dismutase; OMC_{SOD}, superoxide dismutase oxidative modification coefficient.

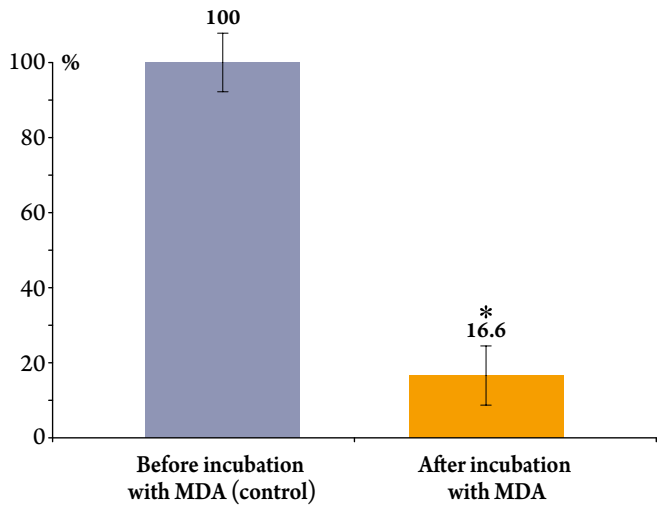
incubated for 3 hours in the isotonic phosphate buffer (pH 7.4), in the presence of 10 mM MDA, a product of acid hydrolysis of its semacetal 1,1,4,4 tetraethoxipropane, following the method described earlier [9].

The findings were statistically processed using SPSS v14.0 software for Windows version 7. Data are presented in the table and figures as a mean and standard deviation. The nonparametric Mann-Whitney test was used for intergroup comparisons. The Spearman's rank correlation test was used to identify and assess the relationship between two rows of comparable quantitative measures. The differences were statistically significant at p<0.05.

Results and Discussion

Key findings are presented in Table 1: the activity of erythrocyte Cu,Zn-SOD was reduced almost equally in all groups of patients with CAD compared to the control group (Figure 1).

Figure 2. Reduced activity of Cu,Zn-SOD in donor erythrocytes (% of control) after incubation of erythrocytes in the isotonic phosphate buffer (pH 7.4) in the presence of 10 mM MDA for 3 hours



The left column is before incubation with MDA (control, 100%); the right column is after incubation with MDA (16.6%).
The confidence interval is 6.12 for control and 7.15 after incubation.
*, p<0.05; significance versus MDA control, i.e., malon dialdehyde.

Thus, the activity of Cu,Zn-SOD in erythrocytes was reduced by 38% in Group 1, 39% in Group 2, and 39% in Group 3 (see Table 1). The levels of this enzyme in erythrocytes in all groups of patients with CAD were not significantly different from those in the control group (see Table 1). These findings suggest that the Cu,Zn-SOD molecules in erythrocytes of patients with CAD in all groups underwent oxidative modification, resulting in inhibition of the enzyme activity. The possible modifying mechanism for the activity of Cu,Zn-SOD is the following: secondary products of free radical oxidation of polyene lipids, such as 4 hydroxinonenal and MDA, are accumulated under oxidative stress and, due to high reaction capacity, can cause changes in the conformation of protein molecules through the reaction of the aldehyde group of dicarbonyls with free protein amino groups [1]. We performed model experiments to verify this suggestion, in which intra-erythrocyte Cu,Zn-SOD was modified in the presence of MDA. The findings (see Figure 2) show that MDA molecules easily penetrate through the cholesterol-rich rigid membrane of erythrocytes and cause highly significant inhibition of the activity of intra-erythrocyte Cu,Zn-SOD.

Thus, the increase in the plasma levels of MDA under oxidative stress can indeed cause carbonyl-dependent modification of the erythrocyte Cu,Zn-SOD molecules and decrease the in vivo activity of this enzyme in patients with CAD (see Table 1, see Figure 1). It should be noted that the estimation of the MDA levels by reaction with 2 thiobarbituric acid (2 TBA), which is usually used in clinical studies as a marker of oxidative stress, is not suitable due to low specificity: 2 TBA can react with different plasma non-MDA substances under the determination conditions [1]. For this reason, it is proposed to use in the literature the term «thiobarbituric acid-reactive substances» (TBARS) – that is, substances reacting with 2 TBA [10]. In the Russian literature, due to inaccurate translation, the meaningless term «TBA-active products» is used instead of TBARS: no activity of 2 TBA can occur in this case, but instead it is a matter of the ability of an oxidized substance to react with 2 TBA to form a colored trimethine complex. In summary, it can be argued that the severity of oxidative stress is more

clearly assessed by the empirical coefficient of oxidative modification of erythrocyte Cu,Zn-SOD (OMC_{SOD}): the ratio of the Cu,Zn-SOD activity (IU/g Hb) to the Cu,Zn-SOD content (ng/g Hb).

Table 1 and Figure 3 show that the difference in OMC_{SOD} level was statistically insignificant between groups of patients with CAD, varying slightly within the range 1.38–1.63, but that levels of OMC_{SOD} in patients with CAD differed statistically significantly from those of the control group, in which OMC_{SOD} levels were significantly higher, at 2.30 (see Table 1, see Figure 3).

The activity of Cu,Zn-SOD can decrease not only due to oxidative modification of enzyme molecules, but also due to other factors, such as mutations in the gene encoding the biosynthesis of Cu,Zn-SOD, and significantly more commonly due to copper and/or zinc deficiency, which present in the active center of the enzyme [1]. In these cases, the reduced activity of Cu,Zn-SOD in erythrocytes is obviously not associated with the oxidative modification of enzyme molecules. Therefore, it is suggested to determine the levels of Cu,Zn-SOD in erythrocytes by an immunochemical method using monoclonal antibodies and calculate the oxidation modification coefficient of erythrocyte Cu,Zn-SOD (OMC_{SOD}) as well as investigate the enzyme activity to clearly establish the presence of oxidation modification of erythrocyte Cu,Zn-SOD. Despite the strong positive correlation (r=0.67; p=0.001) between such measures as the activity of erythrocyte Cu,Zn-SOD and OMC_{SOD} (Figure 4), it is clear that the use of the empirical measure of OMC_{SOD} to assess the severity of oxidative stress in patients with CAD is more accurate than using only changes in Cu,Zn-SOD activity as a test, since OMC_{SOD} eliminates the possible change in the activity of the enzyme under factors other than oxidative stress.

Given the essential role of dicarbonyl-dependent modification of proteins in the pathogenesis of CAD and atherosclerosis [1], it appears that the definition of OMC_{SOD} in patients with CAD can be used not only to assess the severity of oxidative stress but probably also as an indicator of the efficacy of the treatment.

No conflict of interest is reported.

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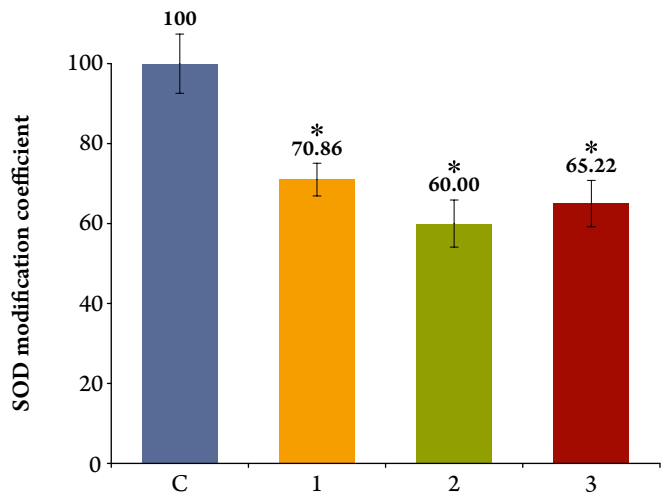
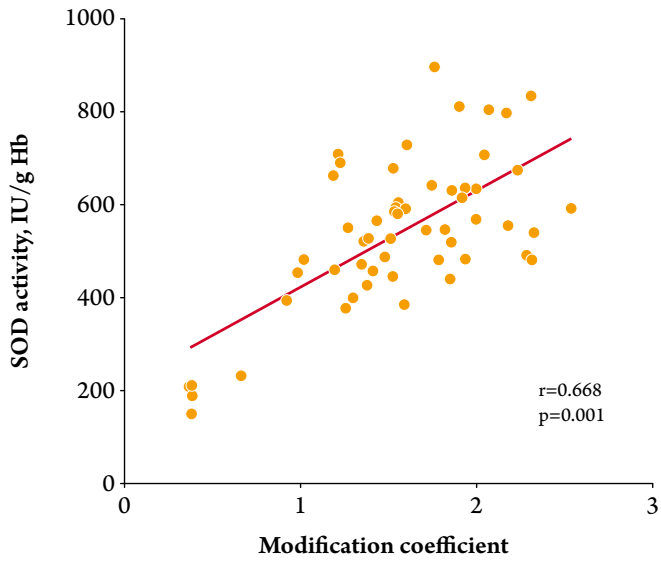
Figure 3. Values of the empirical oxidative modification coefficient of erythrocyte Cu,Zn-SOD (OMC_{SOD}) in the control group and groups of patients with CAD



Column symbols: C = control;
1, 2, 3 = patients with CAD: 1 =patients with acute myocardial infarction; 2 =patients with stable angina; 3 =patients with decompensated CHF. SOD, superoxide dismutase; CAD, coronary artery disease; *, significance versus control, p<0.05.

Figure 4. Correlation between the activity of erythrocyte Cu,Zn-SOD and the oxidative modification coefficient of erythrocyte Cu,Zn-SOD (OMC_{SOD}) in the group of patients with atherosclerosis and coronary artery disease



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