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

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Ceruloplasmin-derived peptide is the strongest regulator of oxidative stress and leukotriene 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
1. Introduction

Ceruloplasmin (Cp) is an acute-phase plasma protein with both antioxidant and pro-oxidant activities (Samygina VR et al. 2013). Cp can participate in protein-protein interactions with a number of leukocyte and pro-inflammatory proteins, including myeloperoxidase (MPO), lactoferrin serprocidins, eosinophilic cationic protein and 5-lipoxygenase (5-LOX) (Sokolov et al. 2007; Sokolov et al. 2010). Complexes of Cp with lactoferrin, MPO, and apoB-100-containing lipoproteins were detected in the plasma of patients with inflammatory diseases and atherosclerosis (Sokolov et al. 2014). At the blood level, Cp plays a number of protective and antioxidant roles (Adamsson et al. 2011). For example, Cp reduces the generation of reactive oxygen species (ROS) and nitrogen species (Shiva et al. 2006). Moreover, Cp maintains plasma nitrite (which acts as a substrate pool for nitric oxide (Varfolomeeva et al. 2016) formation) by catalyzing the oxidation of NO to NO\(^+\) and subsequent hydration to nitrite (Shiva et al. 2006).

Endogenous Cp, which usually plays an anti-inflammatory/antioxidant role, if present at increased concentrations, could exacerbate the damaging effects of pro-inflammatory stimuli in the brain by increasing lipopolysaccharide (LPS)-induced NO production in microglial cells (Lazzaro et al. 2014). Finally, Cp acts as a superoxide dismutase (SOD) to affect the production of ROS by neutrophils (Sokolov et al. 2010).

ROS, such as superoxide and hydrogen peroxide, are generated by free iron (via Fenton and Haber Weiss-type reactions) and inducible enzymes, such as xanthine and NADPH oxidases (Segal 2005). MPO is known to be activated by hydrogen peroxide (Flemmig and Arnhold 2010). MPO undergoes numerous reactions, including halogenation, peroxidase, catalase, and SOD reactions (Arnhold and Flemmig 2010). Ferric (Fe\(^{3+}\)) MPO reacts with superoxide anion radicals and is able to function as a SOD (Winterbourn et al. 1985; Kettle et al. 2007). Among plasma proteins, Cp shows the strongest effects on inhibition of MPO activity (Govorova et al. 1989).
Polymorphonuclear leukocytes (PMNLs) can generate toxic oxygen metabolites by accumulation of NADPH oxidase on cell surfaces (Segal 2005). A powerful trigger of NADPH oxidase in leukocytes is phorbol 12-myristate 13-acetate (PMA). Upon phagocytosis, the activation of NADPH oxidase proceeds in the walls of the phagocytic vacuole, where it forms a conduit for electrons to be pumped from NADPH in the cytosol onto oxygen in the vacuole (Segal 2005), facilitating the release of protease enzymes from cytoplasmic granules. Initiation of NADPH oxidase activity coincides with degranulation, initially for azurophilic (or primary) granules that largely contain MPO and proteins directed toward microbial killing and digestion (e.g., cathepsin G, elastase, and proteinase 3).

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

Cp has been shown to act as a physiological inhibitor of myeloperoxidase (MPO) (Segelmark et al. 1997). The heme protein MPO is highly specific to neutrophils (Klebanoff 2005). MPO is abundantly present in azurophil granules of neutrophils that fuse with phagocytosed bacteria. Moreover, the formation of a specific complex between Cp and MPO has been described (Samygina et al. 2013). In the 2Cp-MPO complex, the MPO heme pocket is shielded by a fragment of Cp (sequence 883–892) (Samygina et al. 2013). A synthetic peptide (RPYLKVFNPR), corresponding to the fragment of Cp at positions 883–892, is a highly efficient inhibitor of MPO and inhibits the peroxidase and chlorinating activities of MPO with half-maximal inhibitory concentrations (IC$_{50}$ values) of 160 and 780 nM, respectively (Samygina et al. 2013; Sokolov et al. 2015b). MPO-derived oxidants have dramatic effects on the product
profiles of human 5-LOX (Zschaler et al. 2015); therefore, targeting of MPO with specific peptides may regulate leukotriene synthesis.

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

2. Materials and Methods

2.1 Bacteria and reagents

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

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

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<th>Abbreviation</th>
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<th>Molecular mass (Da)</th>
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<tr>
<td>P1</td>
<td>RPYLKVFNP (883–892)</td>
<td>1290</td>
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Table 1. Amino acid sequences of the peptides used in this study.
2.2 Ethics statement

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

2.3 Human neutrophil isolation

We isolated PMNLs from freshly drawn citrate-anticoagulated donor blood using standard techniques (Sud'ina et al. 2001). We prepared leukocyte-rich plasma by sedimentation of red blood cells with 3% dextran T-500 at room temperature. We purified granulocytes by centrifugation of leukocyte-rich plasma through Ficoll-Paque (density: 1.077 g/mL) followed by hypotonic lysis of the remaining red blood cells (RBCs). PMNLs were washed twice with phosphate-buffered saline (PBS), resuspended at $10^7$/mL (purity: 96–97%, viability: 98–99%) in Dulbecco’s PBS containing 1 mg/mL glucose (without CaCl$_2$), and stored at room temperature.

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

PMNLs ($2 \times 10^7$ cells) were incubated in 6 mL HBSS/HEPES medium at 37°C with or without tested compounds for 30 min. Thereafter, *Salmonella Typhimurium* was added at 25–50:1 (bacteria:PMNLs) for 20 min. Incubation was terminated by addition of an equal volume of methanol at –20°C. Prostaglandin B$_2$ served as an internal standard. The samples were stored at –20°C. The denatured cell suspensions were centrifuged, and the supernatants were collected as the water/methanol extracts.

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

2.6 Measurement of superoxide anion production by Cyto c reduction

PMNL incubations were performed in collagen-coated 24-well culture plates. The HBSS/HEPES medium was supplemented with 50 µM Cyto c, test compounds, 50 U/mL SOD, or no additive before adding the cells. The plates were incubated at 37°C for 30–60 min in a 5% CO₂ incubator. The reactions were then stopped by cooling to 4°C, and Cyto c reduction was measured as the increase in Δ555/531 (the absorbance at 555 minus the absorbance at 531 nm). Reduction of 10 µM Cyto c caused an increase in the Δ550/535 by 0.18 absorbance units.

2.7 Measurement of ROS

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

2.8 Statistical analysis

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

3. Results and Discussion

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

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

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

PMA stimulates the production of $O_2^-$ by human neutrophils with ED$_{50}$ concentrations of 3.9 ± 2.1 nM (Tauber et al. 1982). P1 and P2 did not induce superoxide formation in neutrophils, as measured by SOD-dependent Cyto c reduction. We stimulated superoxide formation by adding 0.5 nM PMA to PMNLs. In the presence of 20 µM P2, we observed a significant (more than two-fold) increase in PMA-induced superoxide formation (Fig. 2). Application of the superoxide-scavenger SOD (30 U/mL) to the cells completely blocked Cyto c reduction in all samples (data presented for PMA only).

Using carboxy-H2DCFDA as an indicator of oxidative stress in the cell, we observed increased ROS formation in PMNLs stimulated with PMA in the presence of the peptides (Fig. 3). Without PMA, P1 and P2 decreased the level of ROS in PMNLs (Fig. 3).
Treatment with the peptides and PMA yielded a sharp jump in superoxide release and ROS formation, possibly through the activity of the peptide on the antioxidant systems of neutrophils. Antioxidant systems include SOD, catalase, and glutathione (Lubrano and Balzan 2015). P1 is a highly specific inhibitor of MPO (Samygina et al. 2013), which is abundantly expressed in neutrophils, and the observed slight decrease in ROS (Fig. 3) may be connected with this inhibitory activity. When neutrophils ingest bacteria or are exposed to PMA, they produce superoxide. Additionally, ferrie (Fe$^{3+}$) MPO reacts with superoxide anion radicals and is able to function as an SOD (Winterbourn et al. 1985; Kettle et al. 2007). It is possible that inhibition of this activity causes oxidative stress in the cell when the elimination of $O_2^-$ and $H_2O_2$ is blocked (Scheme 1).

P2 peptide, which has two arginines at each end (RRPYLKVFNP RR 882–893), exhibited even stronger activation of PMA-induced oxidative stress. The effects of P1 and P2 on ROS production differ from the effects of the intact Cp protein. The latter can act as an SOD and inhibit the activities of MPO and 5-LOX, thereby decreasing the generation of ROS and HOCl in PMNLs. As we showed in previous studies, limited proteolysis of Cp affects peptide bonds, particularly between K887 and V888, and prevents inhibition of MPO and 5-LOX (Sokolov et al. 2010; Sokolov et al. 2015a). In this study, we demonstrated that inhibition of MPO by P1 and P2 in the context of PMA-induced oxidative stress resulted in increased ROS production and decreased LT synthesis.

The activity of 5-LOX and synthesis of LTs are sensitive to ROS and the ‘peroxide tone’ of the cell (Weitzel and Wendel 1993), including MPO-derived oxidants (Zschaler et al. 2015). In our current study, we analyzed the synthesis of LTs by human PMNLs during their interaction with Salmonella Typhimurium. As part of the innate immune system, PMNLs kill Salmonella in a complement-dependent manner. Priming of PMNLs with PMA enhances LT synthesis induced by bacteria or zymosan particles (Viryasova et al. 2014; Viryasova et al. 2016). In cells challenged with the calcium ionophore A23187, 5-LOX product formation is inhibited by PMA and is not dependent on addition of the MPO inhibitor ABAH (Zschaler and Arnhold 2016).
derived P2 peptide appeared to be a strong inhibitor of 5-LOX product synthesis during the interaction with bacteria *Salmonella* Typhimurium under conditions of oxidative stress.

In summary, our results showed that low-molecular-weight derivatives of Cp exhibited high activity in the regulation of neutrophil cellular responses. We treated PMNLs with two ceruloplasmin-derived peptides, RPYLKVFNPR (883–892) (P1) and RRPYLKVFNPRR (882–893) (P2) and found that neutrophil priming with PMA revealed the regulatory roles of the peptides. P1 and P2 increased PMA-induced ROS formation in PMNLs. ROS are primarily produced via NADPH oxidase and play an important role in the killing of microorganisms by neutrophils. Peptide P2 inhibited 5-LOX product synthesis under conditions of oxidative stress.

In the inflammatory loci, where excessive activation of neutrophils results in oxidative stress in cells, Cp P2 peptide would be expected to suppress LT synthesis. Thus, because LTB4 is a chemoattractant for neutrophils themselves, treatment with P2 would block neutrophil accumulation.

**Acknowledgements**

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**References**


Figure Legends

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 µM P1 or P2 plus PMA (0.5 nM), as indicated, and then added to a solution containing 0.5–1 × 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 ± 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-Šidák multiple comparison tests.

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

PMNLs (1 × 10^6/mL) were seeded in 24-well plates in HBSS-HEPES medium with 50 µM cyto c and 20 µM 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 ± 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-Šidák multiple comparison tests.

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

PMNLs were treated for 1 h with 5 µM carboxy-H2DCFDA, washed, and suspended in HBSS/HEPES medium. Cells (1 × 10^6/mL) were seeded in 24-well plates with 20 µM 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 ± 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-Šidák multiple comparison tests.

Scheme 1. Scheme of P2 peptide activity on MPO-mediated ROS formation.
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Without PMA, P1 and P2 decrease
Scheme 1. Scheme of P2 peptide activity on MPO-mediated ROS formation. P2 peptide activity is blocked (Scheme 1). P2 peptide activity is blocked (Scheme 1).