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Interaction of Ceruloplasmin and 5-Lipoxygenase

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Abstract—The interaction between ceruloplasmin (CP), the multicopper oxidase of human plasma, and 5-lipoxygenase (5-LO), the key enzyme of leukotriene synthesis, is shown for the first time. By Western-blotting and mass spectrometry of tryptic fragments, it is shown that 5-LO from protein extract of human leukocytes binds with immobilized CP. Dose-dependent influence of intact CP on leukotrienes synthesis is found: CP reduced leukotrienes synthesis in leukocytes in a dose above 50 μg/ml (normal CP concentration in plasma is about 300-400 μg/ml). Proteolyzed CP and apo-form of CP is unable to inhibit activity of 5-LO. CP increased activity of 5-LO at low doses (5-10 μg/ml). On the whole, the influence of CP on phagocytosis index of leukocytes coordinates with influence on activity of 5-LO: the index increased in the range of 2-10 μg/ml CP and decreased at doses of CP above 40 μg/ml. The dual role of CP in regulation of cellular response of leukocytes is discussed.

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Ceruloplasmin (CP, ferro:O_2-oxidoreductase) is a copper-containing protein of blood plasma with molecular mass ~132 kDa. From its systematic title one should conclude that the enzyme's peculiar feature is the capability to oxidize Fe^{2+} to Fe^{3+}. However, the physiological role of CP is not likely to be limited to Fe^{2+} oxidation, even though its gene deficiency in humans (aceruloplasminemia) is known to result in oxidative stress, secondary to ferrous iron accumulation in tissues [1]. Possessing the activities of ferroxidase [2], cuproxidase [3], superoxide dismutase [4], glutathione-dependent peroxidase [5], and NO-oxidase [6], CP actively prevents both production and persistence of free radicals. Its gene expression being increased in inflammation, there is every reason to regard CP as an acute-phase reacting protein [7]. The presence and expression of the CP gene is a prerequisite for survival of neurons in their acute-phase response to lipopolysaccharides. CP gene-knockout mice were unable to resist accumulation of iron and demyelination caused by lipopolysaccharide [8]. An increase of CP synthesis by endothelial cells in response to infection by *Streptococcus pneumoniae* is indicative of its participation in modulation of the barrier function that protects the central nervous system from circulating pathogens and potentially toxic molecules accompanying an infection. This hypothesis is supported by the notion that CP gene-knockout mice have an increased synthesis of P-selectin that is responsible for vascular permeability for neutrophils [8]. CP plasma concentration in case of inflammation can increase from 300 to 900 μg/ml, which suggests its participation in regulation of the inflammatory process.

In the last ten years we were the first to describe and partially characterize complexes of CP with cationic proteins of neutrophils, such as lactoferrin (LF), myeloperoxidase (MPO) [9], and species from the serprocidin family (elastase, cathepsin G, proteinase 3, and azurocidin) [10]. Interaction of anionic CP with cationic proteins is somewhat stereotyped, though resulting in certain variety of complexes. We showed the high affinity of components within the complexes formed. For instance, the affinity of CP to LF and to azurocidin is characterized by K_d ~ 13 nM [11, 12]. Both *in vitro* and *in vivo* CP is able to form multimeric complexes including LF and MPO [9]. LF increases ferroxidase activity of CP when forming a complex with the latter [13]. Interaction of CP with

Abbreviations: CP, ceruloplasmin; LF, lactoferrin; 5-LO, 5-lipoxygenase; MPO, myeloperoxidase; OZ, opsonized zymosan.

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MPO results in inhibiting the pro-oxidative activity of the leukocytic enzyme [14]. As shown in our latest studies, the inhibitory effect of CP depends on the integrity of its molecule, i.e. partially proteolyzed CP is not efficient as an inhibitor of the chlorinating activity of MPO [15]. It was shown recently that CP is synthesized by neutrophils of the peripheral blood in patients with localized aggressive periodontitis. In that case CP activates NADPH-oxidase, supplying it with Fe^{3+} [16]. Thus a possibility that CP might perform as a pro-inflammatory agent should be accounted for.

Upon stimulation of neutrophils, particularly in phagocytosis, NADPH-oxidase of plasma membrane is activated [17] and the intracellular content of unbound arachidonic acid grows, which is accompanied by synthesis of leukotrienes [18]. The crucial enzyme for leukotriene synthesis is 5-lipoxygenase (5-LO) catalyzing the first two reactions in the sequence of transformations of arachidonic (5,8,11,14-*cis*-eicosatetraenic) acid. In the course of leukotriene synthesis 5-LO catalyzes the insertion of an oxygen molecule in position 5 of the arachidonic acid molecule. This oxygenase activity of 5-LO requires Fe^{3+} in its active center. As a result, the hydroperoxy acid 5-HPETE is formed first, while the next step brings forth the unstable epoxide LTA_4 (LTA_4-synthase activity of 5-LO). Leukotriene A_4 is the substrate for leukotriene A_4-hydrolase of neutrophils that produces leukotriene B_4 (LTB_4), the strongest chemoattractant.

It has been established that the plasma content of CP is supported mainly by its synthesis in hepatocytes [19]. However, recent studies showed that human neutrophils synthesize and release CP in the intercellular matrix [20]. Because the activity of 5-LO depends on the availability of Fe^{3+} and the "peroxide background" of a cell, one can suggest that CP plays a role of a regulating factor taking care of ferrous iron oxidation and its cellular ingress or egress [21].

The present paper demonstrates the interaction of CP and 5-LO along with the regulatory role of CP in the synthesis of leukotrienes by neutrophils. Partially proteolyzed CP and its apo-form appeared to have no effect on leukotriene synthesis.

MATERIALS AND METHODS

The following reagents were used. Mouse monoclonal antibodies against human 5-LO (BD Biosciences, USA); horseradish peroxidase-labeled goat antibodies against murine immunoglobulins, dry skimmed milk (BioRad, USA); triethylamine, EDTA (Merck, Germany); Sepharose 4B (Pharmacia, Sweden); proteinase inhibitor cocktail (Roche, Germany); arginine, glycerol, Coomassie R-250, mercaptoethanol, ammonium persulfate, Tris (Serva, Germany); SDS, salmon protamine, phenylmethylsulfonyl fluoride (PMSF), 4-chloro-1-naphtol (Sigma, USA); acrylamide, N,N′-methylene bisacrylamide, N,N,N′,N′-tetramethylethylene diamine (Laboratory MEDIGEN, Russia); heparin (SPOFA, Poland). Cyanogen bromide was obtained by brominating KCN in the biphasic water–dichloroethane system. The obtained BrCN in dichloroethane was used to activate Sepharose and subsequently to immobilize on the resin heparin, protamine, arginine, or CP [22]. To evaluate molecular mass of proteins and to accomplish Western blotting, SDS-PAGE was run [23].

Stable preparations of monomeric CP with $A_{610}/A_{280} > 0.049$ containing more than 95% of 132-kDa protein were obtained by affinity chromatography on protamine-Sepharose of human plasma (to which PMSF and EDTA were added to 1 and 0.1 mM, respectively) pre-filtered on columns with arginine-Sepharose and heparin-Sepharose [22]. Apo-form of non-fragmented CP was obtained by adding to 20 μM CP of ascorbic acid (0.1 M), sodium azide (0.1 M), and EDTA (0.5 M). Colorless protein was dialyzed against 0.1 M EDTA (pH 8.0) for 24 h and then three 12-h periods against PBS (at 4°C). Judging by atomic absorption results, CP in such preparation contained less than 0.05 copper atoms per molecule. The absence of trace amounts of chelating agents was proved by special control experiments. Proteolyzed CP was obtained according to a similar protocol, except that no proteinase inhibitors were added to blood plasma and chromatography on heparin-Sepharose was omitted. After a month of storage at 4°C, such a preparation contained about 95% fragments of CP with $M_r \sim 116$ and 19 kDa.

Isolation of neutrophils. Polymorphonuclear leukocytes (neutrophils) were isolated as follows from fresh donor blood to which EDTA was added to provide concentration 1.2 g/liter. Erythrocytes were precipitated at room temperature by 3% dextran T-500 in 0.85% NaCl (40 ml of solution per 100 ml of blood). Plasma enriched with leukocytes and platelets was layered on Ficoll-Paque (density 1.077 g/liter) in a 50-ml centrifuge tube. Granulocyte fraction with admixture of erythrocytes was precipitated upon centrifugation (500*g*, 3 min) in the density gradient. The remaining erythrocytes were lysed in buffer (114 mM NH_4Cl, 7.5 mM $KHCO_3$, 100 μM EDTA), after which cells were washed twice in PBS (1.06 mM KH_2PO_4, 155 mM NaCl, 2.7 mM KCl, 2.96 mM Na_2HPO_4, pH 7.4) and resuspended in D-PBS (0.49 mM $MgCl_2$, 2.7 mM KCl, 1.15 mM K_2HPO_4, 138 mM NaCl, 9.58 mM NaH_2PO_4, pH 7.4) with glucose (1 g/liter). All experiments with neutrophils were accomplished on the same day when blood was collected.

Affinity chromatography of leukocytic extract on CP-Sepharose. Precipitated neutrophils obtained as described above were frozen in three-fold volume of PBS containing proteinase inhibitor cocktail. The mixture was centrifuged upon thawing (15,000*g*, 15 min, 4°C), and the supernatant was subjected to affinity chromatography on

a column (1.5 × 2 cm) packed with CP-Sepharose. The column was equilibrated with PBS, leukocytic extract was loaded, and the resin was washed till absorption of effluent $A_{280} < 0.002$ was achieved. Proteins obtained by elution with 1 M NaCl, 10 mM Tris-HCl, pH 7.4, were analyzed by SDS-PAGE. To reveal the bands of 5-LO, Western blotting was accomplished after electrophoresis [24]. Nitrocellulose membrane with proteins after semidry electric transfer was blocked in BLOTTO-T (3% solution of proteins from dry skimmed milk in PBS containing 0.05% Tween 20) and incubated first in BLOTTO-T with monoclonal murine antibodies against 5-LO (1 : 1000), then with horseradish peroxidase-labeled (1 : 10,000) goat antibodies against murine immunoglobulins. Immunoreactive bands were revealed after incubation in chromogenic mixture containing 6 mg of 4-chloro-1-naphtol in 2 ml of ethanol with 10 ml of 5 mM H_2O_2 in PBS. Proteins were identified by subjecting fragments of their tryptic cleavage to mass spectrometry [25]. To prepare samples for mass spectrometry, proteins were resolved in SDS-PAGE and protein-containing bands were cut out of the gel. Mass spectra were registered on an Ultraflex II tandem MALDI-TOFF spectrometer (Bruker) equipped with a UV laser (Nd). Peptide fingerprints obtained were analyzed using the MASCOT program (http://www.matrixscience.com).

Preparing opsonized zymosan (OZ). Autologous serum needed to opsonize zymosan particles was obtained from a separate blood sample taken from the same donor whose blood was used to isolate neutrophils. Serum was obtained as supernatant after centrifugation (800*g*, 30 min) of an anticoagulant-free blood sample about 2 h past sampling. A portion of zymosan A particles from *Saccharomyces cerevisiae* was suspended in PBS, boiled in a water bath for 5 min, and cooled to room temperature. The suspension obtained was centrifuged (800*g*, 10 min), and ~3 ml of autologous serum (per 200 mg of zymosan) were added to the precipitate. The suspension of zymosan particles in serum thus obtained was incubated for 30 min at 37°C, washed 3 times in PBS, and resuspended in HBSS (Hanks medium: 1.27 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.82 mM $MgSO_4$, 138 mM NaCl, 0.34 mM Na_2HPO_4, 5.55 mM glucose, pH 7.4) containing 10 mM Hepes (HBSS/Hepes).

Measuring phagocytosis coefficient of leukocytes. Cells suspended in HBSS/Hepes ($5 \cdot 10^6$ neutrophils per ml) were dropped into 6-well plates with collagen- or fibronectin-covered coverslips on the bottom of the wells and incubated with CP for 30 min. Then OZ suspension (0.25 mg/ml) was added, and the cells were incubated for another 10 min. After that the cells were carefully washed with PBS and fixed for 30 min in modified HBSS solution containing 10 mM Hepes and 2.5% glutaraldehyde. Finally the samples obtained were carefully washed in PBS and ca. 100 cells on each coverslip were subjected to phase-contrast microscopy. OZ particles within cells were calculated, and phagocytosis coefficient was estimated, i.e. the share of cells that engulfed at least one particle was multiplied by the mean number of OZ particles taken up by cells.

Incubation for studying 5-LO-induced synthesis of arachidonic acid oxidation products in neutrophils. A suspension of neutrophils ($2 \cdot 10^7$ cells) was incubated for 30 min in 6 ml of HBSS/Hepes at 37°C in the presence or in the absence of the species under study, after which cells were stimulated by adding OZ (~10 mg) and again incubated for 20 min. Incubation was arrested by adding an equal volume (6 ml) of methanol cooled to −20°C containing a certain amount of prostaglandin B2 as an internal standard. The samples were stored at −20°C, then centrifuged (800*g*, 10 min), and the supernatant represented by water–methanol extract was collected.

Analysis of lipoxygenase products. Water–methanol extracts were purified by solid-phase extraction on a Sep-Pak C_{18} microcolumn that had been washed with methanol and then equilibrated with bidistilled water. Optimum conditions for purification and concentrating of samples had been elaborated in preliminary experiments. Upon loading a sample, the microcolumn was washed with bidistilled water (~10 ml) and with 300 µl of methanol. Metabolites of 5-LO were extracted by 1.4 ml of methanol, and the samples obtained were dried by evaporation, redissolved in 35 µl of methanol–water mixture (2 : 1), and analyzed by reverse-phase HPLC. Purified samples were applied on a Nucleosil C_{18} 5 µm column (250 × 4.6 mm), and the products were eluted in effluent B gradient (20 to 100%) maintaining elution speed 0.8 ml/min. The eluents had the following content: methanol–acetonitrile–water–acetic acid–triethylamine (25: 25 : 50 : 0.05 : 0.075 v/v (eluent A) and 50 : 50 : 0 : 0.05 : 0.04 v/v (eluent B)). Absorption was registered by a UV-detector with adjustable wavelength at 280 nm (absorption maximum for LTB_4) and at 238 nm (absorption maximum for 5-HETE). The following 5-LO metabolites were registered in the course of analysis: leukotriene B_4 (LTB_4), 5-hydroxyeicosatetraenic acid (5-HETE), 20-hydroxy-LTB_4 (ω-OH-LTB_4), and isomers of LTB_4 identified by comparing with the elution of the respective standard. Amounts of 5-LO products were evaluated by estimating the relation between peak areas to the peak of an internal standard, taking into account the respective extinction coefficients.

Superoxide anion measurements. Neutrophils were incubated in 24-well plates covered with collagen. To assay the production of superoxide anions, 50 µM of cytochrome *c*, 300 units/ml of superoxide dismutase (Cu,Zn-SOD), and/or the species under study in various combinations were dropped in a well prior to placing the cells. The plates were incubated for 30 min at 37°C, then OZ was added and another 30 min of incubation followed. Incubations were arrested by cooling the plates to 4°C, and the degree of cytochrome *c* reduction was assayed by the growth of Δ550/535 that is equal to the difference between

Peptide fingerprint of 78-kDa band (results of search utilizing the MASCOT program)

Fragment of 5-lipoxygenase	Molecular mass, Da		Peptide
	expected	calculated	
145-162	2175.02	2175.03	R.WMEWNPGFPLSIDAKCHK.D propionamide (C)
256-269	1677.82	1677.81	K.LPVTTEMVECSLER.Q propionamide (C)
298-320	2692.42	2692.33	K.TDPCTLQFLAAPICLLYKNLANK.I
473-483	1286.67	1286.70	R.DDGLLVWEAIR.T

absorption values at 550 and 535 nm measured in solutions of samples. Reduction of 10 μM of cytochrome *c* causes an increase of Δ550/535 by 0.18 optical units.

RESULTS AND DISCUSSION

Revealing the interaction between ceruloplasmin and 5-lipoxygenase. Leukocytic proteins eluted from CP-Sepharose by 1 M NaCl were subjected to Western blotting with anti-5-LO, which revealed a band with M_r ~ 78 kDa (Fig. 1). Properly this molecular mass was determined in 5-LO studies [17]. Because we had previously identified a similar band containing LF with the same molecular mass [10], control tests were made to show that in Western blotting LF purified from breast milk does not react with antibodies against 5-LO (data not shown). Mass spectrometry of tryptic fragments of the 78-kDa band detected peptides corresponding to 5-LO (see table).

Desorption of 5-LO from CP-Sepharose induced by 1 M NaCl indicates that ionic forces participate in the interaction of that protein with CP. It is peculiar of cationic proteins that interact with CP to contain amino acid stretches known as heparin-binding motifs [12, 26, 27] XBBXBX and XBBBXXBX, where B stands for K or R (more seldom for H), while X stands for any hydrophobic amino acid [27]. 5-LO is not a cationic protein, yet cationic stretches can be defined in its primary structure: ^{124}IHILKQHRRK^{133}, ^{519}MRGRKS^{524}, ^{634}ARFRKN^{639}, and ^{650}RNKKKQ^{655}. These stretches can be regarded as the likely sites for the contact of 5-LO with CP.

Effect of ceruloplasmin on the 5-LO-induced synthesis of arachidonic acid oxygenation products in neutrophils. The effect of CP on 5-LO activity is evident upon 30 min of preincubation of cells with the protein prior to adding a stimulus, i.e. OZ. This effect is dose-dependent (Fig. 2) and is likely to be linked both with direct interaction of CP with 5-LO and with the influence of CP on endogenous regulator of 5-LO activity. It is known that LTB_4 stimulates phagocytosis of opsonized particles by neutrophils [28], hence double effect of CP on leukotriene production coincides with its influence on phagocytosis coefficient (Fig. 3). It can be suggested that direct interaction of the two proteins underlies the inhibition of 5-LO activity by high amounts of CP, as the inhibitory effect disappears upon limited proteolysis of CP (Fig. 2). A similar phenomenon was observed in our study of an effect of CP on the activity of the neutrophilic pro-oxidative protein MPO, when proteolyzed CP did

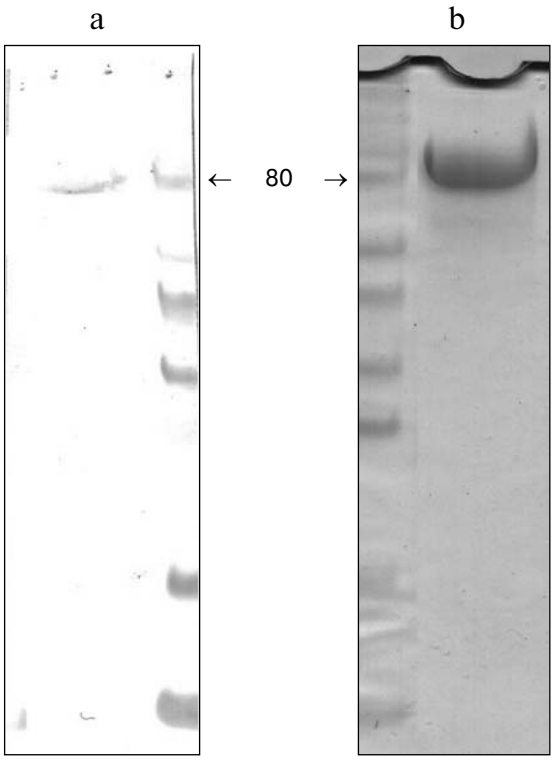

Fig. 1. Analysis of the proteins (20 μg per sample) eluted by 1 M NaCl from CP-Sepharose in the course of affinity chromatography of leukocytic extract. a) Western blotting with antibodies against 5-LO (1 : 1000), dilution of horseradish peroxidase-labeled secondary antibodies (1 : 10,000) (staining with 4-chloro-1-naphtol and H_2O_2); b) results of SDS-PAGE (Coomassie R-250 staining). Arrows show M_r, kDa.

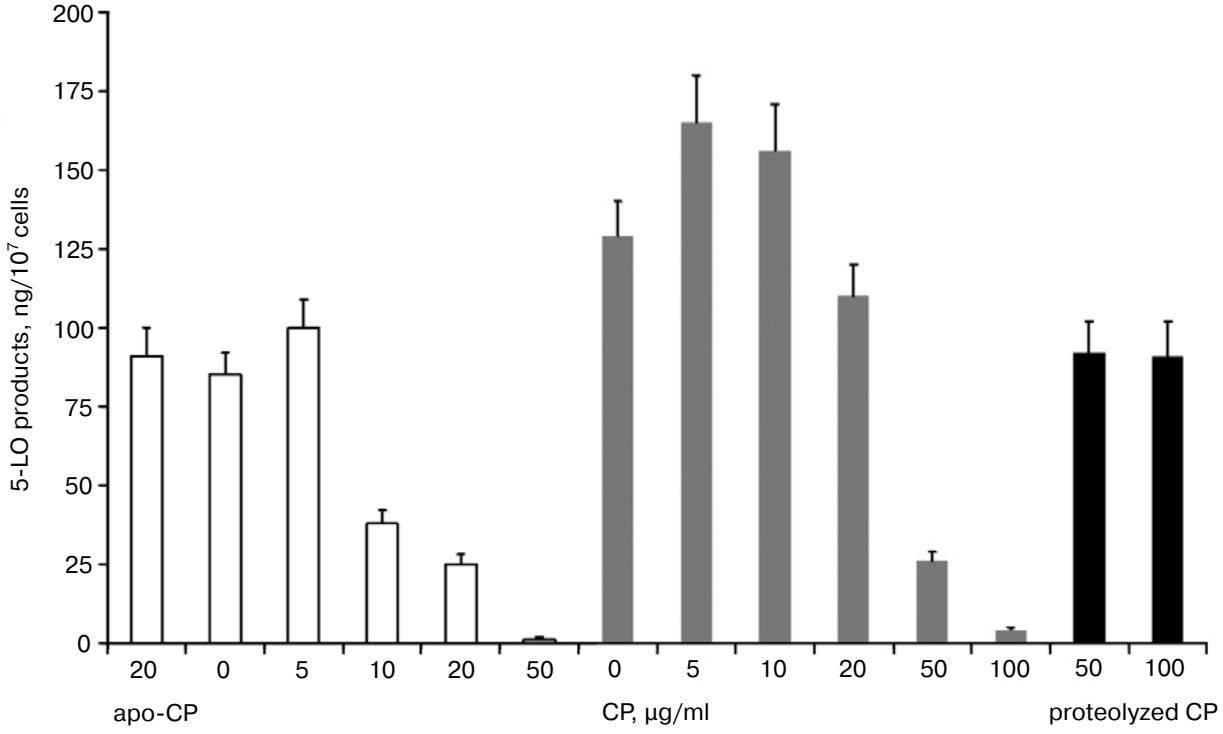

Fig. 2. Effect of CP on production of metabolites of 5-LO in human neutrophils. Cells were incubated for 30 min with varying amounts of CP in protein-free medium (white and black columns) or in a medium supplemented with 0.3 mg/ml of human serum albumin (HSA, gray columns), after which 2 mg/ml OZ was added for 20 min. Amounts of products were determined by reverse-phase HPLC. Apo-CP, apo-form of CP.

not compete with substrates for the catalytic center of the enzyme [15]. Depletion of CP of its copper ions turning it into the apo-form also deprives the protein of the capacity to inhibit both MPO and 5-LO. Apo-CP differs from the holo-form by alterations in tertiary structure resulting in acquiring by the molecule of a peculiar conformation known as "molten globule" [29]. It seems likely that CP in such conformation cannot interact with 5-LO.

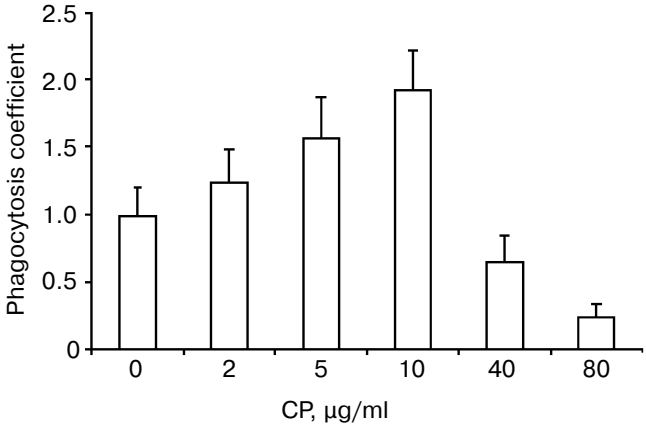

Fig. 3. Phagocytosis of OZ by neutrophils preincubated with CP in various concentrations. After 30 min of incubation of cells in the presence of CP or in its absence, OZ was added (~12 particles per cell) for 3 min and phagocytosis coefficient was determined using phase-contrast microscopy.

Activating effect of low amounts of CP may be connected with its ability to oxidize NO [6], the endogenous inhibitor of 5-LO activity [30, 31]. Concerning the ferroxidase activity of CP making the enzyme an evident antioxidant, it was shown that at least in some cases it can underlie the prooxidant effect of CP. For example, in patients with localized aggressive periodontitis whose neutrophils synthesize iron-oxidizing CP, Fe^{3+} ions were activating NADH-oxidase and caused an increase of superoxide anion-radical production [16]. However, in our experiments superoxide production was quenched (Fig. 4) starting from the dose of 2.5 μg/ml (IC_{50} ~ 2 μg/ml), which confirms the role of CP as a plasma antioxidant possessing superoxide dismutase activity [4] or inhibiting the synthesis of superoxide by an alternative mechanism [32].

The stimulating effect of CP on neutrophilic phagocytosis was first demonstrated in the works of Yaropolov's group [33]. These authors point to the importance of interaction of CP with cells, but reject the notion of CP performing as an opsonin for particles. Indeed, in our experiments preincubation of neutrophils with CP brought a profound effect on such cellular responses of neutrophils as phagocytosis, respiratory burst (superoxide

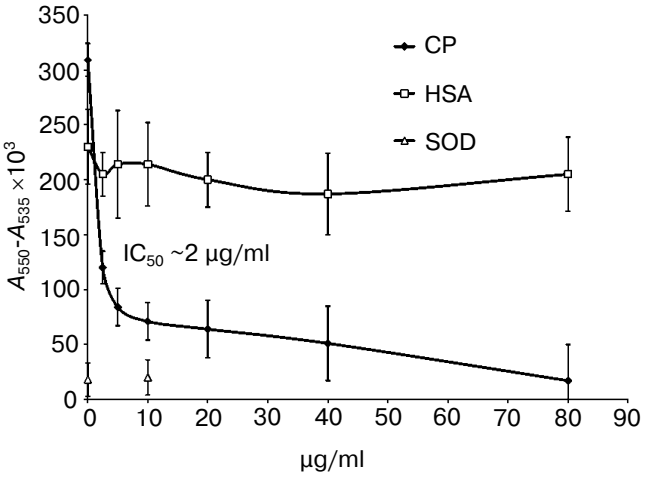

Fig. 4. Effect of CP on production of superoxide anion in human neutrophils. Neutrophils were incubated for 30 min with CP and HSA (negative control) in mentioned concentrations, after which OZ was added for 30 min, optical density (OD) of reduced cytochrome *c* was measured, and the difference between OD at two wavelengths was determined Δ(550/535) nm. Data in the presence of Cu,Zn-SOD (300 units/ml) are given as a positive control.

production), and synthesis of physiologically active metabolites of arachidonic acid, i.e. leukotrienes.

Thus we demonstrate for the first time the effect of CP on 5-LO. Data supporting the notion of direct interaction of these two proteins are obtained. Leukotriene synthesis seems to be extremely sensitive to the integrity of CP. Proteolytic degradation of the latter results in the loss of the efficient influence of CP on that synthesis. It can be suggested that the ability of neutrophils to synthesize CP and to provoke its degradation by proteinases secreted upon activation of these cells is used as a mechanism of fine regulation of cellular responses of neutrophils in inflammation, including the response to infection.

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