Capacity of ceruloplasmin to scavenge products of the respiratory burst of neutrophils is not altered by the products of reactions catalyzed by myeloperoxidase

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Capacity of ceruloplasmin to scavenge products of the respiratory burst of neutrophils is not altered by the products of reactions catalyzed by myeloperoxidase.

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Abstract

Ceruloplasmin (CP) is a copper-containing ferroxidase of blood plasma, acting as an acute phase reactant during inflammation. The effect of oxidative modification of CP induced by oxidants produced by myeloperoxidase, such as HOCl, HOBr and HOSCN, on its spectral, enzymatic and anti-inflammatory properties was studied. We monitored chemiluminescence of lucigenin and luminal along with fluorescence of hydroethidine and scopoletin to assay the inhibition by CP of the neutrophilic respiratory burst induced by phorbol 12-myristate 13-acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLP). Superoxide dismutase activity of CP and its capacity to reduce the production of oxidants in respiratory burst of neutrophils remained virtually unchanged upon modifications caused by HOCl, HOBr and HOSCN. Meanwhile, the absorption of type I copper ions at 610 nm became reduced along with a drop of the ferroxidase and amino oxidase activities of CP. Likewise its inhibitory effect on halogenating activity of myeloperoxidase was diminished. Sera of either healthy donors or patients with Wilson disease were co-incubated with neutrophils from healthy volunteers. In these experiments, we observed a reverse correlation between the content of CP in sera and the rate of hydrogen peroxide production by activated neutrophils. In conclusion, CP is likely to play a role of an anti-
inflammatory factor tempering the neutrophil respiratory burst in the bloodstream despite the MPO-mediated oxidative modifications.

**Keywords:** ceruloplasmin, myeloperoxidase, superoxide dismutase, neutrophils, respiratory burst.


**Introduction**

One of the effector links of innate immunity is represented by polymorphonuclear leukocytes (neutrophils). The primary response to alien microorganisms is stimulation of neutrophils, which is accompanied by the changes of their form, adhesion, taxis towards the focus of microbial accumulation or of a tissue lesion, phagocytosis of microbes, enhanced oxygen consumption (“respiratory burst”) and degranulation. The latter process is characterized by the flux of enzymes and microbicidal proteins from cytoplasmic granules to the phagosome and also their partial secretion into the extracellular space. In order to restrain a premature activation of neutrophils in the bloodstream, some serum proteins belonging to the acute phase proteins are able to inactivate aggressive components released from activated neutrophils. Under inflammatory conditions, acute phase proteins are additionally released into the circulating blood. For example, the molar concentration of ceruloplasmin (CP, ferro:O$_2^-$-oxidoreductase; EC 1.16.3.1), one of the acute phase proteins, raises from 3 µM (basal value) to 10 µM (inflammatory value) (Gitlin 1988). However, no unequivocal concept of functions performed by CP in inflammation has been accepted so far, despite its numerous enzymatic and anti-inflammatory activities. The distinctive feature of CP is its capacity to oxidize Fe$^{2+}$ to Fe$^{3+}$ (Osaki 1966). Indeed, among all substrates of CP the lowest K$_M$ has been determined for Fe$^{2+}$. According to the current concept CP and its homologue hephaestin, play an important role in ferroportin-mediated export of Fe$^{2+}$ from enterocytes, hepatocytes and macrophages, and in subsequent incorporation of Fe$^{3+}$ into transferrin (Musci et al., 2014). Deficiency of the CP gene in humans, manifested as aceruloplasminemia, causes oxidative stress resulting from tissue accumulation of Fe$^{2+}$ (Vassiliev et al. 2005), yet oxidation of iron is unlikely to be the only role of CP. Enzymatic activities of ferroxidase (Osaki 1966), cuproxidase (Stoj and Kosman 2003), superoxide dismutase (Vasil'ev et al. 1988), glutathione-linked peroxidase (Kim and Park 1998)
and NO-oxidase (Shiva et al. 2006) allow CP to prevent production and persistence of oxidants and free radicals. The presence and expression of the CP gene seems essential for survival of neurons in acute-phase response to lipopolysaccharide, as injections of the latter to CP-knockout mice resulted in iron accumulation and demyelination with lethal effect (Glezer et al. 2007). In response to infection with *Streptococcus pneumoniae* synthesis of CP becomes enhanced in endotheliocytes, which suggests its participation in modulation of the blood-brain barrier protecting the central nervous system against circulating pathogens and potentially toxic molecules. In case of CP-gene knockout mice, this notion is supported by an increased synthesis of P-selectin, regulating vascular permeability to neutrophils (Glezer et al. 2007). Such mice do not survive experimental colitis, probably by reason of the absence of anti-inflammatory protection normally provided by CP (Bakhautdin et al. 2013).

Along with soluble CP secreted by hepatocytes into plasma, its glycoprophosphoinositol-anchored form was found in membranes of a number of cells in neural, immune and other tissues (Salzer et al. 1998; Marques et al. 2012). Synthesis of CP is increased in response to hypoxia, iron deficiency (Mukhopadhyay et al. 2000), copper excess (Martin et al. 2005), and such factors as insulin (Seshadri et al. 2002), thrombin (Yang et al. 2006), estradiol (Voronina and Monakhov 1980), and pro-inflammatory cytokines (Mazumder et al. 1997).

In the past ten years we described for the first time and partially characterized the high-affinity complexes of anionic CP (pI 4.7) with cationic proteins of neutrophilic leukocytes, such as lactoferrin, myeloperoxidase (MPO) (Sokolov et al. 2007a), several serprocidins (elastase, cathepsin G, proteinase 3 and azurocidin) (Sokolov et al. 2007b), and 5-lipoxygenase (Sokolov et al. 2010a). For example, the affinity of CP towards lactoferrin and azurocidin is characterized by $K_d \sim 13$ nM (Sokolov et al. 2009, 2010b). Both *in vitro* and *in vivo* CP can form multimeric complexes including lactoferrin and MPO (Sokolov et al. 2007a; Samygina et al. 2013). Ferroxidase activity of CP becomes enhanced as it forms the complex with lactoferrin (Sokolov et al. 2005a). Its interaction with MPO efficiently inhibits the latter (Segelmark et al. 1997), which has an important physiological significance as the excessive activity of MPO in inflammation provokes halogenative stress *via* formation of the hypohalous acids HOCl and HOBr (Panasenko et al. 2013). We have shown that inhibition of MPO by CP decreases the pro-atherogenic modification of low-density lipoproteins (Sokolov et al. 2014a). However, CP does not inhibit the oxidation of thiocyanate by MPO (Sokolov et al. 2014b). Partially proteolyzed CP can inhibit neither chlorinating, nor peroxidase activity of MPO (Panasenko et al. 2008; Sokolov et al. 2008).

Stimulation of neutrophils, occurring at phagocytosis in particular, includes activation of NADPH oxidase in their plasma membrane (Segal 2005), and also the arachidonic acid-induced
leukotriene synthesis (Rådmark et al. 2010). The key enzyme of leukotriene synthesis is 5-lipoxygenase that catalyzes the first two reactions converting arachidonic (\textit{cis},\textit{cis},\textit{cis},\textit{cis-}5,8,11,14-eicosatetraenoic) acid into leukotrienes. In neutrophils, the addition of CP at concentrations higher than 10 µg/mL suppressed the activity of 5-lipoxygenase, decreased the phagocytosis of opsonized zymosan and the production of superoxide anion radicals (Sokolov et al. 2010a). As in the case with MPO, no inhibitory effect on 5-lipoxygenase could be achieved if CP was partially proteolyzed (Sokolov et al. 2010a).

CP acted as an anti-oxidant when added to an \textit{in vitro} system containing activated neutrophils (Krsek-Staples et al. 1993) and monocytes (Betten et al. 2004). Otherwise, low doses of CP can stimulate phagocytosis of neutrophils (Saenko et al. 1994). It is interesting that in patients with localized aggressive periodontitis, whose neutrophils synthesized CP, Fe$^{3+}$ ions caused an increased production of superoxide anion radicals (Iwata et al. 2009). In our recent paper we demonstrate that increase of CP concentration during pregnancy can decrease the respiratory burst of neutrophils in blood (Varfolomeeva et al. 2016).

It is known that CP is damaged by oxidants formed by activated phagocytes (Sharonov et al. 1988; Winyard et al. 1989). However, it remains unknown, how the protective effect of CP is affected by neutrophil-derived oxidants. The ability of HOCI, HOBr, and HOSCN to modify amino acid residues in proteins is well known (Hawkins et al. 2003; Pattison et al. 2004; Skaff et al. 2009).

Damage of proteins was also documented in reactions with hydrogen peroxide (Sokolov et al. 2012a), superoxide anion radicals and HOCI (Sharonov et al. 1988, 1989). Recently, we demonstrated that albumin modified by products of MPO catalysis (HOCI and HOBr) caused an activation of neutrophils (Mikhal’chik et al. 2013; Gorudko et al. 2014).

Therefore, the aim of this study was to investigate the effect of products of reactions catalysed by MPO (HOCI, HOBr, and HOSCN) on enzymatic properties of CP and on the anti-inflammatory activity of CP towards activated neutrophils.

**Materials and methods**

**Chemicals**

The following reagents were used: UNOsphere Q, Bio-Gel A1.5m gel “Bio-Rad” (USA); ammonium persulfate, arginine, Coomassie R-250, D-glucose (D-Glc), glucose oxidase (GO), glycerol, mercaptoethanol, NaN$_3$, Tris “Serva” (Germany); 2,2’-azino-di(3-ethylbenzthiazolinesulphonic acid) disodium salt (ABTS), catalase, celestine blue B (CB), 4-chloro-1-naphtol, cytochrome $c$ (cyt $c$) (type VI from horse heart), $o$-dianisidine dihydrochloride, 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA), dihydrorhodamine 123 (DHR), EDTA,
ethidium bromide, Fe(NH$_4$)$_2$(SO$_4$)$_2$×6H$_2$O, formyl-methionyl-leucyl-phenylalanine (fMLP), horseradish peroxidase type II, lucigenin, luminol, NaBH$_4$, NaOCl, neomycin, phenylmethylsulfonyl fluoride, $p$-phenylenediamine dihydrochloride ($p$-PD), phenyl-Sepharose, phorbol 12-myristate 13-acetate (PMA), resazurin, scoopoletin, SDS, Sephacryl S-200 HR, superoxide dismutase (SOD), triethanolamine (TEA), xanthine, xanthine oxidase “Sigma” (USA); acrylamide, N,N'-methylenebis(acrylamide), N,N,N',N'-tetramethylethylenediamine “MEDIGEN Laboratory” (Russia); heparin “SPOFA” (Poland); Dextran T70 “Roth” (Karlsruhe, Germany). Cyanogen bromide was obtained by bromination of KCN in two-phase system «water/dichloroethane». Solution of BrCN in dichloroethane was used to activate Sepharose (or agarose) and further to immobilize heparin and neomycin on BrCN-activated gel (Sokolov et al. 2005b). Solid phase synthesis of peptide RPYLKVFNPR (corresponding to amino acid 883-892 stretch in CP) was accomplished in the Research Institute of Extra-pure Biopreparations (Saint-Petersburg). Peptide was 99.5 % pure as judged by results of HPLC and mass spectrometry. Hydroethidine was synthesized at the Saint-Petersburg Nuclear Physics Institute by reducing ethidium bromide with NaBH$_4$ (Filatov et al. 1995). To generate HOBr or HOSCN, equal volumes of equimolar solutions (60 mM) of NaOCl and NaBr or NaSCN were mixed in water or 10 mM Na-phosphate buffer (pH 7.4). Bleaching of 5-thio-2-nitrobenzoic acid was measured to assay the production of oxidants (van Dalen et al., 1997). All solutions were prepared using apyrogenic deionized water with specific resistance 18.2 MΩ×cm.

Spectrophotometry

Optical spectra and the rate of absorption changes were monitored on a SF-2000-02 («OKB-Spectr>>, Russia). Spectrophotometry was also used for concentration measurements, and the following absorbance coefficients were used: $\varepsilon_{430}$=178000 M$^{-1}$cm$^{-1}$ (Bakkenist et al. 1978), CP – $\varepsilon_{610}$=9780 M$^{-1}$cm$^{-1}$ (Noyer et al. 1980), H$_2$O$_2$ – $\varepsilon_{240}$=43.6 M$^{-1}$cm$^{-1}$ (Beers and Sizer 1952), ClO$^-$ – $\varepsilon_{292}$=350 M$^{-1}$cm$^{-1}$ (Morris 1966), BrO$^-$ – $\varepsilon_{329}$=345 M$^{-1}$cm$^{-1}$ (Gazda and Margerum 1994).

Protein purification

Stable preparation of monomeric CP with $A_{610}/A_{280}$ > 0.049, containing more than 95 % of 132-kDa protein was obtained by chromatography of blood plasma on UNOsphere Q and neomycin-agarose (Sokolov et al. 2012b). Briefly, 1.4 L of plasma (containing 1 mM phenylmethylsulfonyl fluoride and 0.1 EDTA) was loaded on a PBS-equilibrated UNO-Sphere Q column (30×2.5 cm, flow rate was 10 mL/min). Chromatography was performed using ice-cold solutions. Elution was performed at a rate of 2 mL/min with a linear gradient 0→0.5 M NaCl containing 40 mM TEA-HCl (pH 7.4), the total volume was 200 mL. Blue-colored fractions
were pooled, diluted 1:10 with 20 mM TEA-HCl (pH 7.4) and loaded at a flow rate of 5 mL/min on the neomycin-agarose column (12×2.5 cm) equilibrated with 40 mM TEA-HCl (pH 7.4). Proteins were eluted from the column by 120 mL of a linear gradient 0→100 mM CaCl$_2$ containing 40 mM TEA-HCl (pH 7.4) using at a flown rate of 2 mL/min. Blue-colored fractions were pooled and concentrated in a VivaSpin 20 unit. It was diluted with 18 mL of 40 mM TEA-HCl (pH 7.4), then again concentrated to 2 mL. Activity of purified CP in reaction with Fe$^{2+}$ was characterized by $K_M = 57$ µM and $k_{cat} = 0.98$ s$^{-1}$ (Sokolov et al. 2015c). MPO was purified from human leukocytes by chromatography on heparin-Sepharose, phenyl-Sepharose and gel filtration on Sephacryl S-200 HR, which yielded a preparation with $A_{430}/A_{280}$ (RZ) = 0.85 inherent in homogeneous protein (Sokolov et al. 2010c).

**Ceruloplasmin modification by HOCl, HOBr and HOSCN**

0.1-3 mM HOCl, HOBr, HOSCN in 50 mM Na-phosphate buffer, pH 7.4 was added to CP solution (50 µM), to the molar ratios HOX : CP = (2 - 60) : 1, where X$^-$ is Cl$^-$, Br$^-$ or SCN$^-$. After 30 min of incubation at 37 ºC spectral characteristics of CP, its oxidase activities and the effect on the respiratory burst of neutrophils were analyzed. Oxidative damage of CP was assayed *in vitro* by monitoring the changes of its oxidase activity in reaction with o-dianisidine in polyacrylamide gel after disc-electrophoresis (Davis 1964; Owen and Smith 1961). Reaction mixture contained 1 µM CP, 10 nM GO, 100 µM D-Glc and various combinations of 3 nM MPO, 100 mM NaCl, 1 mM NaBr, 200 µM NaSCN in 0.1 M citrate-sodium-phosphate buffer (pH 6.8). Incubation at 37 ºC lasted for 1 h, after which 200 µM taurine and 10 nM catalase were added to the samples.

**Enzymatic activity**

Oxidase activity of CP in reaction with $p$-PD was determined as the difference between the oxidation by CP of $p$-PD (0.05 %) in Na-acetate buffer, pH 5.5, in the absence and in the presence of NaN$_3$ (0.05 %), an efficient inhibitor of CP. Incubation at 37 ºC lasted for 1 h, after which $A_{530}$ was measured vs. the control aliquot containing NaN$_3$ (Sokolov et al. 2012b). CP concentration in serum samples was assayed by measuring its oxidase activity with $p$-PD in microplates (Varfolomeeva et al., 2016). 10 µl of serum or of purified CP (0-4 µM) were loaded into the wells of a 96-well plate, followed by 240 µl of freshly prepared 0.2 % $p$-PD (m/v) solution in 0.4 M Na-acetate buffer (pH 5.5). The initial $A_{530}$ was measured immediately using plate reader ClarioStar (BMG Labtech, Germany). After 30 min of incubation at 37 ºC $A_{530}$ was measured again. Calibration graph was plotted with purified CP concentration on abscissa and $\Delta A_{530}$ on ordinate ($R^2 = 0.997$) to calculate CP content in tested samples.
Ferroxidase activity was assayed in reaction of 15 nM CP with 150 µM Fe(NH₄)₂(SO₄)₂×6H₂O in 0.4 Na-acetate buffer, pH 5.5. After 10 min incubation, ferrozin was added to the reaction mixture to the final concentration of 150 µM, and A₅₆₄ was monitored vs. the control aliquot with NaN₃ (Pulina et al. 2010).

Superoxide dismutase activity of CP was assayed as its capacity to decelerate resazurin reduction by superoxide anion radicals generated in potassium-phosphate buffer, pH 7.4, containing 100 nM xanthine oxidase, 150 µM xanthine and 50 µM resazurin. The rate of resazurin reduction (A₆₀₄/min) by superoxide anion radicals was measured upon addition of 100, 200 and 400 nM CP. In control experiments, applying the same components except resazurin, the effect of CP on xanthine oxidase activity was evaluated by monitoring urate production (A₂₉₅/min).

To study an effect of CP and synthetic peptide RPYLKVFNPR (corresponding to the fragment 883-892 of the CP sequence) on oxidation of ABTS by MPO (Sokolov et al., 2015a), we used a mixture of 3 nM MPO, 0.1 mM H₂O₂ and 1 mM ABTS in 0.1 M sodium-acetate buffer, pH 6.0 and various amounts of CP or peptide (50-1600 nM).

Isolation of neutrophils

Polymorphonuclear leukocytes (neutrophils) were isolated from fresh donor blood containing 1.2 g/l EDTA as follows. Erythrocytes were precipitated by 3 % dextran T-500 in 0.85 % NaCl (40 mL of solution per 100 mL of blood) at room temperature. Leukocyte- and platelet-enriched plasma was applied on the layer of Ficoll-Paque (density 1.077 g/L) in a 50 mL centrifuge tube. Upon centrifugation for 30 min at 500 g in density gradient the precipitate contained granulocyte fraction with admixture of erythrocytes. Red blood cells were lysed in a hypotonic buffer consisting of 114 mM NH₄Cl, 7.5 mM KHCO₃ and 100 µM EDTA. Granulocytes were twice washed in PBS (1.06 mM KH₂PO₄, 155 mM NaCl, 2.7 mM KCl, 2.96 mM Na₂HPO₄, pH 7.4) and resuspended in D-PBS (0.49 mM MgCl₂, 2.7 mM KCl, 1.15 mM K₂HPO₄, 138 mM NaCl, 9.58 mM NaH₂PO₄, pH 7.4) with D-Glc (1 g/l). All experiments with neutrophils were accomplished on the day of blood sampling.

Measurements of H₂O₂ generation by neutrophils using scopoletin

H₂O₂ production by neutrophils was measured using the scopoletin/peroxidase fluorescent technique (Timoshenko et al. 1998; Gorudko et al. 2011). Briefly, suspension of neutrophils (2 × 10⁶ cells/mL in PBS, containing 1 mM CaCl₂, 0.5 mM MgCl₂) was supplemented with 1 µM scopoletin (a fluorescent substrate of peroxidase), 20 µg/mL horseradish peroxidase, and 1 mM NaN₃ (catalase and MPO inhibitor) and was challenged with native or modified CP at various
concentrations to activate plasma membrane NADPH oxidase. For inhibitor studies neutrophils were pre-incubated for 5 min at 37 °C with CP (0-4.5 µM) or tested serum before the addition of fMLP. H₂O₂-mediated oxidation of scopoletin was recorded as a decrease of its fluorescence at 460 nm (excitation was at 350 nm) during 20 min at 37 °C using a SOLAR CM2203 fluorescence spectrometer (SOLAR). The maximal slope of the recorded traces was calculated and referred to as the rate of H₂O₂ generation by cells.

Assay of O₂⁻ production by neutrophils using cytochrome c

Production of O₂⁻ was continuously recorded by monitoring the SOD-inhibitable reduction of cyt c as previously described (Timoshenko et al. 1998). Neutrophils, resuspended in PBS, containing 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4, were stimulated to produce O₂⁻ by adding native or modified CP at various concentrations. The mixtures containing neutrophils (2 × 10⁶ cells/mL) and 75 µM cyt c were incubated at 37 °C with or without SOD (25 µg/mL). The increase of absorption at 550 nm was monitored using a PB2201 spectrophotometer. Neutrophil O₂⁻ production was finally expressed as nanomoles of O₂⁻ per 2 × 10⁶ neutrophils in 15 min.

Luminol- and lucigenin-dependent chemiluminescence

MPO- and NADPH oxidase-dependent oxygenation activity of neutrophils was assayed on the basis of chemiluminescence arising from luminol and lucigenin as the chemoluminigenic used as substrates. The intensity of emission reflects the MPO-dependent formation of HOCl and NADPH oxidase-dependent formation of O₂⁻ (Panasenko et al. 2016). Briefly, suspension of neutrophils (final concentration 0.4 × 10⁶ cells/mL) was distributed into polystyrene tubes containing Krebs-Ringer buffer (pH 7.4), luminol or lucigenin (0.2 mM), which was followed by adding PMA and then native or modified CP (1-6 µM). Luminescence response was measured using a single-photon luminometer (LKB Wallac Luminometer 1251, Finland).

Monitoring respiratory burst in blood using flow cytometry with hydroethidine

Respiratory burst reaction was monitored not later than 8 h after blood sampling. Such prolonged sample storage did not affect the results (Filatov et al. 1995). The samples (1 mL) were put into test-tubes containing 40 units of heparin. Prior to incubation an equal volume of PBS was added into a test-tube. Finally, hydroethidine was added to achieve the final concentration of 140 µg/mL. The reaction was induced by adding 10 ng/mL of PMA and ran for 80 min at 37 °C. A control portion was the same, but without PMA. Fluorescence intensity spread was measured immediately as the reaction is stopped. A standard flow cytometer
equipped with argon laser (excitation wavelength 488 nm) was used. Sensitivity of the cytometer was calibrated using fluorescent microspheres from Immuno-Brite Standards Kit (Beckman Coulter). Fluorescence peak registered with particles from Level IV (Med-Hi) flask corresponded to the channel 120. Mean value of fluorescence intensity measured in stimulated neutrophils was used to quantify the neutrophils’ capacity for respiratory burst.

Registering respiratory burst of neutrophils by flow cytometry with DHR and DCFH-DA

The effect of native or modified CP (0.2–2 µM) on H₂O₂ production by neutrophils was determined by flow cytometry (Vlasova et al. 2016). Fluorogenic probe DCFH-DA or DHR was added to neutrophils (5 × 10⁶ cells/mL) at a concentration of 10 µM. After 45 min incubation with 50 nM PMA samples were diluted 1:20 in PBS for flow cytometer analysis. Fluorescence intensity was measured using a flow cytometer Navious™ (Beckman Coulter). Ten thousand leukocytes were registered in each sample.

Monitoring the HOCl production by neutrophils

Neutrophils (5×10⁵ cells/mL) were resuspended in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, 10 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 1 mg/mL D-Glc and 20 mM taurine (Sokolov et al. 2015b). An effect of native or modified CP (0.2–2 µM) on HOCl production by cells activated by PMA (100 nM) was studied. Cells in 24-well plates containing various supplements were incubated in a thermal shaker (37 °C) for 60 min. Reaction was stopped by adding catalase (2 nM) and placing the 24-well plates on ice for at least 10 min. Then cells were pelleted by centrifugation (10 min, 5000 g, 4 °C). The supernatant (200 µl) was mixed with 50 µl of 0.5 mM CB (with/without 25 µM KI) in 96-well plates. A₆₅₀ was registered on a plate reader (CLARIOstar, BMG LABTECH, Germany). HOCl production by cells was calculated using calibration plots reflecting dependence between A₆₅₀ and NaOCl (2–40 µM) added in 96-well plates with 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, 10 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 1 mg/mL D-Glc, 20 mM taurine, and 100 µM CB (with/without 5 µM KI).

Statistical analysis

Experiments were repeated at least three times (n ≥ 3) and mean values were calculated as

\[ X_m = \frac{1}{n} \sum X_i, \]

where \( X_i \) is a value of each successive sample. The standard error was expressed as

\[ S^* = \sqrt{\frac{\sum (X_i - X_m)^2}{n-1}} \]

as \( S^*/n \), where \( S^* = \sqrt{\frac{\sum (X_i - X_m)^2}{n-1}} \), and the confidence interval was calculated as \( X_m \pm \)}
(S^*/n^{1/2})_t_{n,1-\alpha/2},\text{ for which } t \text{ was found in the table of values on condition that in our experiments } \alpha = 0.05. \text{ General linear regression and Pearson’s correlation were used to evaluate } \text{IC}_{50} \text{ and the relationship between CP added to serum and the } H_2O_2 \text{ production by activated neutrophils.}

**Results**

*Oxidative modification of CP by products of MPO catalysis i.e. HOCl, HOBr and HO SCN.*

In the first series of experiments, myeloperoxidase-derived hypo(pseudo)halous acids, important products of activated neutrophils, were incubated with CP to investigate their effects on selected CP functions. Increasing concentrations of HOCl and HOBr, but not of HO SCN, decreased the catalytic activity of CP in oxidation of Fe^{2+} (Fig. 1a) and p-PD (Fig. 1b). In contrast, SOD activity of modified CP slightly grew with increasing concentrations of all tested hypo(pseudo)halous acids as compared to non-modified protein (Fig. 1c).

Interaction of CP with HOCl, HOBr and HO SCN was also followed by gradual decrease of the absorption at 610 nm, which is peculiar for type I copper ions (Fig. 2). There was a stronger decrease of absorbance at 610 nm in case of HOCl and HOBr, as compared to HO SCN. The increase of absorption in the range 300 - 400 nm upon modification of CP with HOCl and HOBr, but not in case of HO SCN (Fig. 2), results likely from formation of chloramines and bromamines that absorb in this spectral region (Zgliczyński et al. 1971).

Treatment of CP with HO SCN did not affect its capacity to inhibit the peroxidase activity of MPO. In contrast, HOCl and HOBr reduced this antioxidant activity of CP (Fig. 3, Table 1). Similar results were obtained using the inhibitory CP peptide RPYLKVFNPR(883-892). Thus, the MPO products HOCl and HOBr are able to disturb the catalytic function of CP by modifying critical amino acids.

Effects of HOCl, HOBr and HO SCN, produced in a system containing MPO, D-Glc, GO, halogenides and thiocyanate, on oxidase activity of CP were additionally studied (Fig. 4). Thiocyanate protected CP against the damage caused by hypohalous acids (HOCl and HOBr) generated in the catalytic cycle of MPO.

*Effect of modified CP on respiratory burst of neutrophils.*

Next, we studied the effect of CP, modified by hypo(pseudo)halous acids, on the activation of neutrophils. Doses of native CP corresponding to concentrations of this protein in plasma are known to inhibit the production of O_2• during the respiratory burst of neutrophils (Sokolov et al. 2010a). In our experiments the effect of modified CP on the activity of neutrophils was evaluated by monitoring the production of superoxide anion radicals, hydrogen peroxide and hypochlorous acid (Table 2). Regardless of the type of oxidative modification, CP preserves the capacity to
scavenge oxidants produced during the respiratory burst of neutrophils. For better comparison, the IC_{50} values were calculated for the inhibition of the respiratory burst by non-modified and modified CP. Any method we used to monitor the respiratory burst gave virtually the same value of IC_{50} either for intact CP or for the protein modified by various oxidants (Table 2).

The effect of CP on NADPH-oxidase activity of neutrophils can be the result of its capacity to scavenge oxidants. It can be caused also by the changes occurring in transmembrane signaling following the interaction of CP with the cells (Kataoka and Tavassoli 1985). To clarify the issue, we studied the activator-induced release of hydrogen peroxide by neutrophils pre-incubated with CP. As shown in Fig. 5, we observed no significant difference between the rate of fMLP-induced oxidation of scopoletin by neutrophils in the control group and the cells washed after incubation with CP. This result allows suggesting that CP reduces the respiratory burst due to its interaction with oxidants discharged into the medium upon activation of neutrophils.

When Cu,Zn-SOD was added together with fMLP to the suspension of neutrophils, the rate of scopoletin oxidation increased noticeably (Fig. 5, d). This result favors the notion that fMLP-induced the production of O_2^•, which is followed by dismutation of the latter to H_2O_2. The process was almost completely inhibited by 2 µM CP. When CP was added to the suspension of neutrophils, no significant amounts of hydrogen peroxide were formed, in contrast to the effect of Cu,Zn-SOD.

When fMLP-activated neutrophils were washed with PBS to eliminate CP, the release of hydrogen peroxide resumed (Fig 5, c). Abrogation of the inhibitory effect of CP by washing is contrary to the data about the presence of CP receptors on neutrophils (Kataoka and Tavassoli 1985). Hence, apparently the SOD-like activity of CP is responsible for the inhibition of the respiratory burst. The difference between O_2^• dismutation by Cu,Zn-SOD and CP is the end-product, which is likely to be water in case of CP, but not hydrogen peroxide as with Cu,Zn-SOD.

We observed a reverse correlation between the rate of H_2O_2 production by activated neutrophils and the concentration of CP in sera of either healthy donors or patients with Wilson disease (r = –0.92), if samples of sera were added to neutrophils prior to activation (Fig. 6). Similar reverse correlation between CP concentration and activation of neutrophils was shown in our recent study of pregnant women’s blood (Varfolomeeva et al. 2016).

Flow cytometry with hydroethidine did not show any difference between suppression of the respiratory burst in activated neutrophils by modified and non-modified CP (Fig. 7). Thus, CP inhibits the respiratory burst of neutrophils under conditions that are very similar to those in the bloodstream.
Discussion

In late 1980-ies Lyzlova’s group showed that among plasma proteins CP has the strongest potential as the scavenger of \( \text{O}_2^- \) and HOCl that are, respectively, the substrate and the product of reactions catalyzed by MPO (Govorova et al. 1986; Sharonov et al. 1988, 1989). Shortly after that a protein inhibitor of MPO with \( M \sim 150 \text{ kDa} \) was isolated from serum (Yea et al. 1991), but only some years later it was identified as CP (Segelmark et al. 1997).

All hypo(pseudo)halous acids used for CP modification react with Cys residues. An increase of the SOD activity of modified CP might result from alteration of its type I copper properties caused by oxidation of Cys residues. It is known that for CP to act as SOD, the enzyme needs only part of its catalytic center, i.e. type II and III of copper ions coordinated by His residues (Vasil'ev et al. 1988). HOCl and HOBr interact also with Met, Tyr and other amino acid residues. HOSCN interacts only with Cys at sufficient rate, but not with other residues (Hawkins et al. 2003; Pattison et al. 2004; Skaff et al. 2009). The changes in oxidase properties of CP are probably due to the preferential targets of HOCl and HOBr, which are Met residues. Properly these residues participate in coordination of type I copper that plays the key role in oxidation of \( \text{Fe}^{2+} \), 1,2-PD and o-dianisidine. Electrophoretic mobility of modified CP changed (Fig. 4), most likely due to partial oxidation of its disulfide bonds. A small stimulatory effect of HOSCN on p-PD-oxidase activity of CP might result from the residual HOSCN reacting with p-PD (Fig. 2, b).

In our experiments CP efficiently inhibited the production of \( \text{H}_2\text{O}_2 \) by activated neutrophils, depriving MPO of its substrate. This effect seems to be a prerequisite condition for CP to retain its MPO-inhibiting activity performed essentially by non-fragmented protein, while hydrogen peroxide is able to destroy the molecule (Sokolov et al. 2012a).

The apparent role of CP as a physiological inhibitor of MPO allows suggesting its participation in a number of pathological processes, inflammation being the most vivid. Recently we studied sophisticated interrelations among CP, MPO and thrombin in synovial fluid of rheumatoid arthritis patients and raised again the important issue of CP molecule’s integrity for its functioning (Sokolov et al. 2015c). Yet, many details of that functioning remain unclear, and one of those is the effect of deleterious products formed in inflammation foci on CP. Results of the present study clarify some of such details.

CP readily forms complexes with quite a few proteins released into plasma and other fluids (Sokolov et al. 2007a, 2007b, 2010a, 2015c; Samygina et al., 2013; Kostevich et al. 2015; Skarżyńska et al. 2017). Such interactions mostly help a partner protein to accomplish its functions (Sokolov et al. 2005a, 2014b) and can increase the enzymatic activity of CP. However, some interactions with other proteins or non-protein molecules can entail partial or complete loss of its functions (Hawkins et al. 2003; Dutra et al. 2005; Sokolov et al., 2015c). This study
enlarges our knowledge of the ‘safety margins’ for interactions of CP, at least with respect to MPO.

It was shown that interaction of C-reactive protein with MPO does not prevent CP from efficient inhibition of the latter (Xu et al. 2014). Our study showed that blood plasma, from which CP had been eliminated by immunoprecipitation, has a decreased capacity to inhibit peroxidase activity of MPO, but the latter retains its ability to utilize hydrogen peroxide for oxidation of the more physiological substrate i.e. thiocyanate (Sokolov et al. 2014b). Indeed, thiocyanate oxidation by MPO with the lowest $K_M$ and the highest specificity is not suppressed in the presence of CP (van Dalen 1997). In this study we used a model system with GO generating $H_2O_2$ to show that brominating and chlorinating reactions catalyzed by MPO decrease the oxidase activity of CP (Fig. 3), while thiocyanate prevents its modification. We also observed that HOCl and HOBr produced by MPO, reduced the activity of CP in oxidation of Fe$^{2+}$ and $p$-PD caused by, but its SOD-like activity slightly increased (Fig. 1), which coincided with the capacity of modified CP to inhibit the respiratory burst in neutrophils (Table 2). Decrease of ferroxidase activity of CP after modification by HOCl and HOBr may affect the iron metabolism. However, the impact of this observation in hypoferremia during acute inflammation along with the well-known effect of hepcidin needs to be further investigated.

Neither CP, nor its peptide (883-892) could inhibit MPO, once they were modified by HOCl or HOBr (Fig. 3, Table 1). We showed previously that CP cannot act as an efficient inhibitor of MPO activity in reactions with bromide and thiocyanate (Sokolov et al. 2014b, 2015a). In view that upon treatment with HOBr and HOCl, but not with HOSCN, the capacity of the CP peptide RPYLKVFNP(883-892) to inhibit MPO peroxidase activity decreased, it can be suggested that bromination and/or chlorination damages an amino acid residue that is crucial for interaction with the active center of MPO. It seems likely that such residue is Tyr885. Apparently, the MPO-mediated bromination and chlorination are able to damage an amino acid residue in CP, most likely Tyr885, which is crucial for interaction with the active center of MPO. Indeed, among amino acids of the peptide 883-892 (RPYLKVFNP) tyrosine has a sufficient high rate constant with HOCl and HOBr (Hawkins et al. 2003; Pattison et al. 2004). The CP peptide RPYLKVFNP(883-892) displays a strong regulatory effect in PMA-activated neutrophils as an inducer of superoxide formation and an inhibitor of leukotriene synthesis (Golenkina et al. 2017).

In contrast, mild oxidant HOSCN, the production of which by MPO is not inhibited by CP, caused neither noticeable decrease of any oxidase activity of CP, nor its capacity to inhibit MPO and the respiratory burst of neutrophils. An important conclusion from these results is that CP seems capable of favoring the oxidation by MPO of thiocyanate, but not chloride. As a result,
hypothiocyanite is formed, which is relatively harmless for human cells, but is efficient as an antibacterial agent (Chandler and Day 2012).

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References


Legends to figures.

**Figure 1.** Effect of modification of CP (50 µM) by HOCl, HOBBr and HOSCN on its ferroxidase (a), p-PD-oxidase (b) and SOD (c) activity. Data are presented as mean value and confidence interval (α=0.05), n=6. The activity of CP before modification was set to 1, * indicates significant differences (α=0.05) between the effect HOCl (HOBr) and HOSCN.

**Figure 2.** Absorption spectra of CP (50 µM) after adding HOCl, HOBBr and HOSCN (0 - 600 µM). Optical pathway 1 cm, solutions of HOCl, HOBBr and HOSCN (0-600 µM) were used as the control. Data are presented a mean of 3 curves.

**Figure 3.** Plots used to calculate values of IC₅₀ (nM) for inhibition of MPO-catalyzed peroxidase reaction with ABTS by (a) CP and (b) its peptide (883-892). Data were obtained for non-modified inhibitors and those modified by 4-molar (CP peptide) and 20-molar (CP) excess of HOCl, HOBBr or HOSCN. Logarithmic scale is used to plot concentrations of CP and its peptide. The activity of MPO before adding CP or CP peptide was set to 1.

**Figure 4.** Disc-electrophoresis in polyacrylamide gel of CP (2 µg per lane). 1 µM of CP was incubated for 1 h at 37 ºC with combinations of 10 nM glucose oxidase (GO) and 0.1 mM of D-glucose (D-Glc), 3 nM MPO, 100 mM NaCl, 1 mM NaBr, 0.2 mM NaSCN in 0.1 M citrate-sodium-phosphate buffer (pH 6.8). After incubation 0.2 mM taurine and 10 nM catalase were added. Gel was soaked in o-dianisidine to reveal CP oxidase activity. After three repeats the most typical and demonstrable gel was chosen.

**Figure 5.** Effect of CP on hydrogen peroxide production by neutrophils upon stimulation with 100 nM fMLP. a. Typical fluorescence spectrum of scopoletin obtained after activation of neutrophils in the presence of CP (2 µM) and in its absence (control). b. The rate of H₂O₂ production at various CP concentrations (n=4). c. Scopoletin fluorescence after washing with PBS the suspension of neutrophils from added CP. d. Effects of CP (2 µM) and Cu,Zn-SOD (25 µg/mL) compared.

**Figure 6.** Relationship between H₂O₂ production by stimulated neutrophils and the CP concentration in serum added to neutrophil prior stimulation. All measurements were performed in triplicate.

**Figure 7.** Inhibitory effect of native and modified CP (2 µM) on the oxidative burst of PMA-stimulated neutrophils. Activation of neutrophils was assessed with hydroethidine and expressed as mean fluorescence intensity (a) (n = 5, confidence interval α=0.05) or analysed by flow cytometry (b – f). Typical histograms demonstrate the distribution of hydroethidine fluorescence of neutrophils in the absence of CP (b), or presence of native CP (c), HOCl- (d), HOBBr- (e), or HOSCN- (f) modified CP.
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80x72mm (300 x 300 DPI)
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<table>
<thead>
<tr>
<th>MPO inhibitor</th>
<th>Non-modified</th>
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<th></th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>HOCl</td>
<td>HOBr</td>
<td>HOSCN</td>
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<td>CP</td>
<td>38±6</td>
<td>650±40</td>
<td>840±60</td>
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<tr>
<td>CP peptide (883-892)</td>
<td>158±14</td>
<td>920±60</td>
<td>1260±80</td>
<td>186±18</td>
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**Table 2.** Values of IC$_{50}$ (µM) for inhibition of by CP of neutrophilic respiratory burst, also upon modification of the enzyme by 20-molar excess of HOCl, HOBr or HOSCN.

<table>
<thead>
<tr>
<th>Oxidant produced by activated neutrophils (method of detection)</th>
<th>CP</th>
<th>CP+HOCl</th>
<th>CP+HOBr</th>
<th>CP+HOSCN</th>
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<tr>
<td>O$_2^\cdot$ (cytochrome c)</td>
<td>0.17±0.02</td>
<td>0.14±0.02</td>
<td>0.17±0.02</td>
<td>0.19±0.03</td>
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<td>O$_2^\cdot$ (chemiluminescence of lucigenin)</td>
<td>3.1±0.2</td>
<td>3.2±0.2</td>
<td>3.5±0.3</td>
<td>2.9±0.3</td>
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<tr>
<td>O$_2^\cdot$ (flow cytometry with hydroethidine)</td>
<td>1.1±0.06</td>
<td>1.2±0.07</td>
<td>1.1±0.08</td>
<td>1.3±0.09</td>
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<tr>
<td>H$_2$O$_2$ (flow cytometry with DHR)</td>
<td>0.47±0.04</td>
<td>0.52±0.03</td>
<td>0.48±0.04</td>
<td>0.49±0.03</td>
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<td>H$_2$O$_2$ (flow cytometry with DCFH)</td>
<td>1.7±0.2</td>
<td>1.5±0.1</td>
<td>1.8±0.2</td>
<td>1.9±0.3</td>
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<tr>
<td>H$_2$O$_2$ (scopoletin)</td>
<td>0.94±0.08</td>
<td>0.86±0.09</td>
<td>0.85±0.10</td>
<td>0.99±0.09</td>
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<tr>
<td>HOCl (chemiluminescence of luminol)</td>
<td>4.3±0.3</td>
<td>4.5±0.2</td>
<td>4.8±0.4</td>
<td>4.1±0.3</td>
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<td>HOCl (celestine blue B)</td>
<td>0.66±0.03</td>
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