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Superoxide and hydrogen peroxide suppression by metal ions and their EDTA complexes

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Abstract

Redox-active metal ions such as Fe(II)\(III) and Cu(I)\(II) have been proposed to activate reactive oxygen and nitrogen species (RONS) and thus, perpetuate oxidative damage. Here, we show that concentrations of metal ions and EDTA complexes with superoxide-destroying activities equivalent to 1 U SOD are Fe(III) 5.1 μM, Mn(II) 0.77 μM, Cu(II)-EDTA 3.55 μM, Fe(III)–EDTA 2.34 μM, and Mn(II)–EDTA 1.38 μM. The most active being the aquated Cu(II) species which exhibited superoxide-destroying activity equivalent to 2 U of SOD at 0.29 μM. Hydrogen peroxide-destroying activities were as follows Fe(III)–EDTA ca. 70 U/mg and aquated Fe(III) 141 U/mg. In contrast, DTPA prevented superoxide-destroying activity and significantly depleted hydrogen peroxide-destroying activity. In conclusion, non-protein bound transition metal ions may have significant anti-oxidant effects in biological systems. Caution should be employed in bioassays when chelating metal ions. Our results demonstrate that DTPA is preferential to EDTA for inactivating redox-active metal ions in bioassays.

Keywords: Anti-oxidant; Superoxide; Hydrogen peroxide; Metal ions; Ethylenediaminetetraacetic acid; Diethylenetriaminepentaacetic acid

Oxidative stress contributes to the pathogenesis of a wide number of diseases including rheumatoid arthritis, cardiovascular diseases, and neurological disorders via the overproduction of reactive oxygen and nitrogen species (RONS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and peroxynitrite ($ONOO^-$) [1]. Redox-active metal ions released from storage proteins have been ascribed numerous roles in the exacerbation of oxidative stress [2]. A variety of mechanisms for the generation or further activation of RONS by redox-active metal ions have been proposed. These include the well-known Fenton and Haber–Weiss reactions and the less well-known Udenfriend's system [3,4] where aromatic moieties are hydroxylated by the interaction of ascorbic acid, Fe(II), and O_2 and Weissberger's system [5], where the auto-oxidation of ascorbic acid is catalysed by Cu(II) to generate H_2O_2 (and potentially the hydroxyl radical).

The chelating agent ethylenediaminetetraacetic acid (EDTA) is frequently added during bioassays to suppress the interference of non-protein bound metal ions. For example, addition of EDTA is recommended for the nitroblue tetrazolium (NBT) assay for SOD activity. Conflicting reports on the ability of Fe(III)–EDTA complexes to dismutate $O_2^{\cdot-}$ have been published [6,7]. Previously, we reported superoxide dismutase-like and catalase-like activities of Cu(II) and Fe(III) complexes of EDTA analogues [8].

Here, we report that low-molecular-mass redox-active metal ions and their EDTA chelates have beneficial effects via their anti-oxidant abilities to detoxify H_2O_2 and $O_2^{\cdot-}$. In contrast, diethylenetriaminepentaacetic acid (DTPA) complexes exhibited no superoxide-destroying and minimal H_2O_2-destroying activities and DTPA should be used in preference to EDTA in bioassays to remove interference from metal ions.

Materials and methods

Superoxide-destroying activity. The $O_2^{\cdot-}$-destroying activities of aqueous solutions of Fe(III), Cu(II), and Mn(II) and their EDTA and DTPA complexes were assessed using a modified NBT assay with xanthine oxidase (XO) as the source of $O_2^{\cdot-}$ [9]. All reagents were

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obtained from Sigma–Aldrich Chemical and assays were run in 3 ml of solution. The standard step of addition of EDTA as part of the assay was omitted. Percentage inhibition of NBT reduction was calculated from the controls; NBT + XO and NBT blank. The rates of NBT reduction were set as 0 and 100% inhibition for the controls, respectively. From these data, the calibration plot was constructed for the percentage inhibition against rate of NBT reduction, from which the percentage inhibitions for all test solutions were calculated. Activities were determined with reference to a calibration curve constructed from bovine erythrocyte SOD. Results are given as the concentration of test compound which exhibits superoxide-destroying activity equivalent to 1 U of SOD activity unless stated otherwise.

H_2O_2-destroying activity. The H_2O_2-destroying activities of aqueous solutions of Fe(III), Cu(II), and Mn(II) and their EDTA and DTPA complexes were studied via the evolution of oxygen manometrically as described previously [10]. All reactions were carried out at room temperature and activities were calculated relative to bovine liver catalase (35,200 U/mg). H_2O_2 was added to 25 ml of solutions of metal ions and their EDTA and DTPA complexes in the range of 0.2–0.5 mM.

Results and discussion

The inhibition profile of bovine erythrocyte SOD on the NBT assay was studied over the range of 0.15–4.0 U of enzyme activity (Fig. 1). The calibration graph was used in preference to IC_{50} determinations, where concentrations equivalent to 1 U SOD are taken as 50% of the maximal inhibition of SOD activity. As can be seen in Fig. 1, maximal inhibition is only 80% (at 3–4 U SOD), whereas 1 U SOD exhibits 68% inhibition. Therefore, results are given for the test solution concentrations exhibiting 68% inhibition.

The effects of redox-active metal ions on the reduction of NBT by XO-generated $O_2^{\cdot-}$ radicals are shown in Fig. 2. Under the conditions used, the most active metal ion was the aquated Cu(II) species which exhibited $O_2^{\cdot-}$-destroying activity equivalent to 2 U SOD at 0.29 μM.

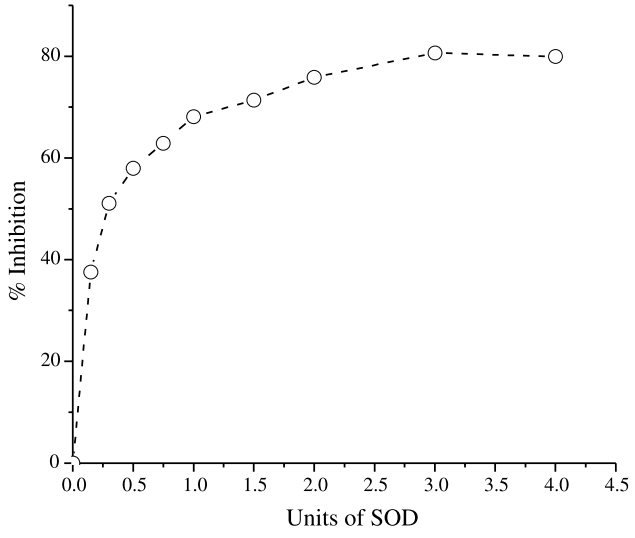

Fig. 1. Plot of inhibition of NBT as a function of SOD activity for bovine erythrocyte SOD.

The relative activities for the other metal ions at this concentration were 0.3 U for Mn(II) and 0 U for Fe(III). The concentrations of metal ions with $O_2^{\cdot-}$-destroying activities equivalent to 1 U of SOD are given in Table 1.

Interestingly, addition of the chelator EDTA had a major effect upon the $O_2^{\cdot-}$-destroying activities of the metal ions studied (Fig. 3 and Table 1). The concentrations of EDTA complexes with activities equivalent to 1 U of SOD are given in Table 1. The solution containing 1:1 ratios of Mn(II):EDTA at a concentration of 0.29 μM exhibited $O_2^{\cdot-}$-destroying activities equivalent to 0.2 U of bovine erythrocyte SOD. The metal ion–EDTA solutions for Cu(II) and Fe(III) exhibited $O_2^{\cdot-}$-destroying activity equivalent to 0.06 and 0.04 U SOD activity, respectively, at the same concentration. $O_2^{\cdot-}$-destroying activities for aquated metal ions were totally depleted on addition of one equivalent of DTPA (Table 1).

A suggested mechanism for $O_2^{\cdot-}$-destroying activity is given in Eqs. (1)–(3). This mechanism is supported by a previous study that shows hydroxyl radicals are not produced during the reaction of superoxide with cupric

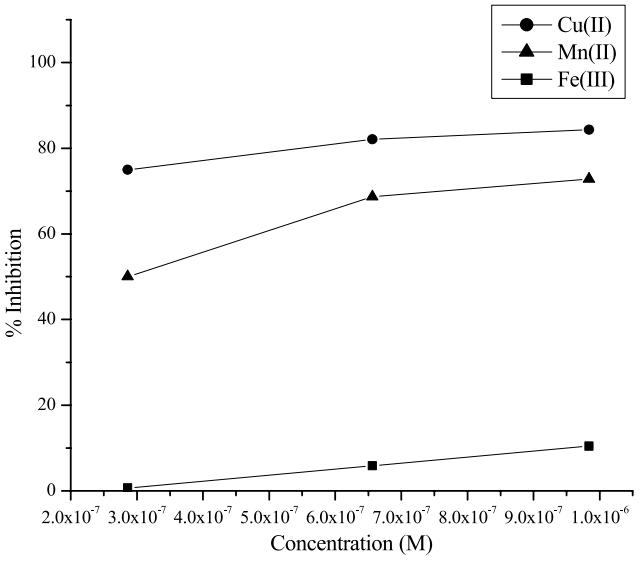

Fig. 2. $O_2^{\cdot-}$-destroying activity profiles of metal ions as assessed by the NBT assay.

Table 1
$O_2^{\cdot-}$-destroying activities for metal ions and their EDTA and DTPA complexes[a]

	Aquated ion	EDTA complex	DTPA complex
Fe(III)	5.1	2.34	NA[c]
Cu(II)	0.29[b]	3.55	NA
Mn(II)	0.77	1.38	NA

[a] Results are given as concentrations [$\times 10^{-6}$ M] with activities equivalent to 1 U of bovine erythrocyte SOD.
[b] Concentration is equivalent to 2 U of SOD activity.
[c] NA, no activity.

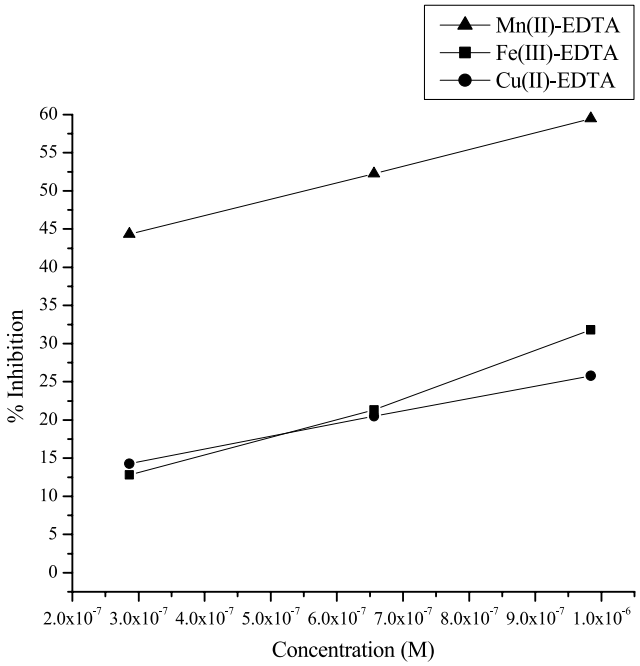

Fig. 3. $O_2^{\cdot-}$-destroying activity profiles of EDTA and its metal ion complexes as assessed by the NBT assay.

complexes of EDTA [11]. The concentration range for aquated metal ions was chosen to reflect levels of non-protein-bound redox-active transition metal ions found in diseased tissues from patients with rheumatoid arthritis ($1.25 \pm 0.95 \times 10^{-7}$ M) [12]. Thus, conducting the NBT-based assay for SOD on synovial fluid from rheumatoid joints would introduce significant $O_2^{\cdot-}$-destroying activity arising from the Cu(II) interaction with added EDTA. The depletion of $O_2^{\cdot-}$-destroying activity by DTPA complexation is supported by the proposal that the metal ion must have at least one co-ordination position available to bind superoxide in two adjacent valence states [13]. This pattern is further reflected by comparison of metal ion $O_2^{\cdot-}$-destroying activities to their respective EDTA complexes. The seven co-ordinate Fe(III) and Mn(II) EDTA complexes exhibited higher activity than the six co-ordinate Cu(II) complex.

$$Cu^{2+}\text{-EDTA} + O_2^{\cdot-} \rightarrow Cu^{+}\text{-EDTA} + O_2 \quad (1)$$

$$Cu^{+}\text{-EDTA} + O_2^{\cdot-} \rightarrow Cu^{2+}\text{-EDTA} - O_2^{2-} \quad (2)$$

$$Cu^{2+}\text{-EDTA} - O_2^{2-} + 2H^{+} \rightarrow Cu^{2+}\text{-EDTA} + H_2O_2 \quad (3)$$

In contrast to the optimal $O_2^{\cdot-}$-destroying activity exhibited by the aquated divalent metal ions (Cu(II) and Mn(II)) and the Mn(II)–EDTA complex, the optimal H_2O_2-destroying activities were exhibited by Fe(III) and its complex with EDTA (Fig. 4). This gave 70 U of activity per milligram of Fe(III) with complexation with EDTA enhancing the activity to 141 U/mg. All divalent metal ions studied and their respective EDTA chelates were ineffective for destroying H_2O_2.

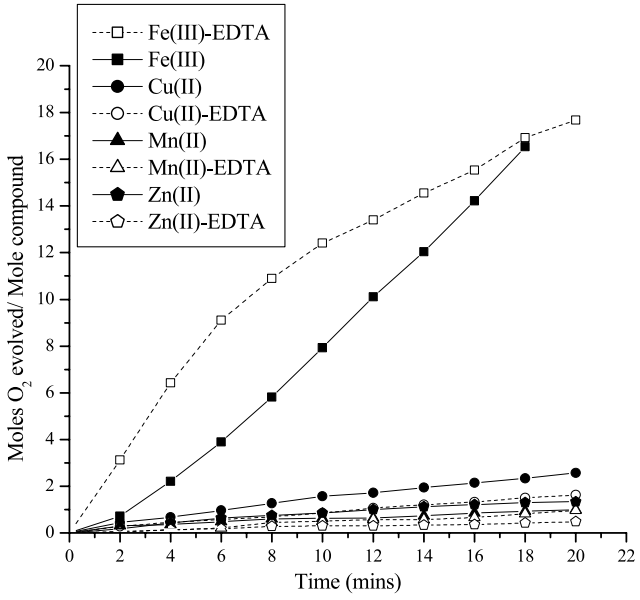

Fig. 4. H_2O_2-destroying activities of metal ions and their EDTA complexes.

DTPA, in contrast to EDTA, has a pronounced negative effect on the H_2O_2-destroying activities of the metal ion species. DTPA effectively depleted the activity of the Mn(II) and Cu(II) species and effected a 4-fold reduction in the activity of the Fe(III) species (Fig. 5). In addition to the effect of DTPA on $O_2^{\cdot-}$-destroying activity, the effect on H_2O_2-destroying activities further supports the use of DTPA in bioassays where RONS are involved.

Proposed mechanisms for the H_2O_2-destroying activities have been given in previous studies [Eqs. (4)–(8)] [14]. It should be noted that some of these reactions result in

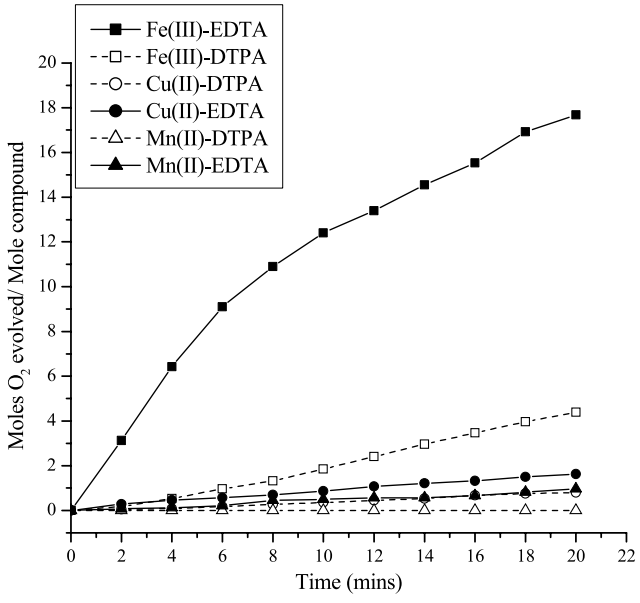

Fig. 5. Comparison of H_2O_2-destroying activities of EDTA and DTPA complexes.

the formation of toxic products such as Fenton-mediated generation of the highly reactive hydroxyl radical (·OH).

$$Fe^{3+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}OOH^{-}\text{-EDTA} + H^{+} \quad (4)$$

$$Fe^{3+}OOH^{-}\text{-EDTA} + H_2O_2 \rightarrow FeO^{2+}\text{-EDTA} + HO_2^{\cdot} + H_2O \quad (5)$$

$$HO_2^{\cdot} \rightarrow H^{+} + O_2^{\cdot-} \quad (6)$$

$$Fe^{3+}\text{-EDTA} + O_2^{\cdot-} \rightarrow Fe^{2+}\text{-EDTA} + O_2 \quad (7)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + {}^{\cdot}OH + OH^{-} \quad (8)$$

In conclusion, both H_2O_2-destroying and $O_2^{\cdot-}$-destroying activities have been exhibited by aquated metal ions. In addition, EDTA complexes of Cu(II) and Fe(III) exhibit significant $O_2^{\cdot-}$-destroying and H_2O_2-destroying activities, respectively, and EDTA complexation actually enhances both activities for the Fe(III) species. These findings should be considered in the design of experiments where redox-active metal ions are present. In particular, for the NBT assay the use of DTPA in place of EDTA should greatly negate the antioxidant effects of metal ions. In addition, this work demonstrates that low-molecular-mass redox-active metal ions may have beneficial effects in oxidative stress.

Acknowledgments

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