Studies of Oxidant-Induced Changes in Albumin Transport Function with a Fluorescent Probe K-35. Metal-Catalyzed Oxidation

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 4, pp. 449-454, April, 2012
Original article submitted November 9, 2010

The dynamics of albumin transport function was studied during metal-catalyzed oxidation of albumin in diluted blood plasma from healthy donors and in the solution of purified albumin using fluorescent probe K-35. The changes were compared with the dynamics of free radical oxidation markers. For oxidation, different concentrations of Cu^{2+}, Fe^{2+}, Fe^{3+} ions as well as EDTA and H_2O_2 were used. Oxidative modification of proteins was assessed by carbonyl and bityrosine fluorescent products. Oxidation of plasma lipids was assessed by the levels of TBA-reactive products. It was found that oxidation markedly decreased effective concentration of albumin characterizing albumin binding capacity, and leads to accumulation of carbonyl products of protein oxidation, bityrosine fluorescent products in proteins, and TBA-active products of lipid oxidation. It was hypothesized that reduced effective concentration of albumin is related to impairment of its binding sites and/or accumulation of free-radical oxidation products filling the binding sites of albumin.

Key Words: *albumin; metal-catalyzed oxidation; fluorescent probe K-35; effective concentration of albumin; free-radical oxidation*

Serum albumin maintains oncotic pressure, performs antioxidant functions, binds and transports low molecular weight compounds (ions, fatty acids, bilirubin, drugs, *etc*.). The binding capacity of albumin is influenced by spatial structure of its binding sites on the molecule. Conformational changes in albumin molecule can modify these binding sites and impair transport function. Plasma albumin concentration is high, 35-50 g/liter. Therefore, different metabolites (glucose, toxins, and free radicals) under various pathological conditions can modify albumin molecule and impair its function.

Fluorescent probes provide a tool to study albumin binding capacity. A method for estimating the binding capacity of albumin using fluorescent probe K-35 was developed at the Institute of Physicochemical Medicine [2]. It was found that albumin binding sites are modified under various pathological conditions, including peritonitis, pancreatitis, cardiovascular diseases, and schizophrenia [2,4,11]. In some cases, threshold values of probe K-35 binding are determined, which are used for prediction of worsening and progression of the disease. However, molecular mechanisms underlying changes in the binding capacity of albumin are poorly understood.

Enhanced production of free radicals, *i.e*. oxidative stress (OS), plays a key role in the genesis and development of many diseases, such as atherosclerosis, hypertension, diabetes, CHD, and neurodegenerative diseases [1,3,12]. Due to its high concentration in blood plasma (600 μM) and the presence of a free sulfhydryl group, albumin prevents OS, *i.e.* acts as an

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0007-4888/12/1534-0463

antioxidant [10]. Interaction with free radicals modifies physicochemical properties of serum albumin, namely degradation of amino acid residues, cross-linking, and conformation changes [7]. This can impair structure and function of binding sites and alter binding capacity.

It can be assumed that damage to albumin molecule is induced by reactive oxygen species generated in the body under pathological conditions, *e.g.* superoxide radicals, hydrogen peroxide, hydroxyl radicals, fatty acid radicals as well as NaOCl, a potent oxidant generated by neutrophil myeloperoxidase activated during inflammation [13].

Metals with alternating valency (iron and copper ions) play an important role in OS [1,9]. These ions are used in model systems to activate OS. At the same time, their involvement in OS development under physiological conditions was shown [1,8,9,14].

Here we studied changes in the albumin binding capacity in a metal-catalysed oxidation (MCO) system.

MATERIALS AND METHODS

Plasma samples were obtained at the Department of Hematology of the Russian Research Center of Surgery RAMS and Sechenov Moscow Medical Academy. We examined 5 citrated plasma samples from 5 healthy donors and a solution of fatty acid-free human serum albumin (A1887, Sigma). The blood was taken in the morning on an empty stomach from the cubital vein into test tubes and stabilized with 3.8% sodium citrate (Sigma) at a 1:9 ratio. The plasma was isolated by centrifugation at 1000*g* for 15 min and the supernatant was stored at -20°C before use.

For oxidation, human serum albumin dissolved in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4, Sigma) and citrate plasma samples from 5 healthy donors diluted 50-fold in PBS were used.

Plasma oxidation was performed in MCO system in a thermostat at 37°C for 24 h in following variants:

1) oxidation induced by 20 μM Cu^{2+} ions ($CuSO_4$; Sigma);
2) oxidation induced by Fe^{2+} ions ($FeSO_4$; Sigma) and H_2O_2 (Fenton system) in concentrations: 400 μM $FeSO_4$+400 μM H_2O_2; 400 μM $FeSO_4$+2 mM H_2O_2. Oxidation was also was carried out in this system in the presence of EDTA (Sigma): 400 μM $FeSO_4$+400 μM H_2O_2+1.6 mM EDTA;
3) H_2O_2 in concentrations: 400 μM $FeCl_3$+400 μM H_2O_2; 400 μM $FeCl_3$+2 mM H_2O_2. The oxidation was also performed in this system in the presence of EDTA (Sigma): 400 μM $FeCl_3$+400 μM H_2O_2+1.6 mM EDTA.

The oxidation of serum albumin solution in PBS was carried out in MCO system in a thermostat at 37°C for 24 h at following variations:

1) oxidation induced by Fe^{2+} ions ($FeSO_4$; Sigma) and H_2O_2 (Fenton System) as follows: 500 μM $FeSO_4$ and 1 mM H_2O_2 were added to 10 mg/ml albumin solution in PBS;
2) oxidation induced by Cu^{2+} ions ($CuSO_4$; Sigma) and H_2O_2 as follows: 400 μM $CuSO_4$ and1 mM H_2O_2 were added to 20 mg/ml albumin solution in PBS.

Oxidative modification of proteins was evaluated by accumulation of carbonyl products of oxidation and formation of bityrosine fluorescent products. Oxidative modification of plasma lipids was estimated by the accumulation of TBA-reactive products and expressed in MDA equivalents. Oxidative modification of albumin was assessed by accumulation of protein carbonyls.

Concentration of carbonyl products was measured as described previously [15] and calculated per 1 mg plasma protein or serum albumin. Total protein was measured by Biuret method using diagnostic kits (Vital-diagnostik). Concentration of bityrosine fluorescent products was determined by the increase in fluorescence intensity at 420 nm at protein concentration of 1 mg/ml [5]. Fluorescence of bityrosine products was recorded on a Perkin Elmer LS-50 spectrofluorometer at excitation and emission wavelengths of 325 and 420 nm, respectively, and 6/6 nm (excitation/emission) bandpasses. MDA content was measured as described elsewhere [6].

Effective albumin concentration (EAC) was measured using K-35 fluorescent probe (Zond-al'bumin diagnostic kit, NIMVTs Zond). Isolated albumin (5 mg/ml) in PBS was diluted 5-fold in reagent 1 to a concentration of 1 mg/ml (Zond-al'bumin). Diluted plasma (1:50 in PBS) was 4-fold diluted with the same buffer. For analysis, 25 μl K-35 probe was added to 2 ml diluted albumin or plasma (reagent 2, Zond-al'bumin) and after stirring fluorescence intensity was measured at excitation and emission wavelengths of 450 and 530 nm, respectively.

Statistical analysis was performed when necessary using Student's *t* test.

RESULTS

First, the dynamics of albumin binding capacity (parameter EAC) was studied during copper-induced oxidation of 50-fold diluted plasma. These changes were compared with parameters characterizing free radical oxidation of plasma (Fig. 1). EAC decreased significantly by about 30% from baseline ($p<0.05$) 24 h after induction of plasma oxidation (Fig. 1, *a*). At the same time, OS significantly increased ($p<0.05$) as soon as after 2-4-h oxidation. The level of carbonyl

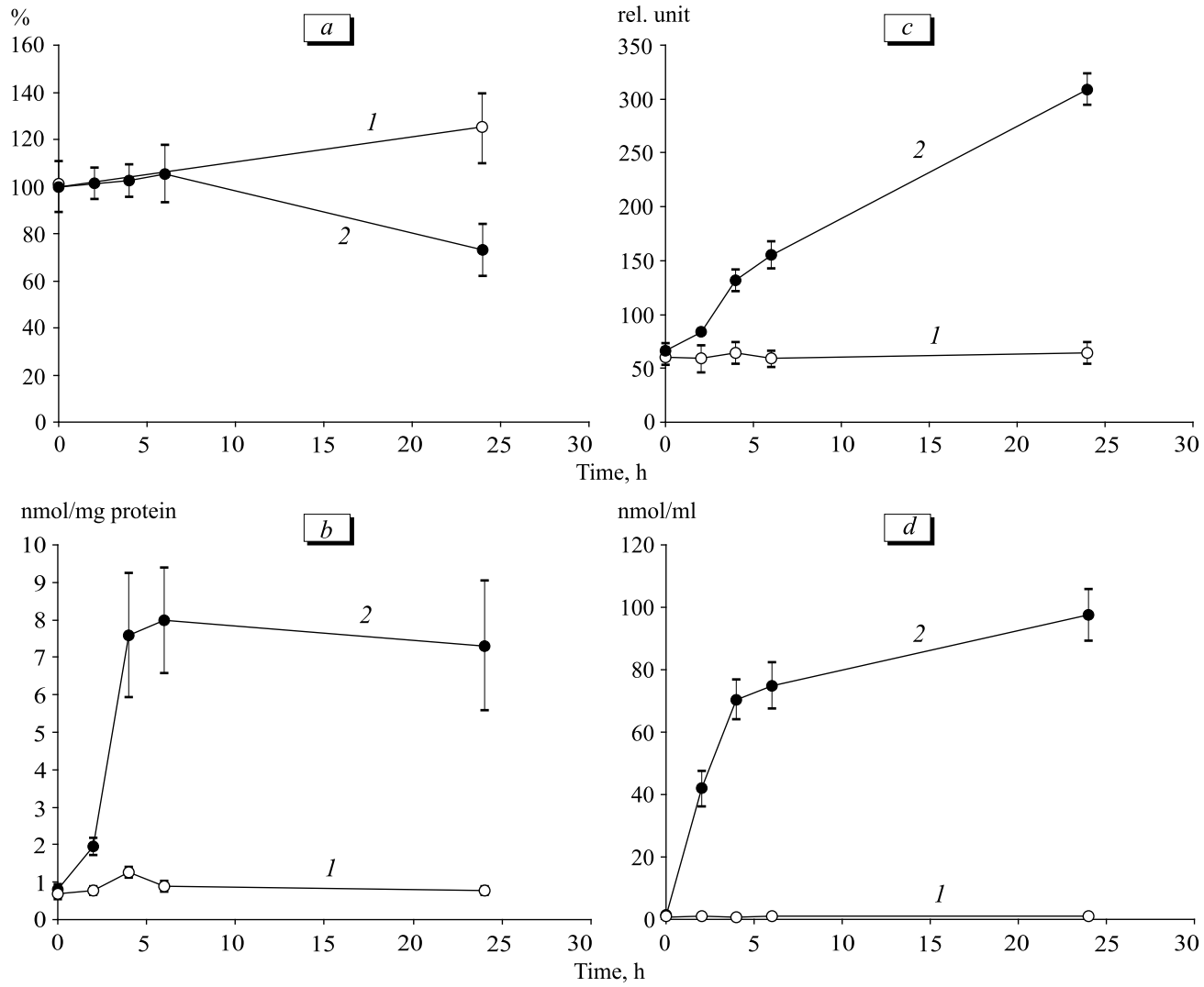

Fig. 1. Dynamics of EAC (*a*), levels of carbonyl products of oxidation (*b*), bityrosine products (*c*), MDA (*d*) during copper-induced oxidation of 50-fold diluted plasma (20 μM $CuSO_4$) as a result of oxidation of plasma samples from 5 donors. *(1)* in the absence of copper ions; (*2*) in the presence of copper ions.

products in proteins increased in 2 h (Fig. 1, *b*) and the level of bityrosine protein fluorescence increased in 4 h after oxidation induction (Fig. 1, *c*). The content of TBA-reactive products (MDA) characterizing lipid oxidation also rapidly increased (in 4 h; Fig. 1, *d*).

Then, EAC dynamics was studied in 50-fold diluted plasma during oxidation induced by the system comprising different concentrations of Fe^{2+} or Fe^{3+} ions as well as H_2O_2 and EDTA. During oxidation induced by Fe^{2+} (or Fe^{3+}) ions and H_2O_2, EAC significantly decreased ($p<0.05$) in the presence of high concentration of iron ions (400 μM; Fig. 2, *a*). Similarly to copper-induced oxidation, reduced EAC was observed 24 h after oxidation induction.

In the system 400 μM Fe^{2+} (or Fe^{3+})+400 μM H_2O_2+1.6 mM EDTA, the decrease in EAC was more pronounced than in the system containing 400 μM Fe^{2+} (or Fe^{3+})+400 μM H_2O_2 without EDTA. Further increase in H_2O_2 concentrations from 400 μM to 2 mM led to greater decrease in EAC. No significant decrease in EAC was found after induction of oxidation in diluted plasma with Fe^{2+} or Fe^{3+} ions alone.

Thus, considerably decreased binding capacity of albumin was revealed when it was oxidized in MCO system in the plasma.

Then, the dynamics of albumin transport function and parameters of OS was studied on albumin MCO in the solution of isolated albumin.

During iron-induced oxidation, the concentrations of albumin Fe^{2+}, and H_2O_2 were 10 mg/ml, 500 μM, and 1 mM, respectively. A significant excess of iron ions over albumin (500 μM *vs.* 150 μM) was observed under these conditions. During copper-induced oxidation, concentrations of albumin, Cu^{2+}, and H_2O_2 were 20 mg/ml, 400 μM, and 1 mM. In this case, there was also an excess of copper (400 μM) over albumin (300 μM).

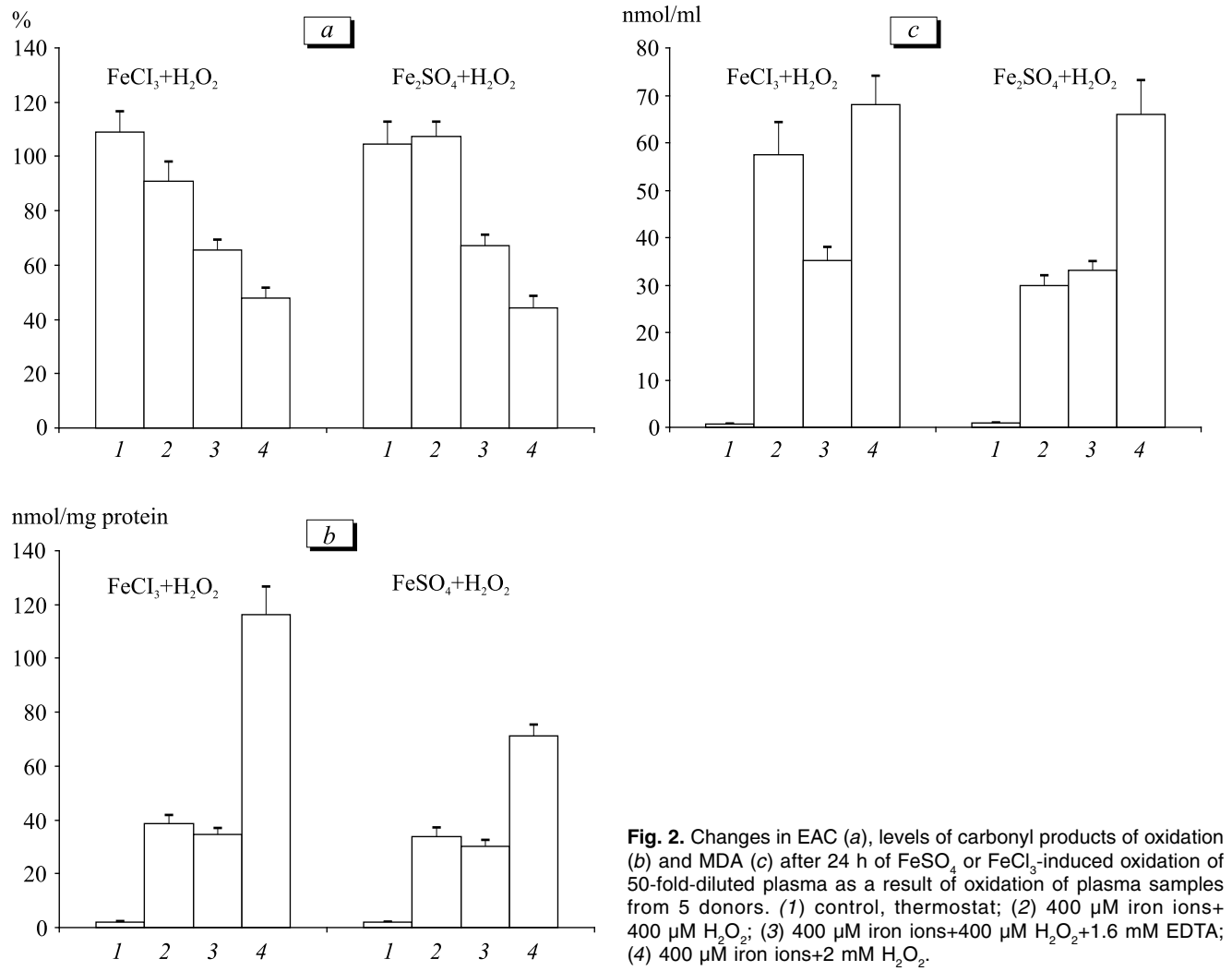

Fig. 2. Changes in EAC (*a*), levels of carbonyl products of oxidation (*b*) and MDA (*c*) after 24 h of $FeSO_4$ or $FeCl_3$-induced oxidation of 50-fold-diluted plasma as a result of oxidation of plasma samples from 5 donors. (*1*) control, thermostat; (*2*) 400 μM iron ions+ 400 μM H_2O_2; (*3*) 400 μM iron ions+400 μM H_2O_2+1.6 mM EDTA; (*4*) 400 μM iron ions+2 mM H_2O_2.

During oxidation of albumin preparation induced by Fe^{2+} and H_2O_2 ions, significant reduction of EAC, *i.e.* reduction in K-35 binding, did not occur within 24 h of oxidation. After 24 h of copper-induced oxidation of albumin preparation, significantly decreased ($p<0.05$) EAC (Fig. 3, *a*) was revealed. Oxidation induced by iron and copper ions similarly increased the content of carbonyl products (Fig. 3, *b*).

Thus, oxidation of diluted plasma induced by 20 μM Cu^{2+} significantly decreased EAC, which was accompanied by potentiation of free radical oxidation. Reduced EAC was also reported during oxidation of diluted plasma in Fenton system (iron ions, H_2O_2). EDTA and increased concentration of H_2O_2 potentiated the effect.

In this study, decreased EAC was found during oxidation of albumin (in PBS) in Cu^{2+}+H_2O_2 system and was not found in Fe^{2+}+H_2O_2 system.

The question arises: why the degree of transport function disturbances differed after albumin oxidation with copper and iron ions? Previous studies showed [5,14] that oxidation in the system Cu^{2+}+H_2O_2 in contrast to the oxidation system Fe^{2+}+H_2O_2+EDTA has a site-specific nature. In case of Cu^{2+}+H_2O_2 system, binding of Cu^{2+} ions to albumin not preventing its interaction with hydrogen peroxide was demonstrated. Therefore, albumin molecule is more damaged at the copper-binding site. In this case, binding sites of albumin are probably damaged to a greater extent, which was confirmed by our results.

We have previously shown that copper ions in low concentrations can induce oxidation of diluted plasma [6]. This study also revealed high intensity of free radical oxidation of both proteins and lipids during oxidation of diluted plasma in the presence of copper and iron ions.

We can assume that not only oxidative damage to albumin, but also lipid peroxidation can affect EAC in oxidized plasma. LPO products apparently bind to albumin, which impairs the function of its binding sites and reduces EAC.

Thus, our findings suggest that reduced EAC for fluorescent probe K-35 observed under various patho-

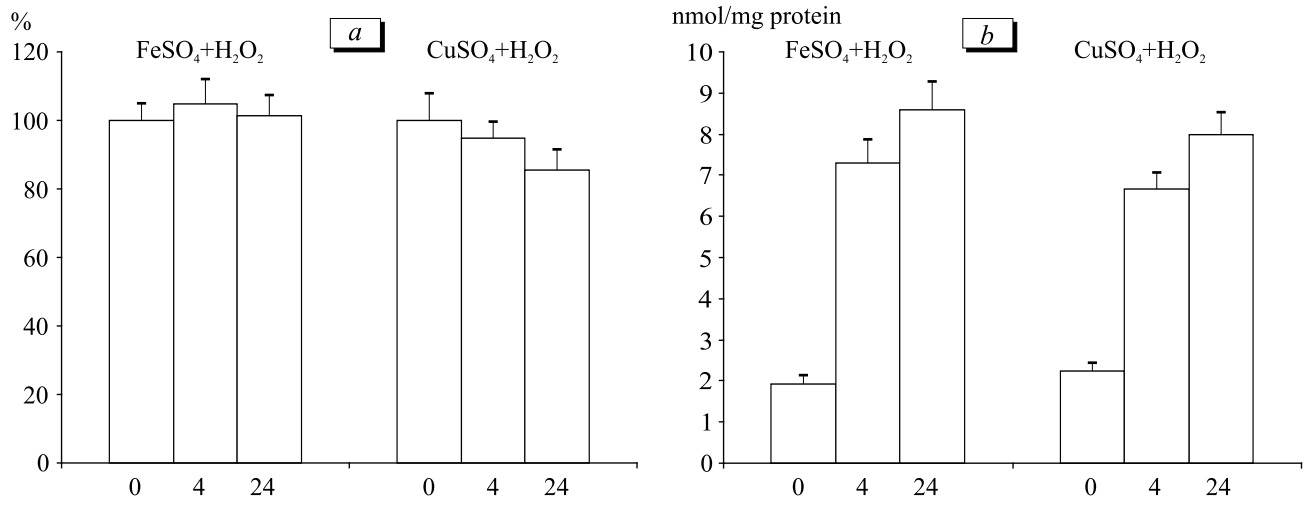

Fig. 3. Changes in EAC (*a*) and the accumulation of carbonyl products (*b*) during oxidation of albumin induced by $FeSO_4$ or $CuSO_4$ and H_2O_2 4 h and 24 h before oxidation.

logical conditions is partially determined by albumin damage by free radicals produced due to OS activation.

REFERENCES

1. O. A. Azizova, G. G. Ivanov, S. V. Drinitsina, and V. I. Sergienko, *Clinical Significance of Free Radical Processes and Electrophysiological Myocardial Remodeling in Coronary Heart Disease* [in Russian], Moscow (2008).
2. *Blood Serum Albumin in Clinical Medicine,* Eds. Yu. A. Gryzunov and G. E. Dobretsov [in Russian], Moscow (1994).
3. Yu. A. Vladimirov, O. A. Azizova, A. I. Deev*, et al*., *Itogi Nauki Tekhniki VINITI AN SSSR. Biofizika,* **29**, 1-252 (1991).

G. E. Dobretsov, Yu. A. Gryzunov, N. V. Smolina, *et al*., *Efferentnaya Fiziko-Khimicheskaya Meditsina*, No. 1, 16-26 (2009).

E. E. Dubinina, S. V. Gavrovskaya, E. V. Kuz'mich, *et al*., *Biokhimiya,* **67**, No. 3, 413-421 (2002).

A. P. Savchenkova, L. B. Dudnik, N. P. Solov'eva, and O. A. Azizova, *Biol. Med. Khimiya*, **49**, No. 6, 576-587 (2003).

7. M. Anraku, K. Yamasaki, T. Maruyama, *et al*., *Pharm. Res.,* **18**, No. 5, 632-639 (2001).
8. C. P. Baron, H. H. Refsgaard, L. H. Skibsted, and M. L. Andersen, *Free Radic. Res*., **40**, No. 4, 409-417 (2006).
9. M. J. Burkitt, *Arch Biochem. Biophys*., **394**, No. 1, 117-135 (2001).
10. G. Candiano, A. Petretto, M. Bruschi, *et al*., *Proteomics*, **73**, No. 2, 188-195 (2009).
11. Yu. A. Gryzunov, G. E. Dobretsov, *Protein Conformation: New Research*, Ed. L. B. Roswell, N.Y. (2008), pp. 125-159.
12. N. Khansari, Y. Shakiba, and M. Mahmoudi, *Recent Pat. Inflamm. Allergy Drug Discov.,* **3**, No. 1, 73-80 (2009).
13. S. J. Klebanoff, *J. Leukoc. Biol*., **77**, No. 5, 598-625 (2005).
14. T. Kocha, M. Yamaguchi, H. Ohtaki, *et al.*, *Biochim. Biophys. Acta*, **1337**, No. 2, 319-326 (1997).
15. R. Levine, J. A. Williams, E. R. Stadman, and E. Shacter, *Methods Enzymol.,* **233**, 346-357 (1994).