Studies of Oxidant-Induced Changes in Albumin Transport Function with a Fluorescent Probe K-35. Effect of Hypochlorite

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The dynamics of changes in albumin transport function during hypochlorite-induced oxidation of isolated albumin in blood plasma and serum was studied with a fluorescent probe K-35. Binding of the probe K-35 to albumin was characterized by effective concentration of albumin. Oxidative modification of proteins was evaluated by the content of carbonyl products of protein oxidation and bityrosine fluorescent products. Oxidation with hypochlorite was accompanied by a decrease in the effective concentration of albumin in albumin, diluted plasma, and serum and accumulation of carbonyl products of protein oxidation and bityrosine fluorescent products. The decrease in the effective concentration of albumin during oxidation with hypochlorite can be explained by oxidative damage to albumin binding sites. Oxidative modification of probe K-35 binding sites with hypochlorite contributes to a decrease in effective concentration of albumin under pathological conditions.

Key Words: *albumin; fluorescent probe; K-35; albumin effective concentration; hypochlorite*

One of the functions of serum albumin in the human body is reversible binding and transport of low-molecular-weight endogenous and exogenous ligands. Albumin binds and transports metabolites (fatty acids and heme derivatives), hormones, bile pigments, metal ions, and drugs. Binding of these substances leads to conformational changes in the albumin molecules, which can be evaluated by changes in spectral characteristics of fluorochromes and chromophores that also bind to albumin.

Recording of binding of a fluorescent probe K-35 (Institute of Physicochemical Medicine) is a common approach to evaluation of albumin transport function [1]. Previous studies showed that K-35 binding is modified under various pathological conditions, including pancreatitis, cardiovascular diseases, and schizophrenia [1,3]. Threshold values of probe binding are sometimes used for the prognosis of disease course and possible complications. However, the molecular mechanisms of changes in transport functions of albumin and their detection with a probe are poorly understood.

Excessive generation of free radicals (*i.e.*, oxidative stress) plays a key role in the development and progression of various diseases, including atherosclerosis, hypertension, diabetes, CHD, and neurodegenerative disorders [2,10]. Albumin is one of the major antioxidants and free radical traps in the body [7]. Due to its high concentration in blood plasma (600 μM) and the presence of a free sulfhydryl group, albumin prevents oxidative stress.

Interaction with free radicals modifies physicochemical properties of serum albumin and leads to degradation of amino acid residues, cross-linking, and conformation change [6]. Oxidative damage to albumin impairs its transport function [14]. Variations in the protein structure during oxidative stress contribute to a change in probe K-35 binding to albumin.

Oxidative modification of albumin can be induced by reactive oxygen species in the body (superoxide radicals, hydrogen peroxide, hydroxyl radicals, and

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fatty acid radicals). Sodium hypochlorite (NaOCl) is one of the potent oxidants inducing free radical damage to proteins. This substance is generated with the enzyme neutrophil myeloperoxidase activated during inflammation [11].

This work was designed to evaluate changes in the transport function of albumin during oxidation of isolated albumin in blood plasma and serum with NaOCl. The study was performed using K-35. The estimated parameters were compared with markers of oxidative damage to albumin.

MATERIALS AND METHODS

For oxidation we used fatty acid-free human serum albumin (Sigma, product A-1887) dissolved in phosphate-buffered saline (PBS, Sigma) to a concentration of 5 mg/ml as well as serum (20-fold diluted in PBS) and citrate plasma (20-fold diluted in PBS) from 3 healthy donors.

Oxidation was induced by adding NaOCl (Sigma) to final concentrations of 1 and 2 mM and subsequent incubation in a thermostat at 37°C. These concentrations of NaOCl were selected due to similarity to the values observed in inflammation and other diseases. Computer modeling showed that the concentration of NaOCl in blood plasma and site of inflammation can reach 150-500 μmol/h and 5 mM, respectively [9].

The kinetics of oxidative stress was evaluated in the zero point (measurement immediately before addition of NaOCl) and 1, 3, and 24 h after induction of oxidation. Oxidative modification of proteins was estimated by accumulation of carbonyl products (CP) of oxidation and formation of bityrosine links. Albumin in the same concentration and blood plasma and serum in the same dilutions were incubated under incubation conditions of experimental samples and used as the control.

Effective concentration of albumin (ECA) was measured using K-35 (Zond-al'bumin diagnostic kit , NIMVTs Zond). Isolated albumin (5 mg/ml) in PBS was diluted 5-fold to a concentration of 1 mg/ml (with reagent 1, Zond-al'bumin). The plasma or serum (diluted by 20 times with PBS) was diluted by 10 times

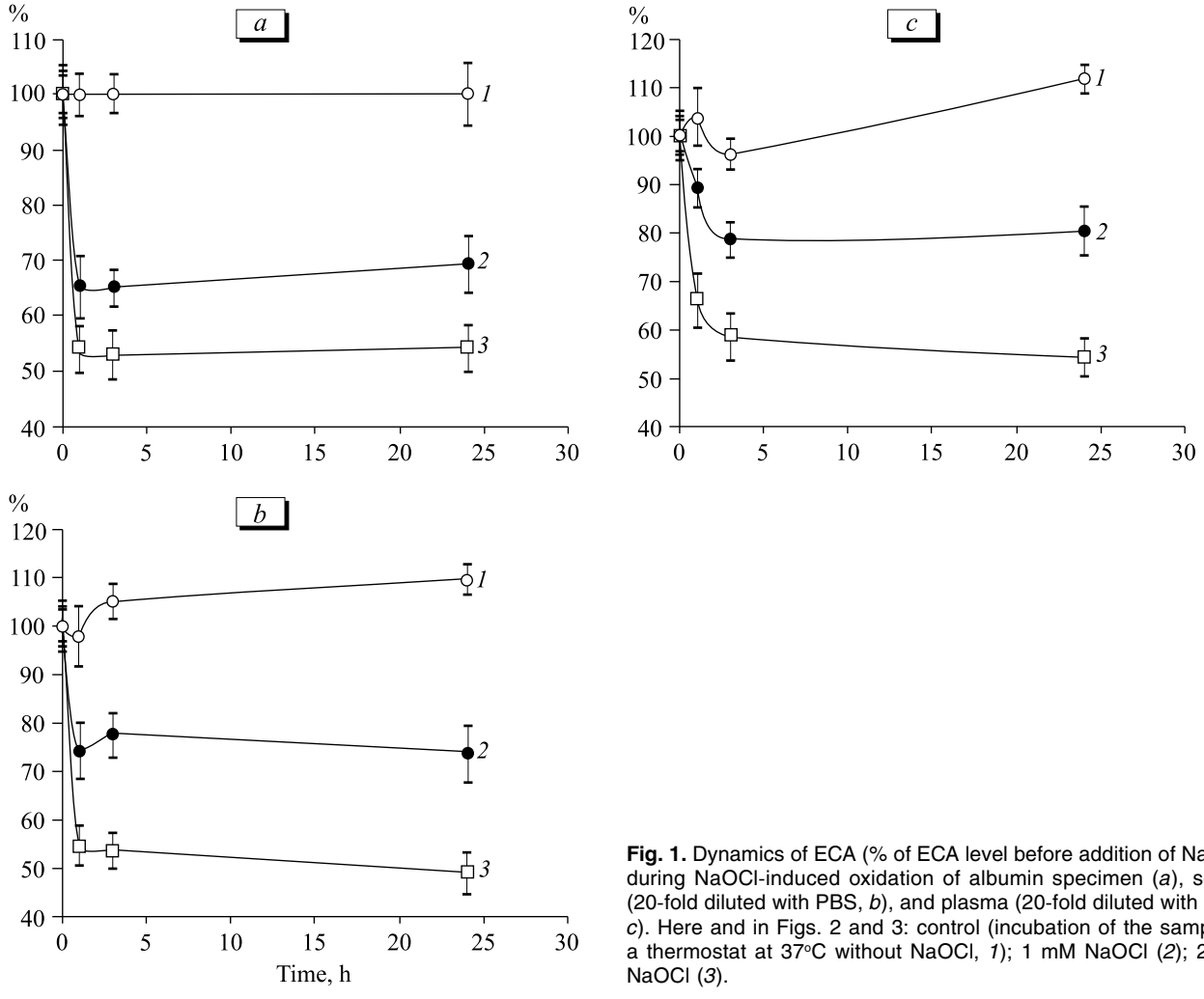

Fig. 1. Dynamics of ECA (% of ECA level before addition of NaOCl) during NaOCl-induced oxidation of albumin specimen (*a*), serum (20-fold diluted with PBS, *b*), and plasma (20-fold diluted with PBS, *c*). Here and in Figs. 2 and 3: control (incubation of the sample in a thermostat at 37°C without NaOCl, *1*); 1 mM NaOCl (*2*); 2 mM NaOCl (*3*).

with reagent 1. Concentrated probe K-535 (25 μl, Zond-al'bumin) was added to 2 ml diluted albumin, serum, or plasma. After agitation the intensity of fluorescence was measured at excitation and emission wavelengths of 450 and 530 nm, respectively.

The content of CP was measured as described elsewhere [10]. HCl (2.5 N, 2 ml) was added to 0.5 ml control sample. 2,4-Dinitrophenylhydrazine (0.0025 M, 2 ml) in 1 ml 2.5 N HCl was added to experimental samples.

The samples were incubated in darkness at room temperature for 1 h, treated with 2.5 ml cold 20% trichloroacetic acid, maintained in a refrigerator for 10 min, and centrifuged at 3000 rpm for 10 min.

The pellet was washed 3 times with ethanol-ethyl acetate 1:1 mixtur for extraction of lipids and 2,4-dinitrophenylhydrazine not reacting with carbonyl groups of oxidized proteins (3000 rpm for 10 min). The pellet was dried to remove the solvent (ethanol and ethyl acetate) and dissolved in 8 M urea. Urea was added to the pellet (2 ml). The mixture was maintained in a boiling water bath until complete dissolution.

Optical density of experimental and control solutions was measured on a Beckman DU-530 spectrophotometer at 370 nm. The amount of carbonyl groups was estimated from the difference between optical densities of the experimental and control samples at 370 nm. Molar extinction coefficient was 22,000 $M^{-1}cm^{-1}$.

Protein concentration in the serum and plasma was measured by the biuret method (diagnostic kit Obshchii belok-01-Vital).

Bityrosine cross-links were evaluated by fluorescence at 420 nm and protein concentration of 2 mg/ml [4]. Fluorescence of bityrosine products was recorded on a Perkin Elmer LS-50 spectrofluorometer at excitation and emission wavelengths of 325 and 420 nm, respectively. The gaps were 6/6 nm (excitation/emission).

The results were analyzed by Student's *t* test (when required).

RESULTS

The changes in K-35 binding to albumin were compared with oxidative damage to the protein. We compared the dynamics of K-35 binding to albumin with the dynamics of oxidative stress during NaOCl-induced oxidation in 3 oxidation systems (isolated albumin, 20-fold diluted blood plasma, and 20-fold diluted serum). The time kinetics of oxidation was evaluated over 24 h (1, 3, and 24 h of oxidation).

ECA characterizing the ligand-binding capacity of albumin [1] was shown to decrease in 3 oxidation systems 1 h after addition of NaOCl (Fig. 1). It should be

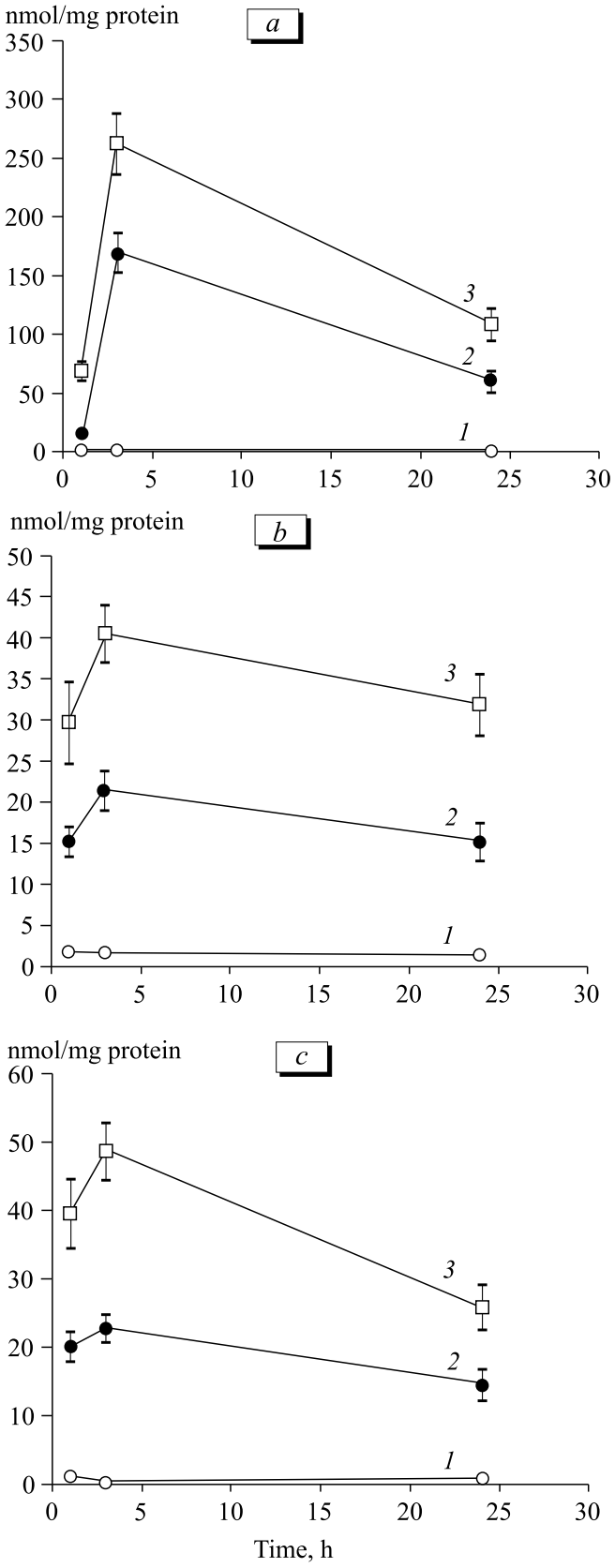

Fig. 2. Dynamics of CP accumulation during NaOCl-induced oxidation of albumin specimen (*a*), serum (20-fold diluted with PBS, *b*), and plasma (20-fold diluted with PBS, *c*).

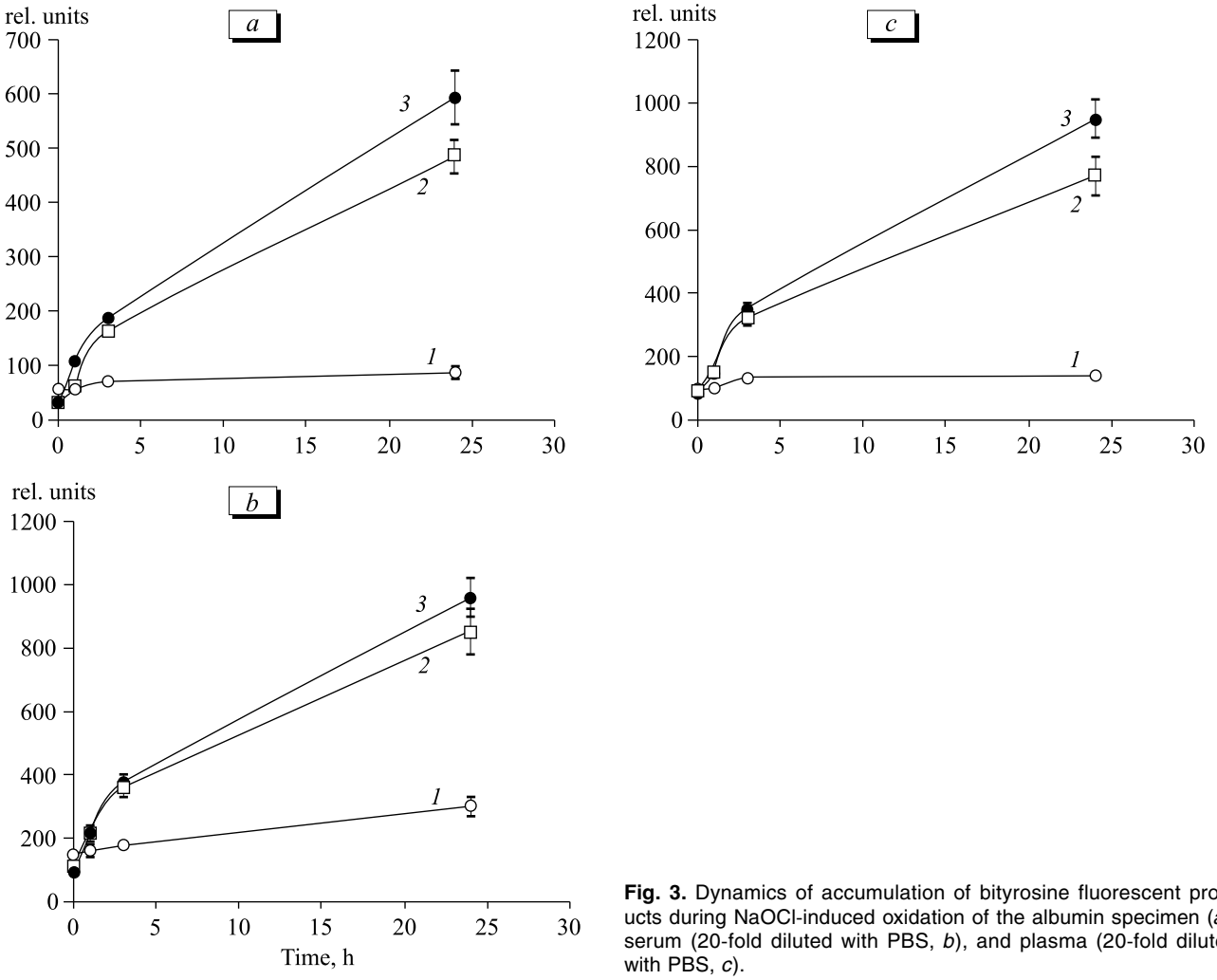

Fig. 3. Dynamics of accumulation of bityrosine fluorescent products during NaOCl-induced oxidation of the albumin specimen (*a*), serum (20-fold diluted with PBS, *b*), and plasma (20-fold diluted with PBS, *c*).

emphasized that this parameter remained unchanged in the follow-up period. ECA in control samples of the serum and plasma increased by 10% over 24-h oxidation.

The decrease in ECA was accompanied by a significant increase in the content of CP. The amount of CP differed significantly during oxidation of isolated albumin and oxidation of proteins in the serum or blood plasma (Fig. 2). CP content in the isolated albumin was much higher than that in oxidized proteins of the serum and plasma. It was probably related to the presence of antioxidants that prevent free radical oxidation of proteins in the serum and plasma. The amount of CP remained practically unchanged during oxidation of control samples with albumin, plasma, and serum in a thermostat at 37°C.

The content of CP was maximum 3 h after induction of oxidation. The amount of CP in oxidation systems decreased significantly after 24 h, which was probably associated with degradation of carbonyl compounds during oxidation and formation of soluble carbonyls at a certain stage of protein oxidation [8].

Fluorescence of bityrosine products formed during NaOCl-induced oxidation of proteins was shown to increase significantly in all systems of oxidation. It was accompanied by a significant increase in the content of bityrosines during oxidation (Fig. 3).

Our results indicate that the decrease in ECA in albumin, diluted plasma, and serum during NaOCl-induced oxidation is accompanied by activation of free radical processes, which results in protein oxidation. Albumin is known to have binding sites for K-35 [1]. They are localized in the so-called drug site 1 or close to this structure [5]. The observed decrease in ECA during NaOCl-induced oxidative damage to albumin is probably associated with injury to binding sites.

Proteins and particularly serum albumin are the main targets of protein oxidation with NaOCl, which occurs under physiological conditions [15]. It can be hypothesized that the decrease in ECA in the diluted serum and plasma is also associated with oxidative damage to albumin binding sites.

NaOCl generation with enzyme myeloperoxidase, which is produced by activated neutrophils, serves as

an important defense mechanism during inflammatory processes [11]. The increased level of myeloperoxidase is an independent risk factor for cardiovascular diseases and complications [13]. The concentration of NaOCl in inflammatory sites can reach 5 mM, which is comparable to the dose of NaOCl used in experimental studies [9]. These data suggest that NaOCl-induced modification of binding sites in K-35 is one of the reasons for ECA decrease, which occurs under various pathological conditions.

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