Glutathione peroxidase-like activity of caeruloplasmin as an important lung antioxidant

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Abstract The copper-containing plasma protein caeruloplasmin (Cp) has been shown to possess several oxidase activities, but with the exception of its ferrous ion oxidising (ferroxidase) activity which so far appear to be of minor biological relevance. Recently, Kim and colleagues (Kim et al. (1998) FEBS Lett. 431, pp. 473–475) observed that Cp can catalytically remove hydrogen peroxide in the presence of thiols. Here, we show that Cp can remove both hydrogen peroxide and lipid hydroperoxides at physiologically relevant concentrations of reduced glutathione known to be present in lung and lung lining fluid. The glutathione peroxidase-like activity of Cp together with its ferroxidase activity would completely remove the primary reactants required for both Fenton chemistry and lipid peroxidation.

Key words: Caeruloplasmin; Antioxidant; Glutathione peroxidase; Fenton reaction; Lipid peroxidation; Free radical

1. Introduction

The major copper-containing protein of normal human plasma is caeruloplasmin (Cp), an acute-phase protein with several oxidase activities (reviewed in [2]) that is synthesised in the liver, although several other tissues can also make the protein [3–5]. In spite of an intensive research interest in Cp, its biological functions remain an enigma. The protein is able to catalyse the oxidation of ferrous ions to the less reactive ferric state (ferroxidase activity) [6,7]. This observation led us several years ago to propose that Cp functions as an important plasma antioxidant when redox active iron is involved in molecular damage [8–9]. Cp contains six tightly bound copper atoms and one loosely associated with the protein which can be removed by chelation (reviewed in [2]). It has recently been suggested that native Cp carries this seventh copper and that this copper makes Cp pro-oxidant towards the oxidation of low density lipoproteins [10]. Debate concerning the pro-oxidant properties of copper associated with Cp continues, however (reviewed in [11]).

Recently, Kim and colleagues observed that Cp destroys hydrogen peroxide (H_2O_2) when thiols are present [1]. Here, we extend this finding to show that Cp can remove H_2O_2 and lipid hydroperoxides (LOOH) at concentrations of reduced glutathione (GSH) reported as present in lung tissue [12] and lung lining fluid [12–15]. The lung has recently been identified as an important site of Cp synthesis in primates [16] with the protein, subsequently, entering lung lining fluid [16]. Lung lining fluid also contains a mixture of the selenium-containing extracellular glutathione peroxidase (EC-GSHPase) and cellular glutathione peroxidase (C-GSHPase) [17] with the former predominating [17]. Since EC-GSHPase requires GSH in the mM range [18] for its activity, Cp may be the more important protein for removing peroxides in lung lining fluid.

2. Materials and methods

2.1. Materials

Cp, linolenic acid, GSH, conalbumin (egg white apotransferrin) and horseradish peroxidase (HRP) were obtained from Sigma (MO, USA). Myeloperoxidase (MPO), 5,5′-dinitro-bis-(2-nitrobenzoic acid) (DTNB), taurine, trichloroacetic acid (TCA), potassium thiocyanate (KSCN), $FeSO_4{\cdot}7H_2O$, HEPES and sodium hypochlorite (HOCI) were purchased from Wako (Osaka, Japan), Sephadex G25 was from Pharmacia (Uppasla, Sweden) and Amplex Red (*N*-acetyl-3-7-dihydroxyphenoxazine) was obtained from Molecular Probes (OR, USA).

2.2. Purification of human Cp

Cp obtained as a solution (50 mg/ml) was further purified by passing it through a column of Sephadex G25 and eluting with 50 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined with the Bradford method using bovine serum albumin as a standard.

2.3. Preparation of LOOHs

A thin film layer of methyl linolenic acid was formed on the inner surface of a flask by rotation [19]. This was allowed to auto-oxidise in air at 37°C. After 48 h, it was suspended in methanol and used without further purification. The amount of hydroperoxide present was calculated from the UV absorption at 234 nm ($\varepsilon = 27\,400\ M^{-1}\ cm^{-1}$).

2.4. Measurement of thiol-linked peroxidase activities

2.4.1. Hydrogen peroxide. The peroxidase activity of Cp was determined by two methods using KSCN [20] and a fluorescence probe [21]. The reaction was started by addition of 500 μM H_2O_2 into a 50 μl reaction mixture containing 0.5–500 μM GSH, 50 mM HEPES buffer (pH 7.0) and an appropriate amount of sample, for incubation at 37°C. After 40 min, the 40 μl reaction mixture was added to 0.8 ml TCA solution to stop the reaction, followed by addition of 200 μl 10 mM $FeSO_4$ and 100 μl KSCN to develop the purple colour. Measurement of the remaining H_2O_2 was performed by monitoring the decrease in absorbance at 480 nm. A 10 μl residue was assayed by the fluorescence method using an Amplex Red probe. Amplex Red, 10 μM, was incubated with the reaction mixture and 1 U/ml HRP in 50 mM Tris, pH 7.4, at room temperature for 5 min before a 10-fold dilution prior to measurement. The fluorescence intensity of the reaction mixture was measured using a fluorescence microplate reader with a filter set for excitation and emission at 560 and 595 nm, respectively (Biolumin 960, Molecular Dynamic).

2.4.2. LOOHs. The reaction was started by addition of 180 μM LOOH into 50 μl of the reaction mixture containing 0.5–500 μM HEPES buffer (pH 7.0) and an appropriate amount of sample for incubation at 37°C. After 40 min, the remaining hydroperoxide was determined as above using the KSCN method.

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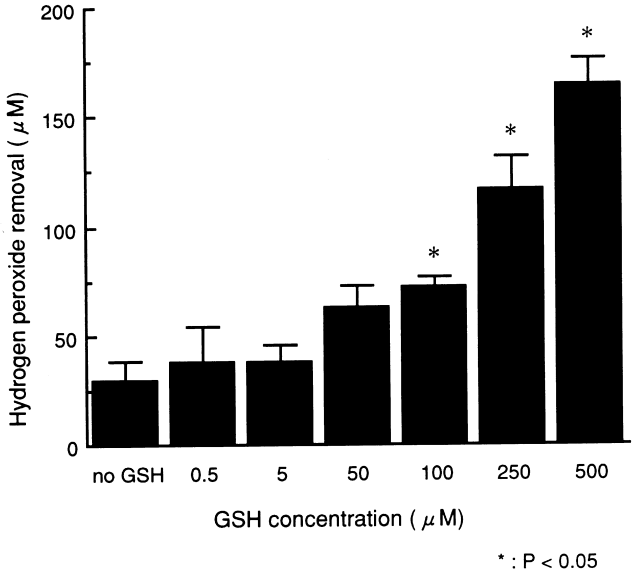

Fig. 1. GSH-dependent removal of H_2O_2 by Cp. 10 μM Cp (65 μg) and 500 μM H_2O_2 were incubated with various GSH concentrations for 40 min. The peroxidase activities were assayed by the KSCN method. The amount of hydrogen peroxide removed was calculated from a standard curve using known amounts of H_2O_2. Data are means ± S.D. of triplicate experiments.

2.5. Measurement of ferroxidase activity of Cp

Ferroxidase activity of Cp was assayed by measuring the oxidation of ferrous ions to the ferric state, at pH 5.5. Ferric ions bind to apotransferrin to produce a pink complex ($A_{460\,nm}$) [22]. In this assay, conalbumin (egg white apotransferrin) was substituted for apotransferrin.

2.6. Measurement of hypochlorous acid formation

Generation of HOCl was determined as taurine-chloramine formation [23]. 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 was titrated to pH 12.0 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 H_2O_2, Cp and NaCl in buffer containing 10 mM taurine and incubated for 10 min at 37°C. TNB was added to the sample solution and incubated for 5 min. The $A_{412\,nm}$ 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 HOCl ($A_{292\,nm}$ $\varepsilon = 350\ M^{-1}\ cm^{-1}$) were added.

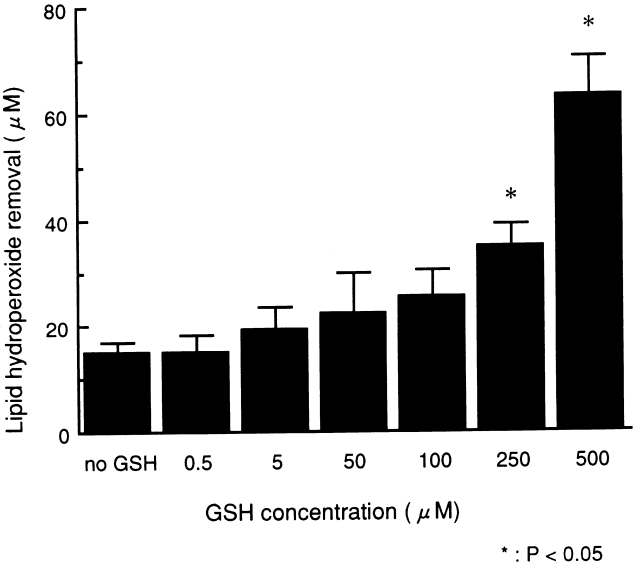

Fig. 2. GSH-dependent removal of LOOH by Cp. 10 μM Cp and 180 μM hydroperoxide were incubated with various GSH concentrations for 40 min. The peroxidase activities were assayed by the KSCN method. The amount of hydroperoxide removed was calculated from a standard curve using known amounts of LOOH that were added. Data are means ± S.D. of triplicate experiments.

3. Results and discussion

Intact human Cp has recently been ascribed a thiol-linked peroxidase activity [1] which we here show can remove H_2O_2 and LOOH at physiologically relevant concentrations of GSH (Figs. 1 and 2) known to be present in lung tissue [12] and lung lining fluid [12–15]. GSH at final reaction concentrations of 50–500 μM supported the peroxidase activities of Cp. Whilst H_2O_2 was being removed, chloride ions enhanced the peroxidase activity as previously observed [1], but made no

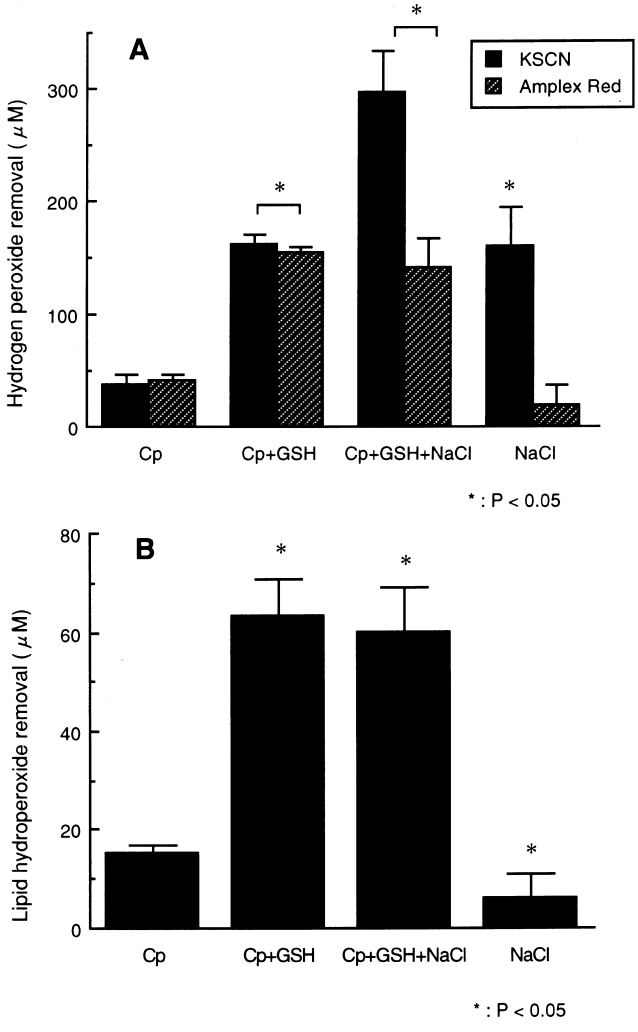

Fig. 3. The effect of chloride ions on peroxide removal (H_2O_2 and LOOH). Reaction conditions were as described under Section 2. (A) H_2O_2: The black bars and hatched bars correspond to the KSCN and the fluorescence assays, respectively. Concentrations in the reaction solution were 10 μM Cp, 500 μM H_2O_2, 500 μM GSH, 150 mM NaCl, respectively. (B) LOOH: Reaction solution contained 10 μM Cp, 180 μM LOOH, 500 μM GSH, 150 mM NaCl, respectively. Data are means ± S.D. of triplicate experiments.

difference to the removal of LOOH (Fig. 3). Chloride enhancement of peroxidase activity was seen only when the KSCN method was employed to measure H_2O_2 (Fig. 3) and did not appear when Amplex Red fluorescence, scopoletin fluorescence and the titanium colourimetric assays were used. It, therefore, appears to be an artefact of the KSCN method. This initial observation led us to consider whether Cp was exerting a MPO-like activity to produce HOCl (Eq. 1). For Cp, however, no evidence for a catalytic role in HOCl production was found (Fig. 4).

$$2H_2O_2 + 2GSH + 2Cl^- \xrightarrow[Cp]{} 2HOCl + GSSG + 2H_2O \quad (1)$$

Normal human plasma contains an EC-GSHPase which can remove H_2O_2 (Eq. 2) and LOOH (Eq. 3) when GSH is present in the low mM concentration range [18].

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad (2)$$

$$LOOH + 2GSH \rightarrow GSSG + LOH + H_2O \quad (3)$$

However, human plasma probably contains less than 1 μM of GSH [24] which would not support the peroxidase activities of either EC-GSHPase or Cp. Very recently, peroxiredoxin IV has been identified as a secretory protein, with glutathione peroxidase-like activity, which acts in the extracellular space [25]. This protein can function at low levels of GSH and may also contribute to peroxide removal [25].

Previous studies [2,8,9] have shown that Cp is a powerful plasma antioxidant when iron-stimulated reactions are involved and this has mainly been ascribed to its ferroxidase activity. Lung lining fluid taken from normal human control subjects was recently found to contain chelatable redox active iron [26] that is likely to participate in Eqs. 4 and 5 leading to the formation of hydroxyl and alkoxyl radicals, respectively.

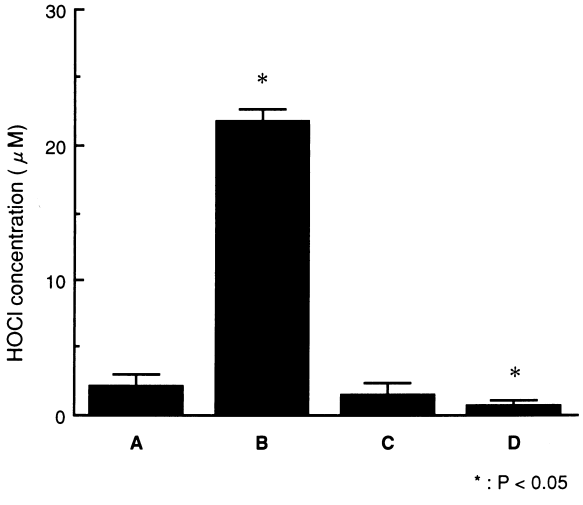

Fig. 4. Formation of HOCl during the thiol-linked peroxidase activity of Cp in the presence of chloride ions. HOCl production was analysed as described in Section 2. Methionine was added as a control to scavenge HOCl. (A) 1 μM Cp (130 μg)+500 μM GSH+50 μM H_2O_2+150 mM NaCl. (B) 1 μg MPO+50 μM H_2O_2+150 mM NaCl. (C) 1 μg MPO+150 mM NaCl. (D) 1 μM PO+50 μM H_2O_2+150 mM NaCl+1 mM methionine. Data are means ± S.D. of triplicate experiments.

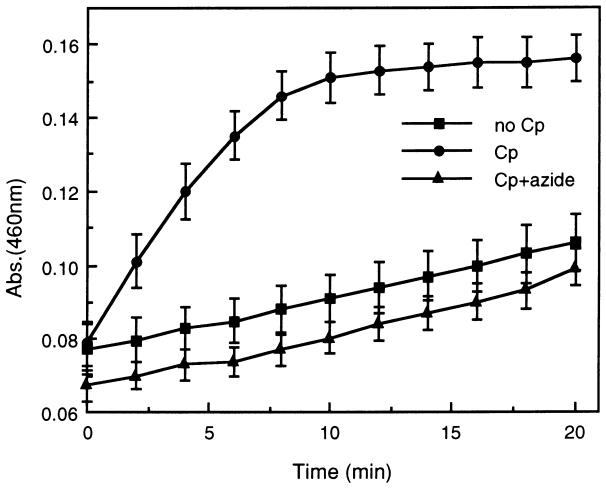

Fig. 5. Ferroxidase activity of Cp. The reaction solution consisted of 0.17 ml buffer (1 M sodium acetate, pH 5.5), 0.27 ml Chelex resin-treated distilled water, 0.25 ml apotransferrin (10 mg/ml), 0.3 ml Fe^{II}-ammonium sulfate (0.4 mM), 0.01 ml sample or water for the blank. The reaction was followed at 460 nm for 20 min at 30°C. The concentration of Cp was 50 nM (6.5 μg/ml) and sodium azide as a ferroxidase inhibitor was 1 mM, respectively. Data are means ± S.D. of triplicate experiments.

The combined glutathione peroxidase (Figs. 1 and 2) and ferroxidase activities (Fig. 5) of Cp would remove the primary reactants required to drive Fenton chemistry (Eq. 4) and hydroperoxide-dependent lipid peroxidation (Eq. 5) and probably other reactions involving a ferrous salt and organic hydroperoxides.

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + {}^{\bullet}OH + OH^- \quad (4)$$

$$Fe^{II} + LOOH \rightarrow Fe^{III} + LO^{\bullet} + OH^- \quad (5)$$

The synthesis of Cp by the lungs would provide a unique antioxidant that deals with the special problems of the lung. Thus, in lung lining fluid interactions between high GSH levels, hydrogen peroxide formation by activated phagocytic cells and low molecular mass iron would be under the control of a single protein expressed in lung tissue.

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