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Antioxidant Binding of Caeruloplasmin to Myeloperoxidase: Myeloperoxidase is Inhibited, but Oxidase, Peroxidase and Immunoreactive Properties of Caeruloplasmin Remain Intact

Y.S. PARK[a], K. SUZUKI[a], SHARON MUMBY[b,c], N. TANIGUCHI[a] and JOHN M.C. GUTTERIDGE[b,*]

[a]*Department of Biochemistry, Osaka University Medical School, Suita, Osaka 5650871, Japan;* [b]*Oxygen Chemistry Laboratory, Directorate of Anaesthesia and Critical Care, Royal Brompton and Harefield NHS Trust, Sydney Street, London, SW3 6NP, UK;* [c]*National Heart and Lung Institute, Imperial College School of Medicine, London, UK*

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The neutrophil enzyme myeloperoxidase (MPO) purposefully makes hypochlorous acid (HOCl) as part of the cells defence against microbial infections. During cell lysis, however, MPO will be released into the extracellular environment where production of HOCl, a powerful oxidant, will lead to molecular damage. Extracellular MPO binds to the copper-containing protein caeruloplasmin (Cp) and prevents MPO making HOCl. Cp has several important antioxidant functions in extracellular fluids associated with its ability to catalyse oxidation of ferrous ions and to remove peroxides. The binding of MPO to Cp did not inhibit these important extracellular antioxidant activities of Cp, but in so doing it provided additional antioxidant protection against formation of HOCl.

Keywords: Antioxidants, reactive oxygen species, caeruloplasmin, myeloperoxidase, hypochlorous acid, iron

Abbreviations: MPO, myeloperoxidase; Cp, caeruloplasmin; RIS, reactive iron species; HSA, human serum albumin; GSH, glutathione reduced form; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, Thio-bis-(2-nitrobenzoic acid); ROS, reactive oxygen species

INTRODUCTION

Caeruloplasmin (Cp) is the major copper-containing protein of human plasma, and its levels respond to tissue injury through the acute phase response. Cp has several oxidase activities *in vitro*, the most important and physiologically relevant of these is its ability to catalyse the oxidation of ferrous ions to the less reactive ferric state (ferroxidase activity).[1] This latter function is considered an important antioxidant property of Cp *in vivo*.[2] More recently, Kim and colleagues[3] described a thiol-dependent peroxidase activity

*Corresponding author. Royal Brompton and Harefield NHS Trust, Sydney Street, London, SW3 6NP, UK. Fax: ++44 0171 351 8524.

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associated with Cp. Since Cp can remove both hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH) at concentrations of reduced glutathione (GSH) found in certain body fluids,[4] it may have a role in controlling peroxide and iron levels in lung lining fluid.[4]

Segelmark and colleagues[5] recently established that native Cp binds to the neutrophil-derived enzyme myloperoxidase (MPO) under physiological conditions of pH and ionic strength, and that such binding inhibits the peroxidase activity of MPO in a dose-dependent manner.[5] MPO is a major intracellular protein of the neutrophil that can be released by exocytosis or by cell necrosis into the plasma, or other extracellular fluids, during an inflammatory response. In the presence of H_2O_2 and chloride ions (Cl^-) MPO intracellularly catalyses the purposeful formation of the powerful oxidant hypochlorous acid (HOCl). HOCl plays an important role in microbial killing by the immune system. When, however, HOCl is released extracellularly in an uncontrolled way it is a powerfully damaging oxidant.[6]

Regulating and controlling unwanted HOCl formation in extracellular fluids by binding Cp to MPO would be biologically advantageous,[5] provided that the important antioxidant functions of Cp, such as its ferroxidase and peroxidase activities, were not significantly impaired. The present study was undertaken to assess the effect of MPO binding on the antioxidant activities of Cp.

MATERIALS AND METHODS

Cp (human) was obtained from Calbiochem (CA, USA) and the purity of the protein was estimated by sodium dodecyl sulfate–polyacrylmide gel electrophoresis. Human serum albumin (HSA), *o*-dianisidine dihydrochloride, GSH, conalbumin (egg white apotransferrin) were obtained from the Sigma (MO, USA) Chemical company. MPO, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), taurine, trichloroacetic acid (TCA), potassium thiocyanate (KSCN), $FeSO_4 \cdot 7H_2O$, Hepes, 4,4′-diaminodiphenylsulfone (dapsone), sodium hypochlorite (NaHOCl), and catalase were purchased from Wako (Osaka, Japan). Microcon-10 was obtained from Amicon (MA, USA). Radial immunodiffusion plates for Cp immunoreactivity were purchased from Dade Behring (Milton Keynes, UK).

Measurement of MPO Activity as HOCl Formation

Generation of HOCl was determined as taurine–chloramine formation;[7] 1 mM Thio-bis-(2-nitrobenzoic acid) (TNB) was prepared by dissolving 2 mM DTNB in 50 mM phosphate buffer, pH 7.4. The solution of DTNB is titrated to pH 12 with sodium hydroxide to promote its hydrolysis, and after 5 min the pH was brought back to pH 7.4 with hydrochloric acid. Reactions were started by adding the complete MPO system (100 μM H_2O_2, 7 nM MPO, 150 mM NaCl) and sample in buffer containing 10 mM taurine, and incubating at 37°C. Reactions were stopped after 10 min by adding 50 μg/ml catalase, followed by addition of TNB (50 μl, 1 mM). After 5 min the absorbance at 412 nm was determined and the amount of HOCl generated was calculated from a standard curve using aliquots of 10 mM taurine buffer to which known amounts of NaHOCl (A292 nm, $\varepsilon = 350\,M^{-1} \cdot cm^{-1}$) had been added.

Cp Ferroxidase Activity

Ferroxidase activity of Cp was assayed by measuring the oxidation of ferrous ions to the ferric state, at pH 5.5.[8] Ferric ions bind to apotransferrin to produce a pink complex (A460 nm). In this assay, conalbumin (egg-white apotransferrin) was substituted for apotransferrin.

Cp Amine Oxidase Activity

Amine oxidase activity of Cp was determined by using *o*-dianisidine.[9] Acetate buffer 0.75 ml (pH 5.0, 0.1 M) and sample 50 μl were pre-incubated

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for 5 min at 37°C. To start the reaction *o*-dianisidine dihydrochloride (200 µl, 7.9 mM) was added to test tubes 1 and 2. The reaction was stopped after 5 min (tube 1) or 15 min (tube 2) by the addition of 2 ml 9 M sulfuric acid. The absorbance of each tube was measured at 540 nm against a water blank, and the activity of Cp calculated from the change in absorbance using the molar extinction coefficient ($\varepsilon = 9.6\,mM^{-1} \cdot cm^{-1}$) of *o*-dianisidine as described by Lehmann *et al.*[9]

Cp Glutathione Peroxidase Activity

The peroxidase activity of Cp was determined by using the KSCN method.[10] The reaction was started by addition of 500 µM H_2O_2 into 50 µl of reaction mixture containing 500 µM GSH, 50 mM Hepes buffer (pH 7.0), and an appropriate amount of sample and incubated at 37°C. After 40 min the 50 µl reaction mixture was added to 0.8 ml TCA solution to stop the reaction, followed by addition of 200 µl of 10 mM $FeSO_4$ and 100 µl of KSCN to develop a purple colour. Measurement of the remaining H_2O_2 was performed by monitoring the decrease in absorbance at 480 nm. To completely block the peroxidase activity of MPO, dapsone was added to MPO.[11] The incubation mixture contained 0.7 µM MPO, 150 mM NaCl, 5 mM H_2O_2, 10 mM taurine, 0.5 mM dapsone in 50 mM phosphate buffer (pH 7.0). Inactivation of MPO by dapsone was performed for 1 h at 37°C and the reaction solution was immediately exchanged using a microcon-10 with 50 mM Hepes buffer (pH 7.0).

Cp Immunoreactivity

Cp immunoreactivity was determined using radial immunodiffusion plates containing a polyclonal antibody. An equimolar mixture of Cp and MPO was freshly prepared, together with controls using albumin or saline instead of MPO. Samples were incubated at 37°C for 30 min.

A 5 µl sample of each mixture was added to the wells of an immunodiffusion plate. After 48 h at 25°C the zones were measured to quantitate the Cp present.

RESULTS

Effect of Cp on HOCl Production by MPO

To determine whether Cp inhibits HOCl production by MPO, we incubated varying concentrations of Cp with the complete MPO–H_2O_2–Cl^- system. Analysis of HOCl production using the taurine chloramine method revealed that Cp had a dose-dependent inhibitory effect of HOCl production by MPO, whilst HSA as a control showed no inhibitory effect (Figure 1). HOCl is known to react with protein,[12] and hatch bars in (Figure 1) show the concentration-dependent removal of HOCl by protein.

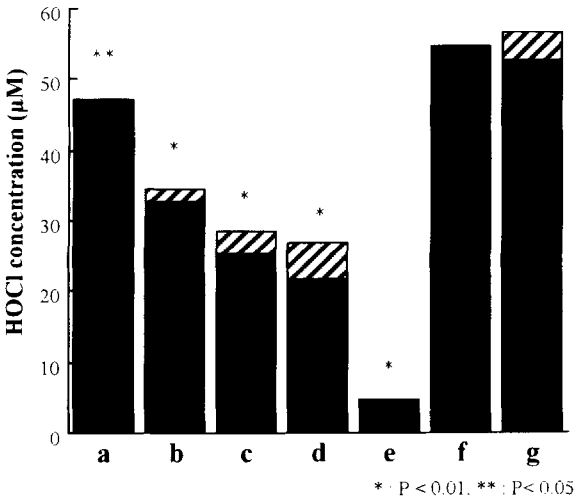

FIGURE 1 Dose-dependent inhibition of MPO HOCl formation by Cp. The experiments are carried out by incubating 1 mM TNB and 10 mM taurine in reaction solution (1 ml) for 10 min at 37°C. Complete MPO system (100 µM H_2O_2, 7 nM MPO, 150 mM NaCl) was incubated with various concentrations of Cp 50 nM (a), 100 nM (b), 250 nM (c), 500 nM (d). Complete MPO system alone (f), (f) + 1 mM methionine as a HOCl scavenger (e) and (f) + 500 nM HSA (g) were used as controls. Hatch bar shows removal of HOCl by non-specific binding to protein. Equal amounts of the above Cp (a–d) and HSA (g) were incubated with 55 µM HOCl, and assayed by the same method. Data are mean ± SD of triplicate experiments.

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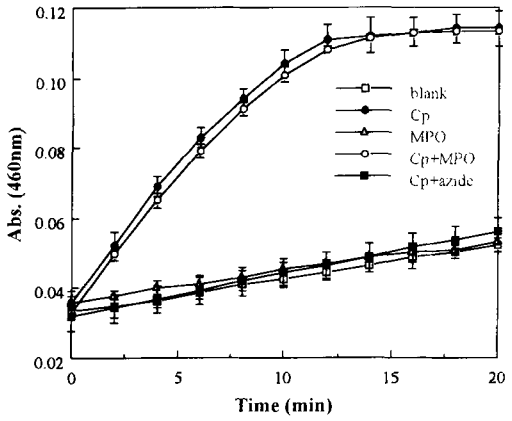

FIGURE 2 Effect of MPO on Cp ferroxidase activity. Reaction solution consisted of 0.17 ml buffer (1 M sodium acetate pH 5.5), 0.27 ml Chelex resin-treated distilled water, 0.25 ml apotransferrin (10 mg/ml), 0.3 ml Fe(II)–ammonium sulfate (0.4 mM), 0.01 ml sample or water for the blank. The reaction was monitored at 460 nm for 20 min at 30°C. The concentration of Cp and MPO were 50 nM and sodium azide, as a ferroxidase inhibitor, was 1 mM. Data are mean ± SD of triplicate experiments.

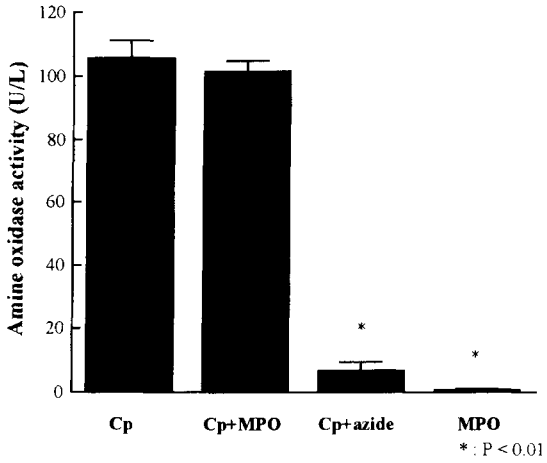

FIGURE 3 Effect of MPO of Cp Amine oxidase activity. The amine oxidase activity was analysed as described in the Materials and Methods section. Reaction solution contained 50 nM Cp, 50 nM MPO or 1 mM sodium azide as an amine oxidase inhibitor. Data are mean ± SD of triplicate experiments.

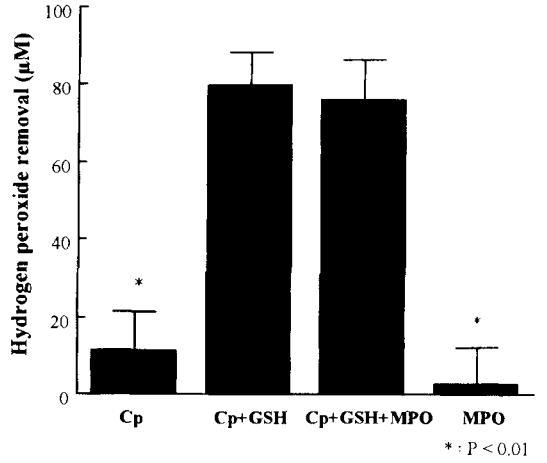

FIGURE 4 Effect of MPO on Cp GSHPase activity. Reaction conditions were described under Materials and Methods. Concentrations in the reaction solution were 1.5 μM Cp (9.6 μg), 500 μM H_2O_2, 500 μM GSH, dapsone treated 1.5 μM MPO (10.5 μg). The amount of hydrogen peroxide removed was calculated from a standard curve using known amounts of H_2O_2. Data are mean ± SD of triplicate experiments.

Effect of MPO on Cp Activity

MPO appeared to have no effect on the ferroxidase activity of Cp (Figure 2). In order to increase binding affinity between Cp and MPO, the samples were preincubated for various times prior to performing the assay. However, we found no significant change in activity (data not shown). We next determined whether the amine oxidase activity of Cp was inhibited by MPO. The activity was assayed by using *o*-dianisidine as the substrate, since p-phenylenediamine, a commonly used substrate was found to interfere with MPO (data not shown). The data show that MPO has no effect on the amine oxidase activity of Cp (Figure 3). When MPO was tested for its effect on the glutathione peroxidase activity of Cp, again, there was no significant inhibition of its ability to remove H_2O_2 (Figure 4).

Effect of MPO on the Immunoreactivity of Cp

The binding of MPO to Cp did not interfere with the immunoreactivity of Cp as observed by the normal development of zones of precipitation (data not shown).

DISCUSSION

Cp binds to MPO and inhibits its peroxidase activity in a dose-dependent way.[5] Cp is an

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anionic protein (pI 4.4), and is likely to bind via electrostatic mechanisms to highly cationic proteins such as MPO.[5] MPO is an abundant protein in neutrophils, and in the presence of H_2O_2 and Cl^- can catalyse formation of reactive oxygen species (ROS) such as HOCl (reviewed in [13]), singlet oxygen ($^{\bullet}O_2$),[14] and tyrosyl radicals (reviewed in [15]) HOCl and $^{\bullet}O_2$ subsequently play important roles in microbial killing by the neutrophil. In addition, HOCl can react with superoxide ($O_2^{\bullet -}$), and with reactive iron species (RIS) to generate further ROS such as hydroxyl radicals ($^{\bullet}OH$).[16] During inflammatory processes, MPO is released into extracellular fluids such as plasma and lung lining fluid[16] where it has the potential to generate unwanted and damaging ROS. Cp is the major copper-containing protein of human plasma which, as an acute phase protein, will be increased during episodes of tissue damage. Cp has oxidase (reviewed in [2]) and peroxidase[3] activities; the ferroxidase,[17] and glutathione peroxidase[4] of which appear to be important as antioxidants in certain tissues and fluids.[4]

Cp, by inactivating extracellular MPO[5] but remaining enzymatically and immunologically active itself, is both inhibiting an enzyme and, at the same time, retaining the ability to remove its substrate. The importance of this dual action of a single protein may reflect the extreme biological hazard of unwanted extracellular HOCl formation.

Acknowledgements

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