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EFFECTS OF CERULOPLASMIN ON SUPEROXIDE-DEPENDENT IRON RELEASE FROM FERRITIN AND LIPID PEROXIDATION

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Ceruloplasmin (CP) effectively inhibited superoxide and ferritin-dependent peroxidation of phospholipid liposomes, using xanthine oxidase or gamma irradiation of water as sources of superoxide. In addition, CP inhibited superoxide-dependent mobilization of iron from ferritin, suggesting that CP inhibited lipid peroxidation by decreasing the availability of iron from ferritin. CP also exhibited some superoxide scavenging activity as evidenced by its inhibition of superoxide-dependent cytochrome *c* reduction. However, superoxide scavenging by CP did not quantitatively account for its inhibitory effects on iron release. The effects of CP on iron-catalyzed lipid peroxidation in systems containing exogenously added ferrous iron was also investigated. CP exhibited prooxidant and antioxidant effects; CP stimulated at lower concentrations, reached a maximum, and inhibited at higher concentrations. However, the addition of apoferritin inhibited CP and Fe(II)-catalyzed lipid peroxidation at all concentrations of CP. In addition, CP catalyzed the incorporation of Fe(II) into apoferritin. Collectively these data suggest that CP inhibits superoxide and ferritin-dependent lipid peroxidation via its ability to incorporate reductively-mobilized iron into ferritin.

KEY WORDS: Ceruloplasmin, ferritin, iron, superoxide, lipid peroxidation.

INTRODUCTION

Considerable evidence suggests that iron, in the form of low molecular weight complexes, functions as catalysts of lipid peroxidation.[1] The concentration of low molecular weight iron complexes *in vivo* is very low, the majority of iron being associated with various proteins including hemoproteins, ferrodoxins, transferrin, and ferritin. The single most concentrated source of physiological iron is ferritin which can bind up to 4500 atoms of iron per molecule as ferric oxyhydroxide.[2] Release of iron from ferritin requires reduction and the availability of an iron chelator.[3] We have previously shown that superoxide ($O_2^{\bar{\cdot}}$), effectively promoted mobilization of ferritin iron and ferritin-dependent lipid peroxidation[4,5] *in vitro*. Similarly, organic radicals such as the paraquat radical,[6] as well as the anion radicals of adriamycin,[7] nitrofurantoin,[7] and alloxan,[8] are all effective in releasing iron from ferritin. Consistent with the *in vitro* results, we have shown that diquat administration to rats results in the release of iron from hepatic ferritin *in vitro*.[9] Thus, ferritin may represent a toxicologically-relevant source of iron for the catalysis of lipid peroxidation.

Ceruloplasmin (CP, ferro-O_2-oxidoreductase) is a copper-containing ferroxidase that oxidizes Fe(II) to Fe(III) and reduces dioxygen to water without the intermediacy of partially reduced forms of oxygen.[10]

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$$\text{CP-[4Cu(II)]} + 4\ \text{Fe(II)} \rightarrow \text{CP-[4Cu(I)]} + 4\ \text{Fe(III)}$$

$$\text{CP-[4Cu(I)]} + O_2 + 4H^+ \rightarrow \text{CP-[4Cu(II)]} + 2H_2O$$

Boyer and Schori reported that CP catalyzed the incorporation of iron into apoferritin.[11] Ceruloplasmin has also been shown to scavenge O_2^-, but the efficiency is several orders of magnitude less than superoxide dismutase.[12] Thus, we hypothesized that CP may inhibit O_2^- and ferritin-dependent lipid peroxidation by catalyzing the reincorporation of iron into ferritin and its marginal O_2^- scavenging potential.

We report that CP effectively inhibited O_2^- and ferritin-dependent lipid peroxidation and O_2^--dependent iron release from ferritin, activities that were independent of scavenging O_2^-. Evidence is also presented suggesting that the ultimate antioxidant effects of CP are primarily due to the ability of CP to catalyze the incorporation of iron into ferritin.

MATERIALS AND METHODS

Materials

2-Thiobarbituric acid, butylated hydroxytoluene, H_2O_2 (30%), xanthine, cytochrome *c* (horseheart, type III), ascorbic acid, and Sephadex G-25 were purchased from Sigma. Analytic reagent grade ferrous ammonium sulfate and ferrous chloride were purchased from Mallinckrodt (Paris, KY), Chelex 100 (100 mesh) from Bio-Rad (Cincinnati, OH), and bathophenanthroline sulfonate (BPS) from Aldrich. Sephadex G-100 was purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). All reagents were prepared in Chelex 100 treated (chromatographed) 50 mM NaCl which was prepared in highly purified water obtained by reverse osmosis and subsequent passaged through a Synbron/Barnstead NANOpure II system.

Proteins

All enzymes and ferritins were purchased from Sigma. Horse spleen apoferritin was desalted over Sephadex G-25 using Chelex 50 treated mM NaCl. Rat liver ferritin was concentrated to ~ 8 mg protein/ml and incubated with 10 mM EDTA at 4° C for one hour followed by chromatography on Sephadex G-25 (equilibrated with Chelex 100 treated 50 mM NaCl) to remove the EDTA and contaminating iron. Ferritin protein was determined using the bicinchoninic acid micro assay[13] and iron content determined by total iron analysis.[6]

Bovine and human CP were purified as per Reif *et al.*[14] Purified CP preparations exhibited 610 nm to 280 nm absorbance ratios of 0.048–0.052 and could be stored at −20° C in the dark for up to 2 months without loss of enzymatic activity or absorbance at 610 nm. CP concentration was determined spectrophotometrically by absorbance at 610 nm ($E_{610} = 10{,}000\ M^{-1}cm^{-1}$).[10] Apoceruloplasmin was prepared as described by Morell and Scheinberg.[15] Xanthine oxidase was purified by Sephadex G-100 chromatography to remove a protease contaminant and assayed by aerobic reduction of cytochrome *c* ($E_{550} = 28\ mM^{-1}cm^{-1}$).[16] Catalase was desalted over Sephadex G-25, to remove the antioxidant thymol, and activity was measured by the method of Beers and Sizer.[17] Superoxide dismutase activity was assayed as described.[16]

Radiolytic Generation of $O_2^{\overline{\cdot}}$

A cesium-137 self-shielded irradiator (American Nuclear Corp., Casper, WY) was used to generate $O_2^{\overline{\cdot}}$. The dose rate ($0.14\,\mathrm{krad \cdot min^{-1}}$) was determined by Fricke dosimetry.[18] The generation of $O_2^{\overline{\cdot}}$ was accomplished by irradiation in the presence of dioxygen and sodium formate as described previously[5] with a yield of $O_2^{\overline{\cdot}}$ equal to $6.26\,\mu M$ $O_2^{\overline{\cdot}}$/krad.

Preparation of Phospholipid Liposomes

Microsomes were isolated from the livers of 250–276 g male Sprague Dawley rats (Simonsen Labs, Gilroy, CA) as described by Pederson and Aust.[19] Microsomal lipid was extracted from freshly isolated microsomes as per Folch *et al.*[20] All solvents were saturated with argon and all steps performed at 4° C to minimize peroxidation of unsaturated lipids. Lipid was quantitated by phosphate analysis,[21] stored at −20° C in argon-saturated chloroform/methanol (2:1), and used within a month of preparation. Phospholipid liposomes were prepared by indirect, anaerobic sonication.

Lipid Peroxidation Assays

Individual lipid peroxidation systems are described in Figure legends. Peroxidation was assayed by malondialdehyde (MDA) formation using the thiobarbituric acid method[22] with butylated hydroxytoluene (0.03%) included in the thiobarbituric acid reagent.

Measurement of Iron Release from Ferritin

Employing xanthine oxidase as the source of $O_2^{\overline{\cdot}}$, initial rates of iron release from ferritin were measured spectrophotometrically by continuously monitoring the formation of the $\mathrm{Fe(II)\text{-}(BPS)_3}$ chromophore ($E_{530} = 22.14\,\mathrm{mM^{-1}cm^{-1}}$) as described by Samokyszyn *et al.*[23] Ferritin iron release in the irradiation system was determined as described by Reif *et al.*,[5] employing 0.1 mM BPS instead of 1 mM.

RESULTS AND DISCUSSION

As shown in Figure 1, irradiation of rat liver ferritin and phospholipid liposomes resulted in lipid peroxidation as evidenced by an increase in MDA. Lipid peroxidation in this system was absolutely dependent on $O_2^{\overline{\cdot}}$ mediated iron release from ferritin because irradiation of phospholipid alone failed to result in detectable MDA.[5] Bovine CP ($2\,\mu M$) completely inhibited both lipid peroxidation (Figure 1) and iron release from ferritin (Figure 2). Ceruloplasmin has been reported to scavenge $O_2^{\overline{\cdot}}$, and under similar conditions as in Figures 1 and 2, CP ($2\,\mu m$) scavenged 35% of $O_2^{\overline{\cdot}}$ as evidenced by inhibition of cytochrome *c* reduction, but inhibited iron release by 70% (Figure 2).

Similarly, we have also shown that CP effectively inhibited $O_2^{\overline{\cdot}}$ and ferritin-dependent liposomal peroxidation and $O_2^{\overline{\cdot}}$-dependent iron relese from ferritin ($200\,\mu M$ Fe) in systems containing xanthine (0.33 mM) and xanthine oxidase (0.025 U/ml) to generate $O_2^{\overline{\cdot}}$.[23] After 30 minutes incubation, $17.5\,\mu M$ and $0.1\,\mu M$ MDA was measured in the absence and presence of $0.1\,\mu M$ human CP, respectively. Inhibition required a catalytically functional ferroxidase because apoceruloplasmin

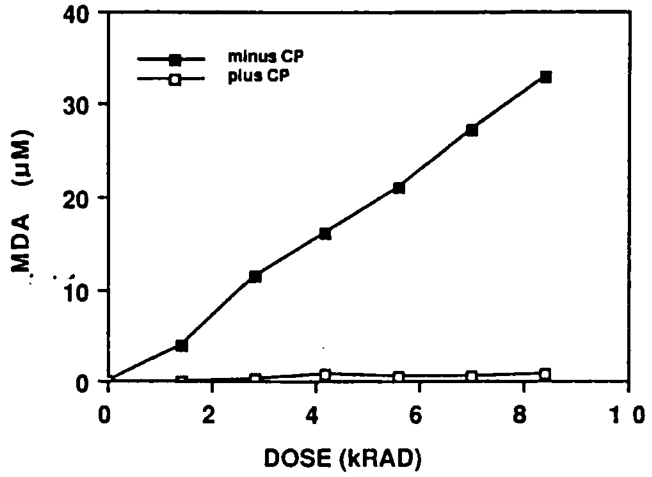

FIGURE 1 Inhibition of ferritin and radiolytically generated superoxide-dependent lipid peroxidation by ceruloplasmin. Reaction mixtures contained phospholipid liposomes (1 µmol lipid phosphate/ml), sodium formate (25 mM), rat liver ferritin (500 mM Fe), and plus or minus bovine CP (2 µM) in 50 mM NaCl (pH 7.0) at ambient temperature and were irradiated for varying lengths of time. Lipid peroxidation was assayed as described in the Experimental Section. Each measurement was performed in duplicate and is representative of repeated trials.

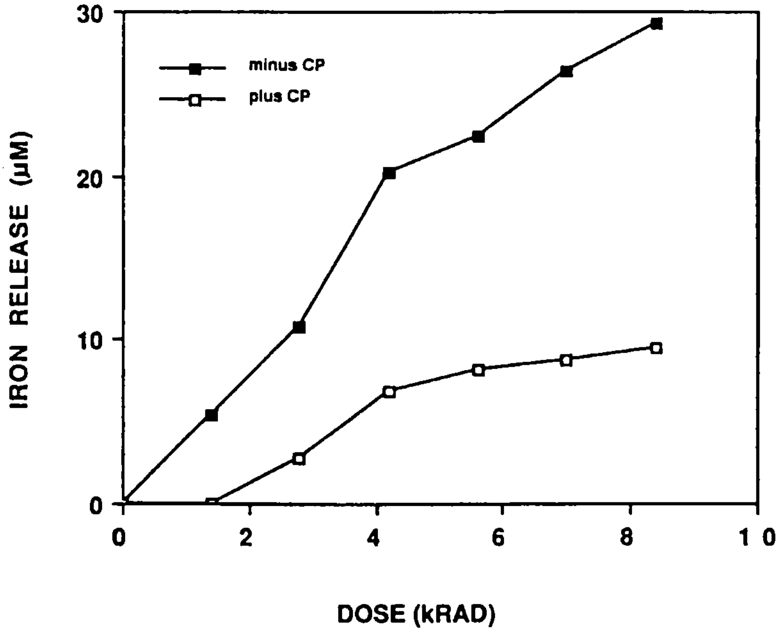

FIGURE 2 Inhibition of radiolytically generated superoxide-dependent iron release from ferritin by ceruloplasmin. Reaction mixtures (2.0 ml starting volume) consisted of sodium formate (25 mM), rat liver ferritin (500 µM Fe), bathophenanthroline sulfonate (100 µM) and plus or minus bovine CP (2 µM) in 50 mM NaCl (pH 7.0) and ambient temperature. Aliquots (0.2 ml) were removed at predetermined time intervals of irradiation, diluted to 1.0 ml, and absorbance of the ferrous bathophenanthroline sulfonate complex was measured at 530 nm ($E = 22.14\,mM^{-1}cm^{-1}$). Each measurement was performed in duplicate and is representative of repeated trials.

TABLE I

Effects of CP, apoCP and SOD on xanthine oxidase-dependent iron release from ferritin and reduction of cytochrome *c*.

System	Iron release[a] (nmol min^{-1} ml^{-1})	Cytochrome *c* reduction[b] (nmol min^{-1} m^{-1})
Control	1.40 ± 0.04	22.1 ± 0.5
+CP (100 nM)	0.16 ± 0.02	21.6 ± 0.3
+apoCP (100 nM)	1.37 ± 0.06	22.1 ± 0.4
+SOD (0.7 U/ml)	0.25 ± 0.01	13.0 ± 0.3

All values represent the mean ± SD of triplicate measurements.

[a] Ferritin (500 µM Fe, 0.4 µM protein), xanthine (0.33 mM), and bathophenanthroline sulfonate (1 mM), were preincubated for 2 min at 37° C in Chelex 100-treated 50 mM NaCl (pH 7.0) in the presence or absence of CP or apoCP (100 nM) followed by the addition of xanthine oxidase (0.02 U/ml). Initial rates of iron release were determined by continuously monitoring the absorbance at 530 nm of the Fe(II)-bathophenanthroline sulfonate chromophore (E_{530} = 22.14 mM^{-1} cm^{-1}).

[b] Cytochrome *c* (0.41 mM) and xanthine (0.33 mM) were preincubated for 2 min at 37° C in the presence or absence of CP or apoCP (100 nM) in Chelex 100-treated 50 mM NaCl (pH 7.0) followed by the addition of xanthine oxidase (0.02 U/ml). Initial rates of cytochrome c reduction were determined by continuously monitoring the absorbance at 550 nm (E_{550} = 28 $\text{mM}^{-1}\text{cm}^{-1}$).

Data from Ref.[23].

failed to inhibit lipid peroxidation. Under similar conditions, CP (0.1 µM) inhibited iron release from ferritin by 90% (Table I). Inhibition of iron release from ferritin by CP was concentration-dependent and, under the conditions employed (Table I), exhibited an IC_{50} of 25 nM.[23] As in the irradiation system, $O_2^{\cdot-}$ scavenging by CP in the xanthine oxidase system could not account for the inhibitory effects of CP on iron mobilization from ferritin. Ceruloplasmin (0.1 µM) scavenged only 2% of xanthine oxidase-derived $O_2^{\cdot-}$, but under similar conditions inhibited iron release by 90% (Table

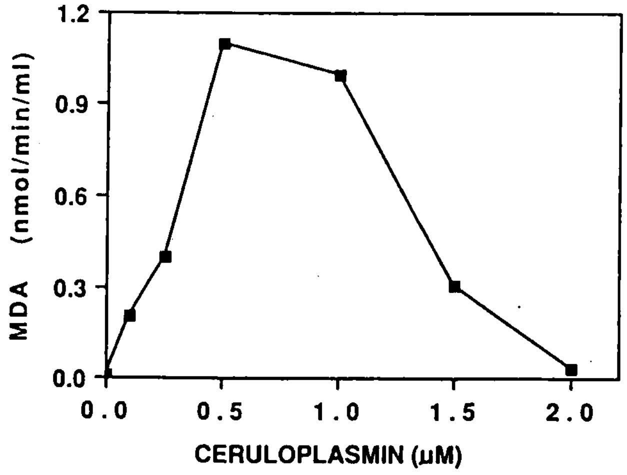

FIGURE 3 Effects of ceruloplasmin on ferrous iron-catalyzed lipid peroxidation. Phospholipid liposomes (1 µmol phosphate/ml) and increasing concentrations of ceruloplasmin were preincubated for 2 min at 37° C in Chelex-treated 50 mM NaCl (pH 7.0) followed by the addition of ferrous chloride (200 µM). Lipid peroxidation was assayed via MDA analysis as described in the Experimental Section, and each data point represents the amount of MDA detected at 25 min after the addition of ferrous chloride. Data from Ref.[23].

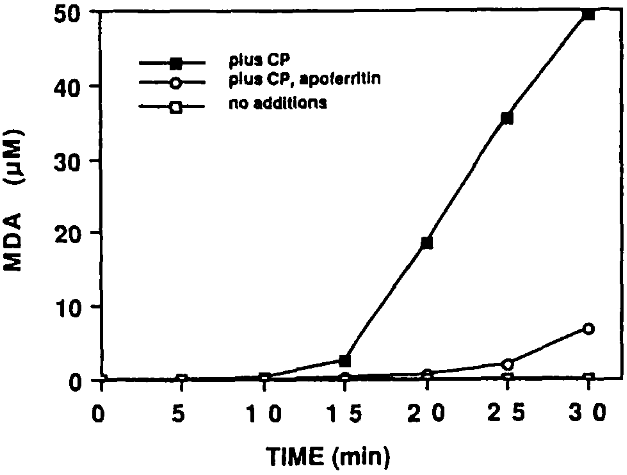

FIGURE 4 Inhibition of ceruloplasmin and ferrous iron-catalyzed lipid peroxidation by apoferritin. Phospholipid liposomes (1 μmol phosphate/ml) were preincubated for 2 min at 37°C in Chelex-treated 50 mM NaCl (pH 7.0) in the presence or absence of CP (0.5 μM) or CP (0.5 μM) plus apoferritin (50 μg/ml). Lipid peroxidation was initiated by the addition of ferrous chloride (200 μM) and assayed via MDA formation employing the thiobarbituric acid assay.

I). In contrast, a concentration of superoxide dismutase that inhibited iron mobilization by 82% inhibited cytochrome c reduction by 41%.

Collectively, these results suggest that CP inhibits O_2^- and ferritin-dependent lipid peroxidation primarily via the ability of CP to catalyze the reincorporation of O_2^--mobilized iron into ferritin. Unfortunately, under the conditions employed, the maximum amount of O_2^--dependent iron release represents less than 1% of the total ferritin iron and we could not accurately determine the degree of iron reincorporation into ferritin by CP. Therefore, we investigated the effects of CP on iron-catalyzed lipid peroxidation in systems containing phospholipid liposomes and added ferrous iron, and subsequently the effects of adding apoferritin. As shown in Figure 3, CP exhibited both prooxidant and antioxidant effects when incubated with ferrous iron and phospholipid liposomes. Stimulation at 0.5 μM CP resulted from partial Fe(II) oxidation whereas inhibition at 2 μM CP resulted from the rapid and complete oxidation of Fe(II). These results are consistent with our previous reports demonstrating that iron-catalyzed lipid peroxidation, in hydroperoxide free systems, are dependent on both Fe(II) and Fe(III).[1] However, as shown in Figure 4, addition of horse spleen apoferritin (50 μg/ml) to mixtures of Fe(II), phospholipid liposomes, and the concentration of CP (0.5 μM) that yielded a maximal rate of lipid peroxidation in Figure 3, inhibited lipid peroxidation.

We have subsequently confirmed the results of Boyer and Schori[11] and demonstrated that CP catalyzes the incorporation of iron into apoferritin.[14,23] Collectively, these results[14,23] and the results presented here support a model that CP inhibits O_2^- and ferritin-dependent lipid peroxidation by catalyzing the incroporation of iron into ferritin. Thus, systems containing CP and ferritin may represent an additional protective mechanism against iron-catalyzed oxidative damage *in vivo*.

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