Ceruloplasmin-derived peptide is the strongest regulator of oxidative stress and leukotriene synthesis in neutrophils

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1 Ceruloplasmin-derived peptide is the strongest regulator of oxidative stress and leukotriene
2 synthesis in neutrophils

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Abstract

Ceruloplasmin, an acute-phase protein, can affect the activity of leukocytes through its various enzymatic activities and protein-protein interactions (with lactoferrin, myeloperoxidase, eosinophil peroxidase, serprocidins, and 5-lipoxygenase (5-LOX), among others). However, the molecular mechanisms of ceruloplasmin activity are not clearly understood. In this study, we tested the ability of two synthetic peptides, RPYLKVFNPR (883–892) (P1) and RRPYLKVFNPRR (882–893) (P2), corresponding to the indicated fragments of the ceruloplasmin sequence, to affect neutrophil activation. Leukotriene (LT) B₄ is the primary eicosanoid product of polymorphonuclear leukocytes (PMNLs, neutrophils). We studied leukotriene synthesis in PMNLs upon interaction with *Salmonella enterica* serovar Typhimurium. Priming of neutrophils with phorbol 12-myristate 13-acetate (PMA) elicited the strong regulatory function of P2 peptide as a superoxide formation inducer and leukotriene synthesis inhibitor. Ceruloplasmin-derived P2 peptide appeared to be a strong inhibitor of 5-LOX product synthesis under conditions of oxidative stress.

Keywords

Ceruloplasmin; 5-lipoxygenase; myeloperoxidase; superoxide; neutrophil

Abbreviations

PMNL, polymorphonuclear leukocyte; Cp, ceruloplasmin; MPO, myeloperoxidase; LTB₄, leukotriene B₄; 5-LOX, 5-lipoxygenase; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; P1, RPYLKVFNPR; P2, RRPYLKVFNPRR; LPS, lipopolysaccharide; NO, nitric oxide

41 1. Introduction

42 Ceruloplasmin (Cp) is an acute-phase plasma protein with both antioxidant and pro-
43 oxidant activities (Samygina VR et al. 2013). Cp can participate in protein-protein interactions
44 with a number of leukocyte and pro-inflammatory proteins, including myeloperoxidase
45 (MPO), lactoferrin serprocidins, eosinophilic cationic protein and 5-lipoxygenase (5-LOX)
46 (Sokolov et al. 2007; Sokolov et al. 2010). Complexes of Cp with lactoferrin, MPO, and apoB-
47 100-containing lipoproteins were detected in the plasma of patients with inflammatory diseases
48 and atherosclerosis (Sokolov et al. 2014). At the blood level, Cp plays a number of protective
49 and antioxidant roles (Adamsson et al. 2011). For example, Cp reduces the generation of reactive
50 oxygen species (ROS) and nitrogen species (Shiva et al. 2006). Moreover, Cp maintains plasma
51 nitrite (which acts as a substrate pool for nitric oxide (Varfolomeeva et al. 2016) formation) by
52 catalyzing the oxidation of NO to NO⁺ and subsequent hydration to nitrite (Shiva et al. 2006).
53 Endogenous Cp, which usually plays an anti-inflammatory/antioxidant role, if present at
54 increased concentrations, could exacerbate the damaging effects of pro-inflammatory stimuli in
55 the brain by increasing lipopolysaccharide (LPS)-induced NO production in microglial cells
56 (Lazzaro et al. 2014). Finally, Cp acts as a superoxide dismutase (SOD) to affect the production
57 of ROS by neutrophils (Sokolov et al. 2010).

58 ROS, such as superoxide and hydrogen peroxide, are generated by free iron (via Fenton
59 and Haber Weiss-type reactions) and inducible enzymes, such as xanthine and NADPH oxidases
60 (Segal 2005). MPO is known to be activated by hydrogen peroxide (Flemmig and Arnhold
61 2010). MPO undergoes numerous reactions, including halogenation, peroxidase, catalase, and
62 SOD reactions (Arnhold and Flemmig 2010). Ferric (Fe³⁺) MPO reacts with superoxide anion
63 radicals and is able to function as a SOD (Winterbourn et al. 1985; Kettle et al. 2007). Among
64 plasma proteins, Cp shows the strongest effects on inhibition of MPO activity (Govorova et al.
65 1989).

66 Polymorphonuclear leukocytes (PMNLs) can generate toxic oxygen metabolites by
67 accumulation of NADPH oxidase on cell surfaces (Segal 2005). A powerful trigger of NADPH
68 oxidase in leukocytes is phorbol 12-myristate 13-acetate (PMA). Upon phagocytosis, the
69 activation of NADPH oxidase proceeds in the walls of the phagocytic vacuole, where it forms a
70 conduit for electrons to be pumped from NADPH in the cytosol onto oxygen in the vacuole
71 (Segal 2005), facilitating the release of protease enzymes from cytoplasmic granules. Initiation
72 of NADPH oxidase activity coincides with degranulation, initially for azurophilic (or primary)
73 granules that largely contain MPO and proteins directed toward microbial killing and digestion
74 (e.g., cathepsin G, elastase, and proteinase 3).

75 Following the onset of infection, PMNLs mount the first antimicrobial attack via
76 phagocytosis. 5- LOX is activated during the phagocytosis of microorganisms and foreign
77 particles by neutrophils (Williams et al. 1985). Upon cell activation, human neutrophils readily
78 produce leukotriene (LT) B₄. LTs are lipid mediators derived from arachidonic acid via the 5-
79 LOX pathway. In a previous study, we investigated the interaction of Cp with 5-LOX and found
80 that Cp inhibits 5-LOX (Sokolov et al. 2010).

81 Cp has been shown to act as a physiological inhibitor of myeloperoxidase (MPO)
82 (Segelmark et al. 1997). The heme protein MPO is highly specific to neutrophils (Klebanoff
83 2005). MPO is abundantly present in azurophil granules of neutrophils that fuse with
84 phagocytosed bacteria. Moreover, the formation of a specific complex between Cp and MPO has
85 been described (Samygina et al. 2013). In the 2Cp-MPO complex, the MPO heme pocket is
86 shielded by a fragment of Cp (sequence 883–892) (Samygina et al. 2013). A synthetic peptide
87 (RPYLKVFNPR), corresponding to the fragment of Cp at positions 883–892, is a highly
88 efficient inhibitor of MPO and inhibits the peroxidase and chlorinating activities of MPO with
89 half-maximal inhibitory concentrations (IC₅₀ values) of 160 and 780 nM, respectively (Samygina
90 et al. 2013; Sokolov et al. 2015b). MPO-derived oxidants have dramatic effects on the product

91 profiles of human 5-LOX (Zschaler et al. 2015); therefore, targeting of MPO with specific
92 peptides may regulate leukotriene synthesis.

93 In this study, we tested the ability of two synthetic peptides, RPYLKVFNP (883–892)
94 and RRPYLKVFNP (882–893), to modulate superoxide production and leukotriene synthesis
95 in neutrophils.

96

97 **2. Materials and Methods**

98 *2.1 Bacteria and reagents*

99 *Salmonella* Typhimurium strain IE 147 was obtained from the Collection of Gamaleya
100 Research Centre of Epidemiology and Microbiology (Moscow, Russia). Bacteria were grown in
101 Luria-Bertani broth, washed twice using physiological salt solution, and centrifuged at $2000 \times g$.
102 The concentration of the stock suspension was 1×10^9 CFU/mL. The bacteria were opsonized
103 with fresh serum from the same donor whose blood was used to prepare neutrophils. Serum was
104 isolated by centrifuging clotted blood. For opsonization, bacteria were incubated for 30 min in
105 Dulbecco's salt solution containing 5% serum. Hank's balanced salt solution with calcium and
106 magnesium but without Phenol Red and sodium hydrogen carbonate (HBSS), Dulbecco's PBS
107 with magnesium but without calcium, PMA, and cytochrome c (Cyto C) were purchased from
108 Sigma (Steinheim, Germany). Dextran T-500 was from Pharmacosmos (Holbæk, Denmark). In
109 this study, we used synthetic amino acid sequences purified by high-performance liquid
110 chromatography (HPLC; NPF Verta, Russia).

111 The amino acid sequences of the peptides used in this study are presented in Table 1.

112 Table 1. Amino acid sequences of the peptides used in this study.

Abbreviation	Amino acid sequence	Molecular mass (Da)
P1	RPYLKVFNP (883–892)	1290

P2	RRPYLKVFNP RR (882–893)	1600
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113

114 2.2 Ethics statement

115 We prepared neutrophils from the blood of healthy volunteers. Blood was collected via
116 venous puncture, as approved by the Ministry of Public Health Service of the Russian
117 Federation. Experimental and participant consent procedures were approved by the Institutional
118 Ethics Committee of the A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State
119 University.

120 2.3 Human neutrophil isolation

121 We isolated PMNLs from freshly drawn citrate-anticoagulated donor blood using
122 standard techniques (Sud'ina et al. 2001). We prepared leukocyte-rich plasma by sedimentation
123 of red blood cells with 3% dextran T-500 at room temperature. We purified granulocytes by
124 centrifugation of leukocyte-rich plasma through Ficoll-Paque (density: 1.077 g/mL) followed by
125 hypotonic lysis of the remaining red blood cells (RBCs). PMNLs were washed twice with
126 phosphate-buffered saline (PBS), resuspended at 10^7 /mL (purity: 96–97%, viability: 98–99%) in
127 Dulbecco's PBS containing 1 mg/mL glucose (without CaCl_2), and stored at room temperature.

128 2.4 Analysis of arachidonic acid metabolism via the 5-LOX pathway

129 PMNLs (2×10^7 cells) were incubated in 6 mL HBSS/HEPES medium at 37°C with or
130 without tested compounds for 30 min. Thereafter, *Salmonella* Typhimurium was added at 25–
131 50:1 (bacteria:PMNLs) for 20 min. Incubation was terminated by addition of an equal volume of
132 methanol at –20°C. Prostaglandin B₂ served as an internal standard. The samples were stored at –
133 20°C. The denatured cell suspensions were centrifuged, and the supernatants were collected as
134 the water/methanol extracts.

135 2.5 Analysis of 5-LOX products

136 The water/methanol extracts were purified by solid-phase extraction using Sep-Pak C18
137 columns (500 mg; Macherey-Nagel, Dueren, Germany), which were conditioned sequentially
138 with methanol and water. Metabolites of 5-LOX were extracted with 1.5 mL methanol. The
139 samples were evaporated, dissolved in 35 μ L methanol/water (2:1), and analysed by reversed-
140 phase HPLC, as described previously (Viryasova et al. 2014).

141 *2.6 Measurement of superoxide anion production by Cyto c reduction*

142 PMNL incubations were performed in collagen-coated 24-well culture plates. The
143 HBSS/HEPES medium was supplemented with 50 μ M Cyto c, test compounds, 50 U/mL SOD,
144 or no additive before adding the cells. The plates were incubated at 37°C for 30–60 min in a 5%
145 CO₂ incubator. The reactions were then stopped by cooling to 4°C, and Cyto c reduction was
146 measured as the increase in $\Delta 555/531$ (the absorbance at 555 minus the absorbance at 531 nm).
147 Reduction of 10 μ M Cyto c caused an increase in the $\Delta 550/535$ by 0.18 absorbance units.

148 *2.7 Measurement of ROS*

149 Intracellular formation of ROS was monitored by fluorometric measurements of green
150 fluorescence (excitation: 485 nm, emission: 538 nm) after incorporation of carboxy-H₂DCFDA
151 (5 μ M; ThermoFisher Scientific, USA), in accordance with the manufacturer's protocol.

152 *2.8 Statistical analysis*

153 Results are reported as means $\pm$ standard errors of the means (SEMs) or standard
154 deviations (SDs). Analysis of statistical significance was performed by one-way analysis of
155 variance (ANOVA) followed by Holm-Šidák tests for multiple comparisons using GraphPad
156 Prism 6 software. Differences with *P* values of less than 0.05 were considered statistically
157 significant.

158

159 **3. Results and Discussion**

160 *3.1 Priming of PMNLs with PMA elicited the effects of P2 on 5-LOX product formation upon*
161 *treatment with Salmonella Typhimurium*

162 Addition of P1 and P2 to PMNLs did not induce 5-LOX product formation in neutrophils
163 (data not shown). During phagocytosis of bacteria such as *Salmonella* Typhimurium, human
164 neutrophils synthesize 5-LOX metabolites (LTB₄ and its isomers, ω-OH-LTB₄ and 5-HETE)
165 (Golenkina et al. 2011). PMNL incubation with opsonised *Salmonella* Typhimurium at a ratio of
166 bacteria:PMNLs in the range 25–50:1 resulted in the production of 5-LOX metabolites; the data
167 are presented in Fig. 1. Pretreatment of PMNLs with P1 or P2 did not influence the synthesis of
168 5-LOX products in PMNLs exposed to bacteria, except for increased LTB₄ synthesis after
169 treatment with P1 (Fig. 1). Pre-incubation (priming) of cells with 0.5 nM PMA augmented the
170 synthesis of 5-LOX products. At concentrations below 1 nM, PMA is essentially nontoxic for
171 neutrophils (Takei et al. 1996). In the presence of PMA, P2 appeared to exert strong suppressive
172 effects on LT synthesis (Fig. 1).

173
174 *3.2 P2 activated superoxide formation in neutrophils upon their interaction with PMA*

175 PMA stimulates the production of O₂^{•−} by human neutrophils with ED₅₀ concentrations of
176 3.9 ± 2.1 nM (Tauber et al. 1982). P1 and P2 did not induce superoxide formation in neutrophils,
177 as measured by SOD-dependent Cyto c reduction. We stimulated superoxide formation by
178 adding 0.5 nM PMA to PMNLs. In the presence of 20 μM P2, we observed a significant (more
179 than two-fold) increase in PMA-induced superoxide formation (Fig. 2). Application of the
180 superoxide-scavenger SOD (30 U/mL) to the cells completely blocked Cyto c reduction in all
181 samples (data presented for PMA only).

182 Using carboxy-H₂DCFDA as an indicator of oxidative stress in the cell, we observed
183 increased ROS formation in PMNLs stimulated with PMA in the presence of the peptides (Fig.
184 3). Without PMA, P1 and P2 decreased the level of ROS in PMNLs (Fig. 3).

185 Treatment with the peptides and PMA yielded a sharp jump in superoxide release and
186 ROS formation, possibly through the activity of the peptide on the antioxidant systems of
187 neutrophils. Antioxidant systems include SOD, catalase, and glutathione (Lubrano and Balzan
188 2015). P1 is a highly specific inhibitor of MPO (Samygina et al. 2013), which is abundantly
189 expressed in neutrophils, and the observed slight decrease in ROS (Fig. 3) may be connected
190 with this inhibitory activity. When neutrophils ingest bacteria or are exposed to PMA, they
191 produce superoxide. Additionally, ferric (Fe^{3+}) MPO reacts with superoxide anion radicals and is
192 able to function as an SOD (Winterbourn et al. 1985; Kettle et al. 2007). It is possible that
193 inhibition of this activity causes oxidative stress in the cell when the elimination of O_2^- and H_2O_2
194 is blocked (Scheme 1). P2 peptide, which has two arginines at each end (RRPYLKVFNPRR
195 882–893), exhibited even stronger activation of PMA-induced oxidative stress.

196 The effects of P1 and P2 on ROS production differ from the effects of the intact Cp
197 protein. The latter can act as an SOD and inhibit the activities of MPO and 5-LOX, thereby
198 decreasing the generation of ROS and HOCl in PMNLs. As we showed in previous studies,
199 limited proteolysis of Cp affects peptide bonds, particularly between K887 and V888, and
200 prevents inhibition of MPO and 5-LOX (Sokolov et al. 2010; Sokolov et al. 2015a). In this
201 study, we demonstrated that inhibition of MPO by P1 and P2 in the context of PMA-induced
202 oxidative stress resulted in increased ROS production and decreased LT synthesis.

203 The activity of 5-LOX and synthesis of LTs are sensitive to ROS and the ‘peroxide tone’
204 of the cell (Weitzel and Wendel 1993), including MPO-derived oxidants (Zschaler et al. 2015).
205 In our current study, we analyzed the synthesis of LTs by human PMNLs during their interaction
206 with *Salmonella* Typhimurium. As part of the innate immune system, PMNLs kill *Salmonella* in
207 a complement-dependent manner. Priming of PMNLs with PMA enhances LT synthesis induced
208 by bacteria or zymosan particles (Viryasova et al. 2014; Viryasova et al. 2016). In cells
209 challenged with the calcium ionophore A23187, 5-LOX product formation is inhibited by PMA
210 and is not dependent on addition of the MPO inhibitor ABAH (Zschaler and Arnhold 2016). Cp-

211 derived P2 peptide appeared to be a strong inhibitor of 5-LOX product synthesis during the
212 interaction with bacteria *Salmonella* Typhimurium under conditions of oxidative stress.

213 In summary, our results showed that low-molecular-weight derivatives of Cp exhibited
214 high activity in the regulation of neutrophil cellular responses. We treated PMNLs with two
215 ceruloplasmin-derived peptides, RPYLKVFNP (883–892) (P1) and RRPYLKVFNP (882–
216 893) (P2) and found that neutrophil priming with PMA revealed the regulatory roles of the
217 peptides. P1 and P2 increased PMA-induced ROS formation in PMNLs. ROS are primarily
218 produced via NADPH oxidase and play an important role in the killing of microorganisms by
219 neutrophils. Peptide P2 inhibited 5-LOX product synthesis under conditions of oxidative stress.
220 In the inflammatory loci, where excessive activation of neutrophils results in oxidative stress in
221 cells, Cp P2 peptide would be expected to suppress LT synthesis. Thus, because LTB₄ is a
222 chemoattractant for neutrophils themselves, treatment with P2 would block neutrophil
223 accumulation.

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315

316

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Draft

318 Figure Legends

319 Fig. 1. Effects of P1 and P2 on the formation of 5-LOX products in human PMNLs.

320 PMNLs (2×10^7) were incubated for 30 min at 37°C without additives or with 20 μ M P1 or P2
321 plus PMA (0.5 nM), as indicated, and then added to a solution containing $0.5-1 \times 10^9$ *Salmonella*
322 Typhimurium bacterial cells for 20 min. The products of arachidonic acid transformation by 5-
323 LOX were extracted from the medium and separated using HPLC. LTs; the sum of LTB₄, its
324 isomers, and ω -OH-LTB₄. Values indicate means $\pm$ SEMs for six independent experiments
325 performed in duplicate. * $P < 0.05$ compared with the control. # $P < 0.05$ for pairs of data
326 compared as indicated by one-way ANOVA followed by Holm-Šídák multiple comparison tests.

327 Fig. 2. Effects of P1 and P2 on superoxide formation in PMNLs.

328 PMNLs (1×10^6 /mL) were seeded in 24-well plates in HBSS-HEPES medium with 50 μ M cyto
329 c and 20 μ M P1 or P2, with or without 0.5 nM PMA and 30 U/mL superoxide dismutase (SOD).
330 Cells were then incubated at 37°C for 40 min. Cyto c reduction was measured by determining
331 changes in absorbance at 555 and 531 nm. Values indicate the means $\pm$ SDs from six
332 independent experiments performed in duplicate. # $P < 0.05$ for pairs of data compared as
333 indicated by one-way ANOVA followed by Holm-Šídák multiple comparison tests.

334 Fig. 3. Effects of P1 and P2 on ROS formation in PMNLs.

335 PMNLs were treated for 1 h with 5 μ M carboxy-H₂DCFDA, washed, and suspended in
336 HBSS/HEPES medium. Cells (1×10^6 /mL) were seeded in 24-well plates with 20 μ M P1 or P2,
337 with or without 0.5 nM PMA, and incubated at 37°C for 60 min. ROS formation was evaluated
338 by measurement of fluorescence. Values indicate the means $\pm$ SDs from three independent
339 experiments performed in duplicate. ** $P < 0.01$ compared with the control. ## $P < 0.01$ for pairs
340 of data compared as indicated by one-way ANOVA followed by Holm-Šídák multiple
341 comparison tests.

342 Scheme 1. Scheme of P2 peptide activity on MPO-mediated ROS formation.

343

Fig. 1. Effects of P1 and P2 on the formation of 5-LOX products in human PMNLs. PMNLs (2×10^7) were incubated for 30 min at 37°C without additives or with 20 μ M P1 or P2 plus PMA (0.5 nM), as indicated, and then added to a solution containing $0.5-1 \times 10^9$ *Salmonella Typhimurium* bacterial cells for 20 min. The products of arachidonic acid transformation by 5-LOX were extracted from the medium and separated using HPLC. LTs; the sum of LTB₄, its isomers, and ω -OH-LTB₄. Values indicate means $\pm$ SEMs for six independent experiments performed in duplicate. *P < 0.05 compared with the control. #P < 0.05 for pairs of data compared as indicated by one-way ANOVA followed by Holm-Šídák multiple comparison tests.

In the presence of PMA, P2 app
215x145mm (300 x 300 DPI)

Fig. 2. Effects of P1 and P2 on superoxide formation in PMNLs. PMNLs ($1 \times 10^6/\text{mL}$) were seeded in 24-well plates in HBSS-HEPES medium with $50 \mu\text{M}$ cyto c and $20 \mu\text{M}$ P1 or P2, with or without 0.5 nM PMA and 30 U/mL superoxide dismutase (SOD). Cells were then incubated at 37°C for 40 min. Cyto c reduction was measured by determining changes in absorbance at 555 and 531 nm. Values indicate the means $\pm$ SDs from six independent experiments performed in duplicate. # $P < 0.05$ for pairs of data compared as indicated by one-way ANOVA followed by Holm-Šídák multiple comparison tests. superoxide formation (Fig. 2). 111x73mm (300 x 300 DPI)

Fig. 3. Effects of P1 and P2 on ROS formation in PMNLs.

PMNLs were treated for 1 h with 5 μ M carboxy-H₂DCFDA, washed, and suspended in HBSS/HEPES medium. Cells (1×10^6 /mL) were seeded in 24-well plates with 20 μ M P1 or P2, with or without 0.5 nM PMA, and incubated at 37°C for 60 min. ROS formation was evaluated by measurement of fluorescence. Values indicate the means $\pm$ SDs from three independent experiments performed in duplicate. **P < 0.01 compared with the control. ##P < 0.01 for pairs of data compared as indicated by one-way ANOVA followed by Holm-Šídák multiple comparison tests.

Without PMA, P1 and P2 decreases

97x77mm (300 x 300 DPI)

Scheme 1. Scheme of P2 peptide activity on MPO-mediated ROS formation. is blocked (Scheme 1). P2 pept
291x117mm (300 x 300 DPI)