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Ceruloplasmin (ferroxidase) oxidizes hydroxylamine probes: deceptive implications for free radical detection

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Abstract

Ceruloplasmin (ferroxidase) is a copper-binding protein known to promote Fe2+ oxidation in plasma of mammals. Besides its classical ferroxidase activity, ceruloplasmin is known to catalyze the oxidation of various substrates, such as amines and catechols. Assays based on cyclic hydroxylamine oxidation are used to quantify and detect free radicals in biological samples ex vivo and in vitro. We show here that human ceruloplasmin promotes the oxidation of the cyclic hydroxylamine 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride (CPH) and related probes in Chelex-treated phosphate buffer and rat serum. The reaction is suppressed by the metal chelators DTPA, EDTA and Desferal, while heparin and bathocuproine have no effect. Catalase or SOD additions do not interfere with the CPH-oxidation yield, demonstrating that free radicals are not involved in the CPH oxidation mediated by ceruloplasmin. Plasma samples immunodepleted of ceruloplasmin have lower levels of CPH oxidation, which confirms the role of ceruloplasmin (ferroxidase) as a biological oxidizing agent of cyclic hydroxylamines. In conclusion, we show that the ferroxidase activity of ceruloplasmin is a possible biological source of artifacts in the cyclic hydroxylamine-oxidation assay used for ROS detection and quantification.

Keywords

Ceruloplasmin; Free radicals; Electron Spin and Paramagnetic Resonance; Spin probes

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) are involved in physiological and pathological responses of living organisms [1]; various strategies have been employed in attempts to detect and quantify these species [2–4]. However, this task has been shown to be far from simple because of the low steady-state concentrations and short half-lives of free radicals *in vivo* [5].

Electron paramagnetic resonance (EPR) is the primary and most unambiguous technique for detection of free radicals, although it has low sensitivity relative to the reactivity of most free radicals. Recently, diamagnetic cyclic hydroxylamines, which can be oxidized by ROS to paramagnetic nitroxides, have been proposed as probes suitable to quantify free radicals [3]. It is known that this approach lacks specificity regarding the oxidant/free radical that oxidizes the probe and the chemical identity of the possible free radical [4]; thus, additional experiments must be carried out to validate these measurements [3]. One typical example is the use of the cyclic hydroxylamine oxidation assay in cellular systems *in vitro* for superoxide detection. Those studies use the total nitroxide formation in the presence of DTPA minus that in samples containing SOD to estimate specifically the level of superoxide production [6–8].

Although the use of spectroscopic probes seems to be a simple means for the detection and quantification of ROS in cellular systems, there are other inherent limitations of this methodology and many sources of artifacts, such as: (i) accessibility and adequate concentration of the probes at cellular sites of ROS production; (ii) perturbation of the system studied by the probes; (iii) production of ROS by the probes themselves; (iv) low stability of some probes in the intracellular *milieu* and/or products formed that may be additionally metabolized in cellular systems; and (v) undesired probe reactions in complex biological systems. A point of considerable concern is the potential reduction or oxidation of spectroscopic probes by redox-active components of biological systems without ROS involvement [4].

Ceruloplasmin (ferroxidase, EC 1.16.3.1) is a blue-copper plasma glycoprotein that binds up to 7 copper atoms. This protein is a member of the multicopper oxidase family, such as the well-known laccase and ascorbate oxidase. Although it exhibits structural homology to the copper oxidases, it was first described because it binds approximately 95% of the total circulating copper and is strongly related to metal homeostasis of iron and copper [9]. Among the different functions of ceruloplasmin [10], the most important *in vivo* is its ferroxidase activity that promotes Fe^{2+} one-electron oxidation coupled to the reduction of molecular oxygen directly to water [10, 11]. This activity is known to be fundamental for iron incorporation into transferrin and, therefore, for iron homeostasis [9]. Patients diagnosed with aceruloplasminemia, a rare genetic disease that is characterized by a 50% reduction of ceruloplasmin levels in plasma, have higher intracellular-hepatic levels of iron and generally develop neurological degenerative diseases due to iron deprivation in the nervous-system cells [9, 12]. It is generally known that ceruloplasmin also exhibits other oxidase activities directed to a wide range of substrates such as aromatic amines, phenols and catecholamines [13–15]. Enzymatic competition kinetic experiments [14, 15] and recent X-ray crystallographic studies [16] showed that there are different binding sites in ceruloplasmin for different categories of compounds.

Ceruloplasmin is a positive acute-phase protein, which means that its level in plasma is elevated in disorders accompanied by inflammation [17, 18]. Higher serum levels of ceruloplasmin are associated with different pathological and physiological states (Table 1), such as during viral infections [19], rheumatoid arthritis [20], alcoholic liver steatosis [21]

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and nonalcoholic steatohepatitis [22], cardiovascular diseases [23–28], cancer [29–31], diabetes type 1 and 2 [32–34], other diseases [35–37] and pregnancy [38]. It is noteworthy that the pathological conditions are associated with higher ROS formation [39–45].

In this work, we have investigated the ability of ceruloplasmin (ferroxidase) to catalyze the oxidation of cyclic hydroxylamine probes, with the aim of unmasking possible artifacts occurring during ROS measurements using this methodology. Here we show that ceruloplasmin (ferroxidase) mediates the CPH-nitroxide formation in serum or Chelex-treated phosphate buffer samples in a non-free radical-mediated process through the recycling of contaminant trace iron present ubiquitously in the solutions; thus, we reveal a possible relevant source of artifacts in the hydroxylamine-oxidation assays used for ROS detection.

MATERIALS AND METHODS

Chemicals

The spin probes 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride (CPH), 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CMH), 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine hydrochloride (PPH), 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H), 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride hydrochloride (CAT1-H), 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine hydrochloride (TMH), and N-(1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide hydrochloride (TMTH) were obtained from Alexis Biochemicals (San Francisco, CA, USA). Human ceruloplasmin (EC 1.16.3.1), diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate salt (Desferal), ethylenediaminetetraacetic acid (EDTA), heparin sodium salt, bathocuproine disulfonic acid, ammonium iron(II) sulfate, and *o*-dianisidine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catalase from beef liver, 65,000 U/mg crystalline suspension in water (EC 1.11.1.6) and SOD from bovine erythrocytes, 5000 U/mg lyophilized (EC 1.15.1.1), were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Polyclonal antibody antihuman ceruloplasmin, affinity purified, from goat was obtained from Bethyl Laboratories (Montgomery, TX, USA). A coimmuniprecipitation kit and polyacrylamide desalting columns were obtained from Pierce®;, Thermo Scientific (Rockford, IL, USA). Chelex 100 resin was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All the reactions were carried out in 100 mM sodium phosphate buffer, pH 7.4. The buffer was treated with Chelex 100 resin to diminish the concentration of transition metal ions and thereby minimize trace-metal interference.

Preparation of CPH, CMH, PPH, TEMPONE-H, CAT1-H, TMH, TMTH stock solutions

Stock solutions of CPH, CMH, PPH, TEMPONE-H, CAT1-H, TMH, TMTH (2 mM) in Chelex-treated 100 mM phosphate buffer, pH 7.4, were prepared daily shortly before use.

Animals

CD male rats weighing 200–250 g (Charles River Breeding Laboratories, Raleigh, NC, USA) were used. Rats were housed in a room with air conditioning and a 12/12h light/dark cycle, fed a standard rat chow (NIH open formula, Ziegler Brothers, Gardner, PA, USA), and had access to water *ad libitum*. All studies were approved by the institutional review board and adhered to NIH guidelines for the care and handling of experimental animals.

Ex vivo studies with rat serum samples

Animals were deep-anesthetized using pentobarbital injected ip (50–75 mg/kg). After confirmation of a deep anesthetic state, the heart was exposed and blood was drawn with a

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needle and syringe. The blood was allowed to clot in silicone-coated vacutainers for 30 min at 37°C followed by 3h at 4°C. Vacutainers were centrifuged (2000 rpm for 10 min at 4°C) and the serum was collected and frozen for later analysis.

Measurement of rat plasma ceruloplasmin levels

Plasma activity of ceruloplasmin was measured by the method of Ravin [46] modified by Schosinsky *et al.* [47]. Briefly, ceruloplasmin activity was measured using the difference in the absorbance of the oxidized *o*-dianisidine in samples incubated for 5 and 20 min (ϵ_{540nm} = 9.6 $mM^{-1}cm^{-1}$, after acid addition).

Ceruloplasmin serum immunodepletion

Serum samples were depleted of ceruloplasmin using a polyclonal antibody antihuman ceruloplasmin, affinity-purified, produced in goats (Bethyl Laboratories, Montgomery, TX, USA) and a commercial kit for coimmunoprecipitation according to the instructions of the manufacturer (Pierce®, Thermo Scientific, Rockford, IL, USA). Briefly, immunoaffinity columns for ceruloplasmin were prepared by covalent immobilization of the purified antibody onto a funtionalized-agarose support (AminoLink® Plus). Control samples were prepared in parallel using a control-agarose resin provided in the kit. Columns prepared with 50 μL of resin were incubated for 24h with 200 μL of serum diluted 1:1 (V/V) with water. The flow-through was collected by centrifugation (2000 g for 30 s) and used for ceruloplasmin activity measurements and the cyclic hydroxylamine oxidation assay.

Electron paramagnetic resonance experiments

EPR spectra were recorded on a Bruker EMX EPR spectrometer equipped with an ER 4122 SHQ cavity (Billerica, MA, USA). The following settings were used: microwave power, 20 mW; modulation amplitude, 1 G; magnetic field modulation, 100 kHz; conversion time, 163.84 ms; and time constant, 40.96 ms. Relative EPR signal intensity was measured from the first integral area of the first line of the recorded nitroxide spectra. All experiments were incubated for 10 min at 25°C.

Statistical analysis

All experiments were carried out in duplicate or triplicate each day and repeated twice on different days. Data were means ± SEM. Data were analyzed using a Student's *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

Samples containing ceruloplasmin (1 μM) and the hydroxylamine CPH (10 μM, 50 μM or 1 mM) produced EPR signals (Fig. 1D, F, H) in a CPH concentration-dependent manner. Control studies demonstrated that a much weaker signal was observed in the absence of ceruloplasmin (Fig. 1C, E, G). The signal observed when no ceruloplasmin was added to the incubation mixture was probably the result of trace quantities of CPH nitroxide present in the stock solution or due to the oxidation of the probe by trace metals still present in the Chelex-treated buffer. In fact, autoxidation of the hydroxylamine stock solution prepared in Chelex-treated buffer and incubated in ice resulted in an increase of 10 ± 2% in the area of the nitroxide every 3h. All the spectra shown in all the figures were recorded using the same probe solution, and samples were prepared in time to have all the samples scanned in less than 2h. Because spectra with good signal-to-noise were obtained with 1 mM CPH, the investigation was pursued using this concentration [48, 49].

In the literature, the use of CPH as a spin probe for free radical quantification is most often used with plasma and blood samples. The possible interference of heparin and EDTA, the

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most common additives used for plasma collection, were evaluated in samples of CPH (1 mM) and ceruloplasmin (1 μM) (Fig. 2). The ceruloplasmin-dependent signal was not affected by heparin addition (Fig. 2B), in contrast to the effect of EDTA which inhibited the reaction (Fig. 2C). In the absence of ceruloplasmin, neither heparin (Fig. 2E) nor EDTA (Fig. 2F) had any effect.

Besides EDTA, other metal chelators, DTPA and Desferal, significantly inhibited the CPH-nitroxide formation mediated by ceruloplasmin (Fig. 3) but, again, had no effect on CPH nitroxide formation in samples with CPH alone. The same behavior was observed for the other cyclic hydroxylamines tested: CMH, PPH, TEMPONE-H, CAT1-H, TMH and TMTH (1 mM) (data not shown).

The possible contamination of copper in our ceruloplasmin solution was investigated using bathocuproine, a chelator with high affinity for Cu(I) [50]. We also prepared a desalted solution of ceruloplasmin to investigate the possible interference of unbound and loosely bound copper, or loosely bound iron, in the ceruloplasmin used. Reactions carried out with the desalted ceruloplasmin or prepared with bathocuproine showed no inhibition in ceruloplasmin-dependent CPH-nitroxide formation, in contrast to the effect of DTPA addition (Fig. 4).

The effect of iron in the ceruloplasmin (ferroxidase)-catalyzed oxidation of CPH was studied in samples with and without DTPA and bathocuproine (Fig. 5). This metal induced significantly higher levels of CPH-nitroxide formation in samples without ceruloplasmin (4.5 times higher than the control), but this effect was significantly higher in the presence of the ferroxidase (8.5 times compared to the control). The ceruloplasmin-dependent enhancement of the iron-mediated CPH oxidation was completely blocked by DTPA (200 μM), in contrast to the modest effect of bathocuproine (200 μM). Indeed, this chelator is well-known for its high affinity to Cu(I), but has a weak complexation with different metals, such as iron [50, 51].

Samples prepared with serum showed the same behavior as samples prepared with Chelex-treated buffer, in which CPH oxidation was increased by ceruloplasmin (0.25–4 μM, Supp. Fig. 1). As in Chelex-treated phosphate buffer, the increased CPH-nitroxide formation in serum samples with added ceruloplasmin was blocked by the metal chelator DTPA (200 μM) (Fig. 6A, lines b and d). At the same time, it is obvious that serum samples with added ceruloplasmin had smaller signals than samples prepared with Chelex-treated buffer (Fig 6A lines a and c, Supp. Fig. 1), perhaps due to lower concentrations of nonchelated trace metals present in the serum.

Catalase (650 U/mL) and/or SOD (50 U/mL) did not affect the formation of CPH nitroxide mediated by ceruloplasmin in Chelex-treated buffer or rat serum (Supp. Fig. 2). It proves that neither H_2O_2 nor superoxide anion is involved in the oxidation of CPH mediated by ceruloplasmin.

As already mentioned, the basal level for CPH oxidation (samples without ceruloplasmin addition) was not changed by the addition of metal chelators in the samples prepared in Chelex-treated buffer (Fig. 6B, lines a and b), but it was indeed inhibited in samples of serum in which ceruloplasmin was already present (Fig. 6B, lines c and d) and is consistent with CPH oxidation by the endogenous ferroxidase.

Samples prepared with Chelex-treated buffer or rat serum were compared for the effect of DTPA and bathocuproine on ceruloplasmin-mediated CPH-nitroxide formation (Fig. 7). As expected from the previous experiments, bathocuproine did not inhibit cyclic hydroxylamine oxidation in samples prepared with rat serum or Chelex-treated buffer. Ceruloplasmin-

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mediated CPH-nitroxide formation in samples prepared with Chelex-treated buffer or rat serum was totally blocked by DTPA.

To further demonstrate the role of ceruloplasmin in the *ex vivo* oxidation of cyclic hydroxylamines, we prepared serum samples that were immunodepleted of ceruloplasmin (Fig. 8). The ceruloplasmin immunodepletion procedure resulted in a reduction of 69 ± 10% in the ceruloplasmin serum activity (n = 6) (Fig. 8B). CPH nitroxide formation in the ceruloplasmin-immunodepleted samples was significantly lower by 43 ± 9% (Fig. 8A, C).

DISCUSSION

ROS are known to be involved in various pathologies [39–45], and the majority of these disorders have an inflammatory basis that is known to be associated with high ceruloplasmin levels in serum (Table 1). Here we investigated the hypothesis that ceruloplasmin, a major copper-carrying protein and a ferroxidase enzyme, might be able to oxidize cyclic hydroxylamine probes, leading to possible misinterpretation of *in vivo* free radical formation.

Among the cyclic hydroxylamines, 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride (CPH) is easily the most popular probe for *in vivo* and *in vitro* free radical quantification [3, 48, 49, 52–55]. In fact, our *in vitro* studies showed increasing EPR signals in the presence of increasing concentrations of CPH and ceruloplasmin. The weaker signal observed when no ceruloplasmin was present in the system is not surprising, because it is known that even trace amounts of transition metal ions are able to catalyze the oxidation of hydroxylamines [56].

We found that H_2O_2 and superoxide anion are not involved in the CPH oxidation mediated by ceruloplasmin in Chelex-treated buffer or serum. It has been shown that the oxidation of different ceruloplasmin substrates, such as 6-hydroxydopamine [57] and iron [15], is coupled to the direct reduction of O_2 to water.

Ceruloplasmin (ferroxidase) is unique among the multicopper oxidases because it contains three Type 1 copper sites, as well as a Type 2 copper and a Type 3 copper pair in close proximity that can constitute a 'trinuclear' copper cluster [58]. It is supposed that substrates are oxidized at the first center, a reaction mediated by a one-electron reduction of Cu(II). The electron is then transferred to the closely associated tricluster, which coordinates oxygen until enough electrons are present to reduce the oxygen to water [59, 60]. In contrast to other multicopper oxidases, for which the canonical substrate is well established, ceruloplasmin has the peculiar ability to utilize a number of structurally unrelated molecules as direct electron donors, including Fe(II), aromatic amines, catechols [61], and even nitric oxide [62, 63]. Employing partial removal of copper from ceruloplasmin, Musci *et al.* [64] showed that the three ceruloplasmin sites may be functionally active independently.

However, our results show that the CPH oxidation by ceruloplasmin is inhibited by different metal chelators (DTPA, EDTA and Desferal), demonstrating the involvement of metals in the hydroxylamine oxidation mediated by ceruloplasmin (ferroxidase). Since addition of the Cu(I) chelator bathocuproine or the use of a desalted ceruloplasmin solution did not cause inhibition in CPH-nitroxide formation in samples prepared in Chelex-treated buffer or rat serum, we conclude that our ceruloplasmin solution is essentially free of unbound or loosely bound copper. Therefore, we propose that CPH-nitroxide formation is increased through ferroxidase activity by catalyzing the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), which ultimately oxidizes the hydroxylamine to its nitroxide (Scheme 1). Furthermore, iron addition to samples containing ceruloplasmin showed higher CPH-nitroxide yields that could be totally blocked by DTPA but not by bathocuproine. These results are corroborated

by previous data of Curzon *et al.* [65] and McDermott *et al.* [15], who reported ceruloplasmin acting as a nonspecific oxidase towards various phenols, catechols, and biogenic amines.

Serum samples that were submitted to an immunodepletion procedure for ceruloplasmin showed significantly lower CPH-nitroxide formation than control samples, indicating that ceruloplasmin is one species in the serum responsible for oxidizing the hydroxylamine. This result is also supported by the inhibition of the CPH-nitroxide formation in neat serum samples with added DTPA.

Blood collection for plasma separation involves the addition of additives, such as heparin or the metal chelator EDTA. This transition metal chelator inhibited CPH oxidation, but the addition of heparin to samples with ceruloplasmin did not interfere with the CPH-nitroxide yield.

In agreement with our conclusions, previous work by Erel and co-workers [66, 67] showed that the ferroxidase activity of ceruloplasmin is highly correlated to the oxidation of alchilamine, a chromogenic amine compound used in the commercially available oxidative stress assay “d-ROM test”.

The cyclic hydroxylamine-oxidation approach to measure free radicals in serum has inherent limitations. In serum samples, ceruloplasmin (ferroxidase) can contribute to the oxidation of CPH in a non-free radical-mediated process. Since many physiological and pathological states are associated with increased serum levels of ceruloplasmin, such as those listed in Table 1, our results call into question a significant body of work where increases in the hydroxylamine-oxidation yield in the serum of patients has been interpreted as an increase in the ROS formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LIST OF ABBREVIATIONS

ROS	Reactive Oxygen Species
EPR	Electron Spin and Paramagnetic Resonance
CPH	1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride
CMH	1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride
PPH	1-Hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine hydrochloride
TEMPONE-H	1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride
CAT1-H	1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride hydrochloride

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TMH	1-Hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine hydrochloride
TMTH	N-(1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide hydrochloride
DTPA	Diethylenetriaminepentaacetic acid
Desferal	Deferoxamine mesylate salt
EDTA	Ethylenediaminetetraacetic acid

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HIGHLIGHTS

Artifactual oxidation of hydroxylamines mediated by ceruloplasmin

Trace metals are involved in the hydroxylamine oxidation mediated by ceruloplasmin

Ceruloplasmin in serum is a possible source of artifactual hydroxylamine oxidation

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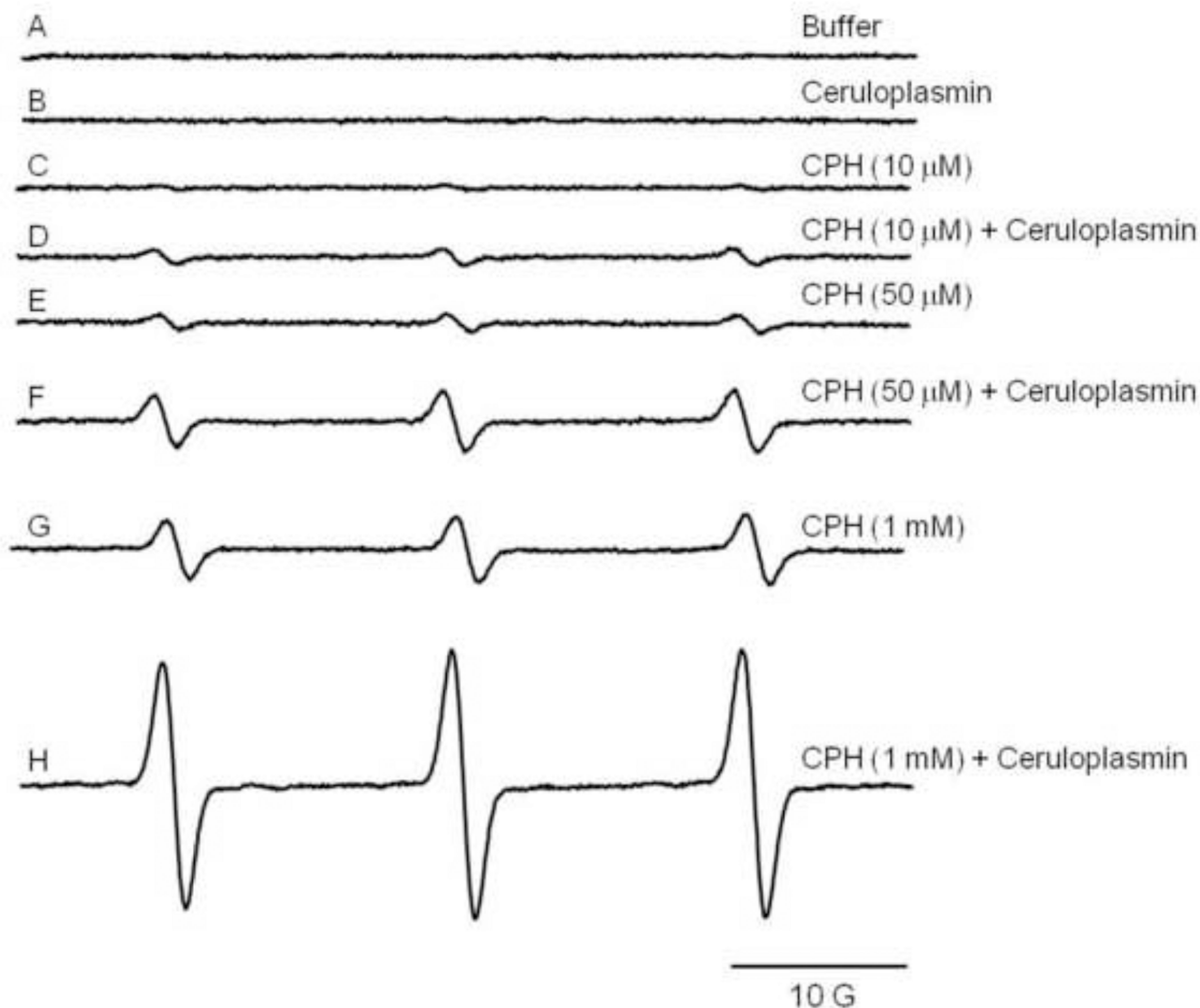

Figure 1.
Ceruloplasmin catalyzes CPH-nitroxide formation in Chelex-treated phosphate buffer. Shown are representative EPR spectra of samples containing (A) buffer, (B) 1 μM ceruloplasmin, (C) 10 μM CPH and (D) in the presence of 1 μM ceruloplasmin, (E) 50 μM CPH and (F) in the presence of 1 μM ceruloplasmin, (G) 1 mM CPH and (H) in the presence of 1 μM ceruloplasmin.

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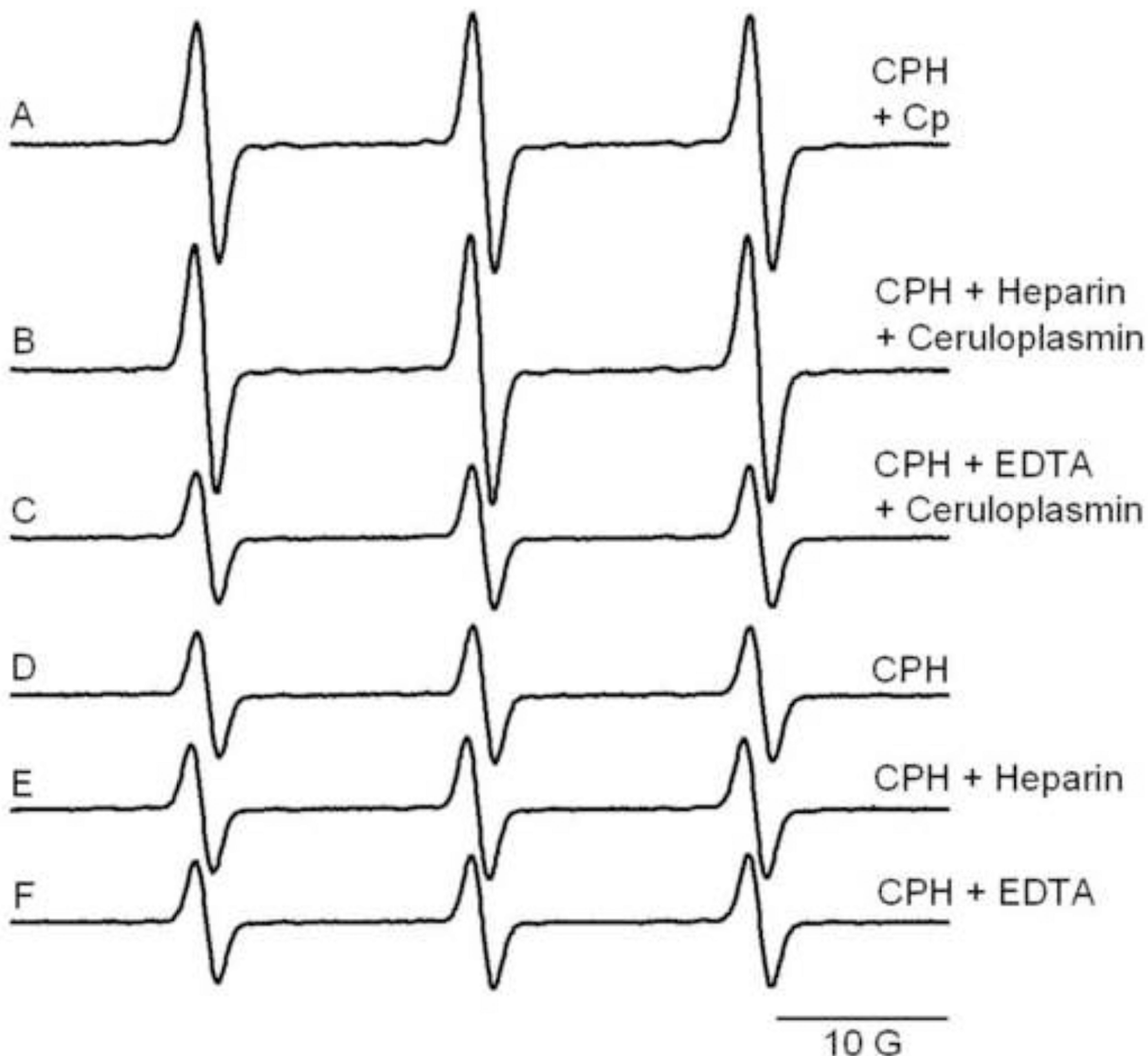

Figure 2.
Effect of heparin and EDTA on CPH-nitroxide formation catalyzed by ceruloplasmin in samples prepared with Chelex-treated phosphate buffer. Representative EPR spectra of samples containing (A) 1 mM CPH and 1 μM ceruloplasmin, and in the presence of (B) Heparin (50 U/mL) or (C) EDTA (200 μM) are shown. Control samples in the absence of ceruloplasmin (D, E and F) are shown.

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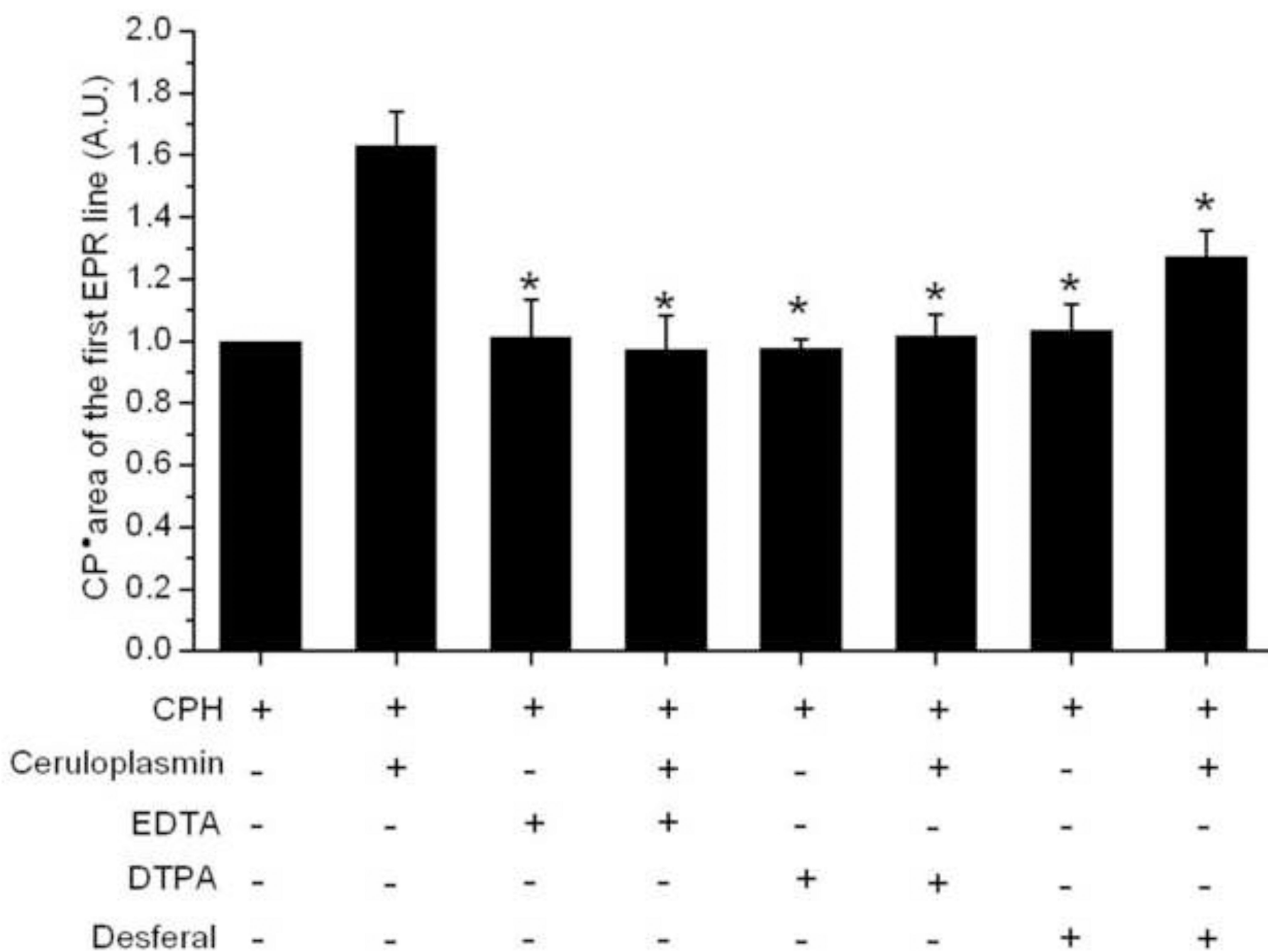

Figure 3.

Effect of different transition metal chelators on the CPH-nitroxide formation catalyzed by ceruloplasmin in samples prepared with Chelex-treated phosphate buffer. Spectra were taken for reaction mixtures containing CPH (1 mM), or CPH (1 mM) and ceruloplasmin (1 μM) in the presence of the transition metal chelators EDTA, DTPA and Desferal (200 μM). The areas of the first EPR line were normalized to the area of the sample containing only 1 mM CPH. * $P < 0.05$, compared to the area of the sample CPH + ceruloplasmin.

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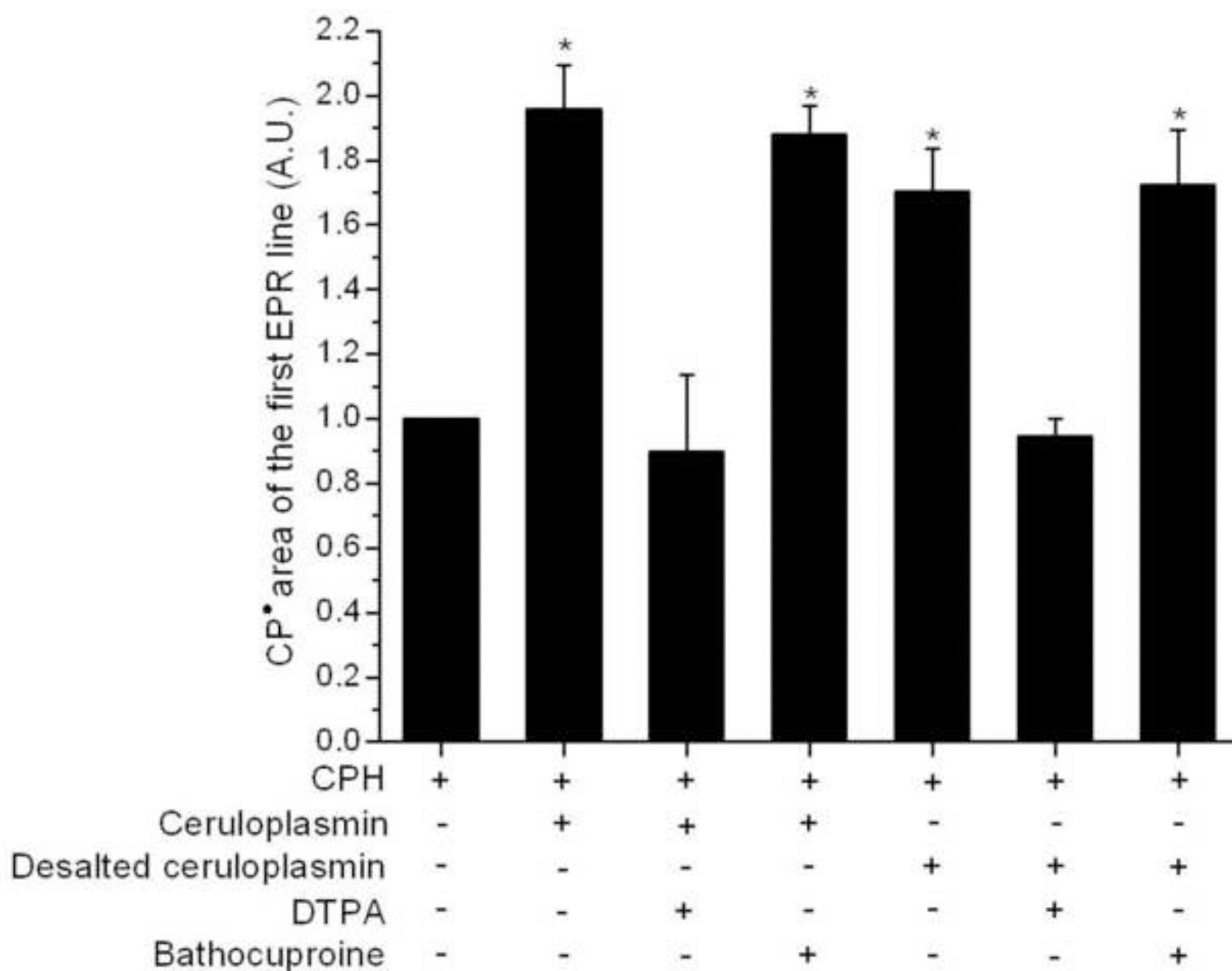

Figure 4.
Effect of transition metal ion chelators in the CPH-nitroxide formation catalyzed by ceruloplasmin and desalted ceruloplasmin in samples prepared with Chelex-treated phosphate buffer. Spectra were taken for reaction mixtures containing CPH (1 mM) and ceruloplasmin (2 μM) in the presence of DTPA (200 μM) or bathocuproine (200 μM). The areas of the first EPR line were normalized to the area of the sample containing only 1 mM CPH. Statistically significant differences compared to the sample of only CPH are shown (* $P < 0.05$).

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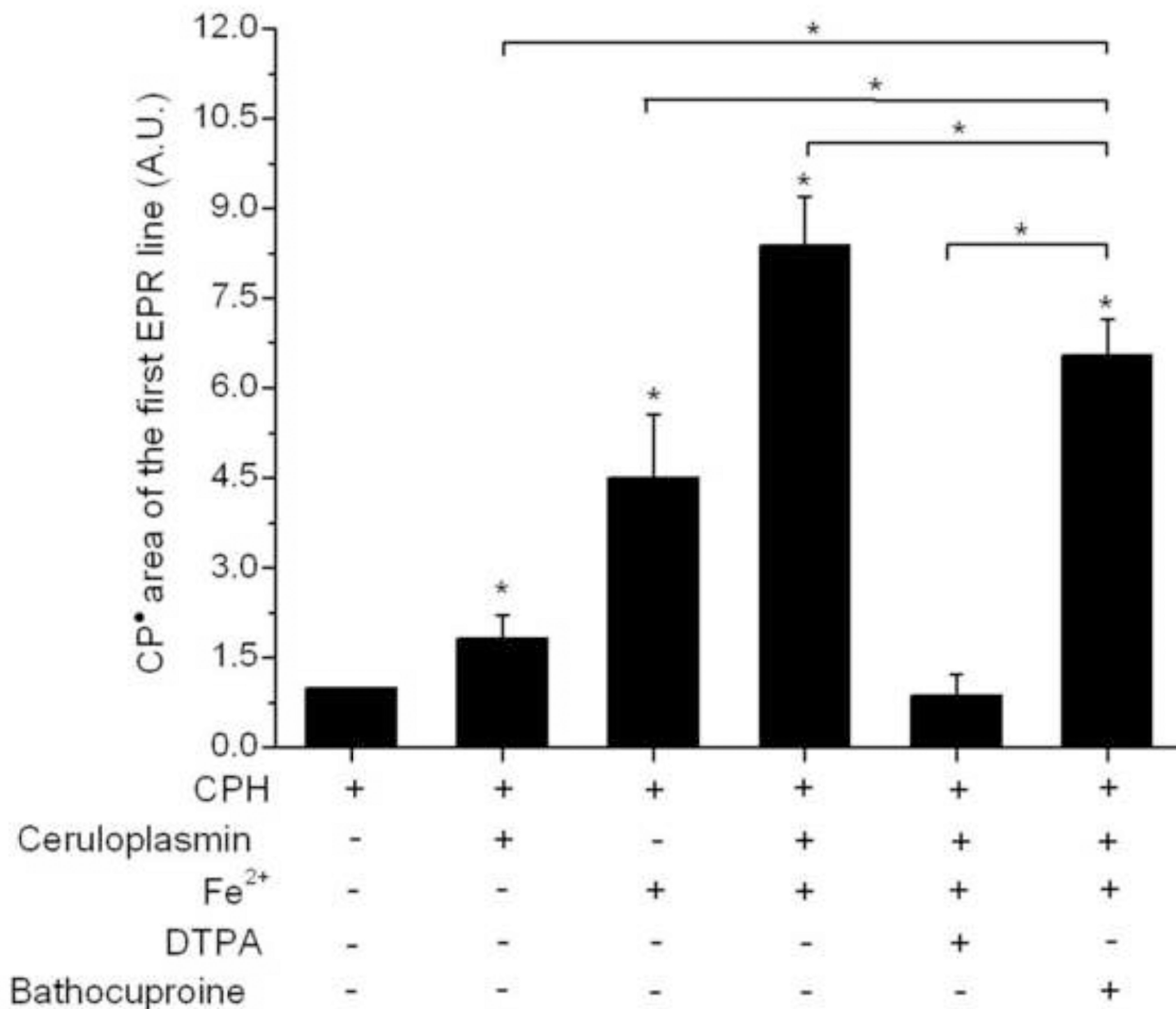

Figure 5.

The effect of iron (Fe^{2+}), DTPA and bathocuproine in the CPH-nitroxide formation in samples of ceruloplasmin. Spectra were taken for reaction mixtures containing CPH (1 mM) and ceruloplasmin (2 μM) in the presence of Fe^{2+} (0.1 μM), with or without DTPA (200 μM) or bathocuproine (200 μM). The areas of the first EPR line were normalized to the area of the sample containing only 1 mM CPH. Asterisks on the top of the bars represent statistically significant differences compared to the sample of only CPH, whereas brackets show statistically significant differences between the groups pointed out (* $P < 0.05$).

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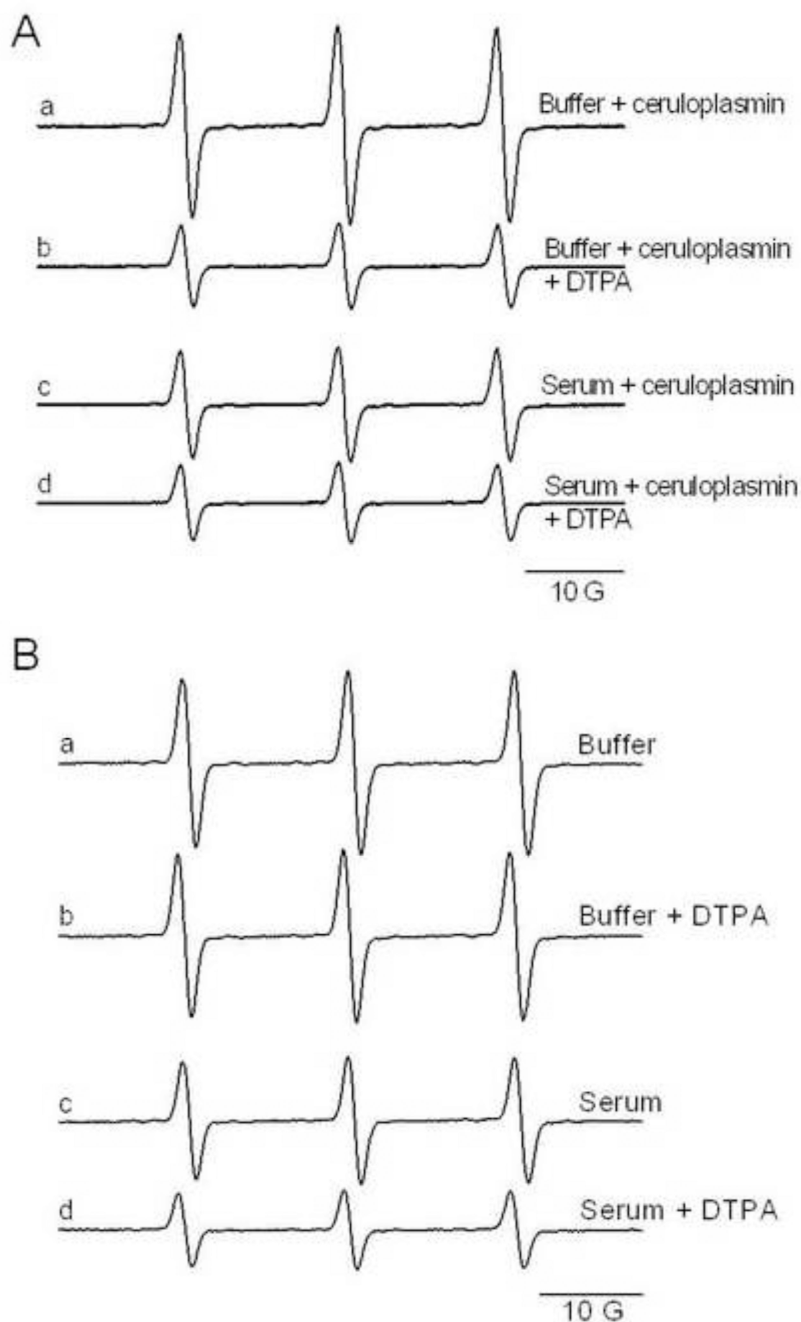

Figure 6.
Effect of ceruloplasmin and DTPA in the CPH-nitroxide formation in Chelex-treated phosphate buffer or rat serum. In (A) reaction mixtures were composed of 1 μM ceruloplasmin and1 mM CPH prepared with Chelex-treated phosphate buffer or rat serum, with and without DTPA (200 μM). In (B) samples were prepared as in (A) but without ceruloplasmin. The spectra were recorded after incubation for 10 min at 25°C.

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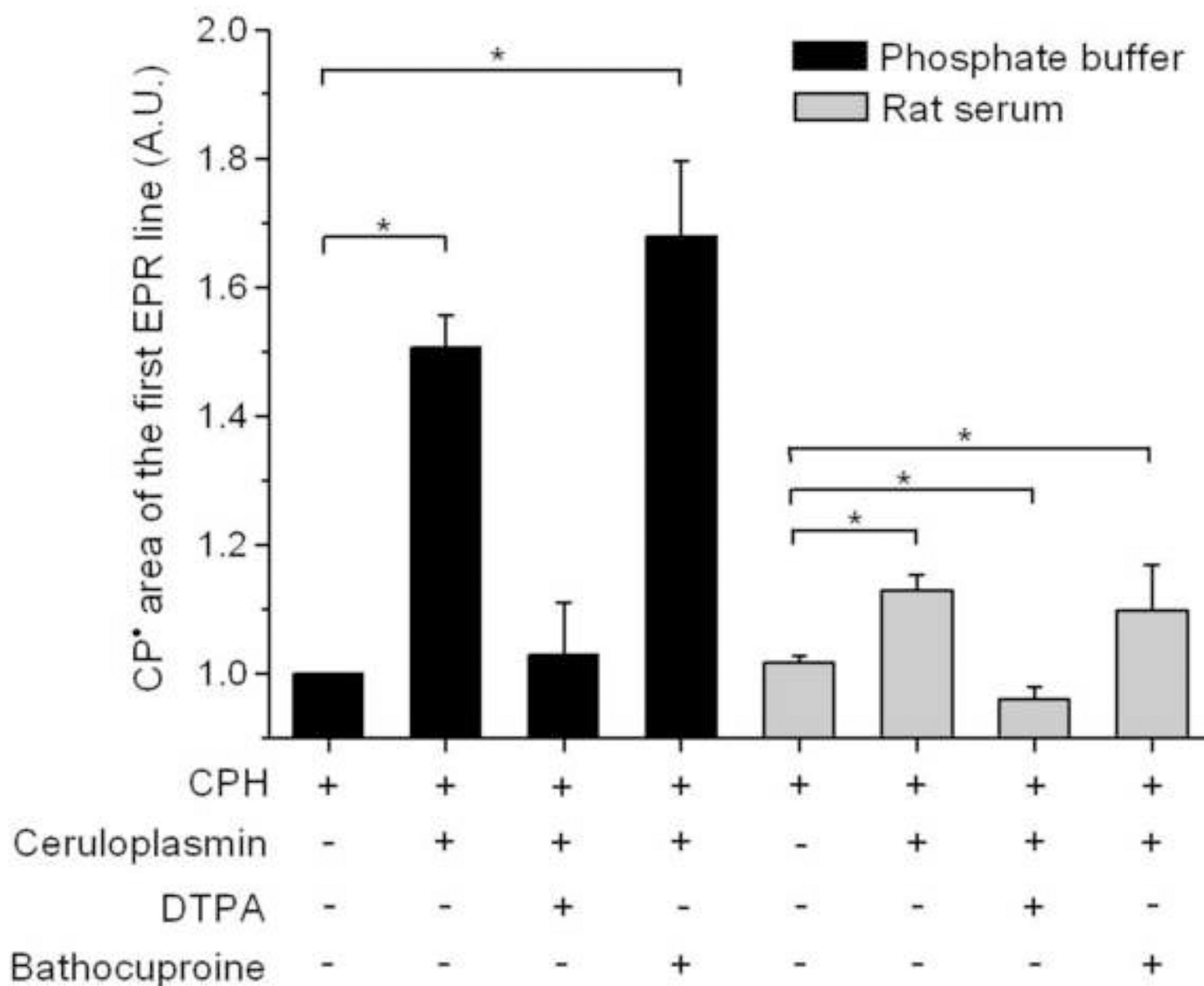

Figure 7.
Effect of bathocuproine and DTPA on CPH-nitroxide formation in samples of ceruloplasmin prepared with Chelex-treated phosphate buffer or rat serum. Reaction mixtures were composed of 1 mM CPH and 1 μM ceruloplasmin prepared with Chelex-treated phosphate buffer or rat serum with and without DTPA or bathocuproine (200 μM). Statistically significant differences are shown (* $P < 0.05$).

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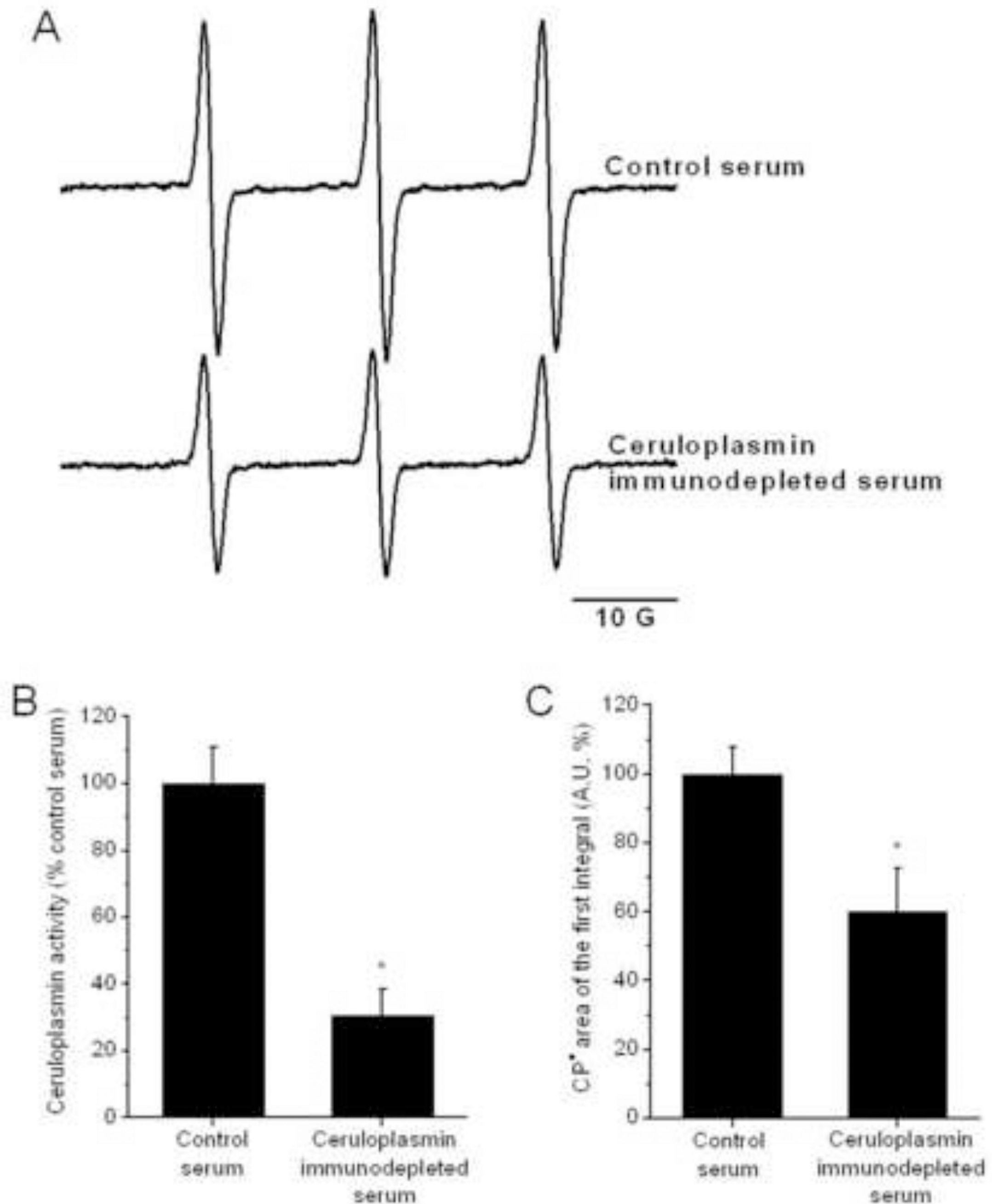

Figure 8.

CPH-nitroxide yield in samples prepared with ceruloplasmin-immunodepleted rat serum. Panel (A) shows the EPR-spectra of CPH-nitroxide signals detected in samples of control and ceruloplasmin-immunodepleted rat serum with 1 mM CPH. The spectra were recorded after incubation for 10 min at 25°C. The activity of ceruloplasmin (B) was determined in the control and immunodepleted rat serum samples. The calculated areas of the CPH-nitroxide signals are shown in (C). The data in panels (B) and (C) are expressed as percentage of the average control rat serum sample measured. Statistically significant differences are shown (* $P < 0.05$).

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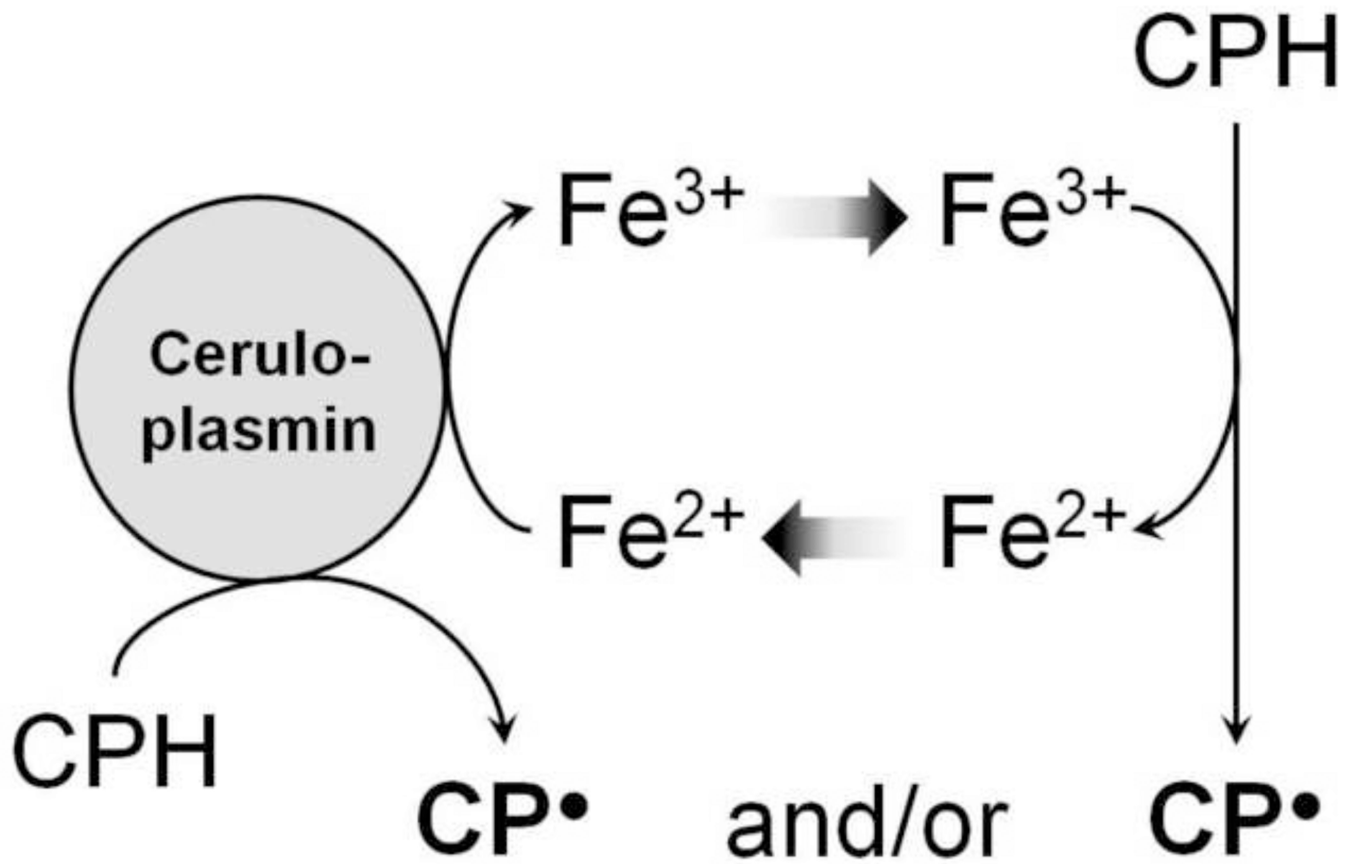

Scheme 1.

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Table 1

Human physiological or pathological states associated with increased levels of ceruloplasmin.

Physiological/ Pathological state	Fold increase in serum levels of ceruloplasmin	Ref.
Rheumatoid arthritis	1.62	[20]
Alcoholic liver steatosis	1.39	[21]
Non-alcoholic steatohepatitis	1.30	[22]
Coronary artery disease	1.61	[23]
Cardiovascular disease	1.83	[28]
Lymphocytic leukemia	2.25	[31]
Diabetes	Without diabetic complications: 1.37	[32]
	With diabetic complications: 1.66	
Schizophrenia	1.19	[35]
Lupus	1.51	[36]
Metabolic syndrome, insulin resistance	1.17	[37]
Pregnancy	3.3	[38]