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Catalytic superoxide scavenging by metal complexes of the calcium chelator EGTA and contrast agent EHPG

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Abstract

Metal ion chelators widely used in experimental protocols and clinical diagnosis are generally assumed to be inert. We previously reported that the ubiquitous chelator EDTA has high levels of superoxide suppressing activity. Here, we report that the common chelators calcium chelator EGTA and contrast agent EHPG have significant activities in suppressing superoxide levels depending on the nature of metal ion chelated. The most active species is Mn(II)-EGTA which exhibited an IC_{50} value of 0.19 μM for superoxide destruction. In addition, IC_{50} values for Mn(II)-EHPG and 2Cu(II)-EGTA were 0.69 and 0.60 μM, respectively. In conclusion, Mn(II) and Cu(II) complexes of the common chelators EGTA and EHPG exhibit considerable superoxide scavenging activities. Caution should be employed in their use in biological systems where superoxide has a key role and they may be useful for the development of catalytic anti-oxidants.

Keywords: Chelator; Superoxide dismutase; Metal ions; Ethylenebis(hydroxyphenylglycine); Ethylenebis(oxyethylenenitrilo)tetraacetic acid

Superoxide ($O_2^{\bullet-}$) is the one electron reduction product of O_2. It is produced from several sources, by (i) the loss of electrons from the mitochondrial electron transport chain onto O_2 [1], (ii) the oxidation of molecules such as glyceraldehydes and $FADH_2$ in the presence of O_2, (iii) activated phagocytes as part of their protective mechanism, (iv) via the action of drugs, radiation, and poison, and (v) production from tumours which exhibit an increased metabolism. Superoxide itself can result in the release of metal ions from transport proteins, increase neutrophil adhesion and infiltration, and regulate proinflammatory cytokines. It is not itself highly reactive but can react to form the two highly reactive oxygen species $^{\bullet}OH$ and $ONOO^-$, which are thought to be the main perpetrators of oxidative damage.

Oxidative damage resulting from $O_2^{\bullet-}$ overproduction contributes to many conditions including stroke [2], ischaemia [3], asthma [4], atherosclerosis [5], neurodegenerative diseases [6] as well as many other inflammatory conditions [7]. Therefore, there is much interest in antioxidant therapies to negate the action of superoxide.

The native enzyme superoxide dismutase (SOD) is unsuitable for the treatment of these conditions due to its short plasma half-life, its inability to cross cell membranes, and the risk of immunogenic responses. CuZnSOD itself can also have a pro-oxidant effect at high concentrations, which are postulated, to be through its reaction with hydrogen peroxide (H_2O_2). This can result in Fenton generation of $^{\bullet}OH$ [8] and also inactivation of CuZnSOD [8]. This is demonstrated by its bell-shaped dose–response curve [9,10]. Therefore, a large amount of research has been based on the development of SOD mimics. These have the advantage that they can be designed with certain specifications such as; selective reactivity, i.e., inactive with H_2O_2, best redox potential for $O_2^{\bullet-}$ disproportionation, and with small molecular weights for entry into cells.

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The development of therapeutic mimics of anti-oxidant enzymes has attracted vigorous activity for the past decade, resulting in a range including porphyrins [11,12], manganese salens [2,13], macrocyclics [3,14], nitroxides [15], and many other catalytic antioxidants [16,17]. Despite the demonstration of protective effects in a variety of model systems, this field has presented a large number of challenges. From the entire range of SOD mimic chelators under development, problems encountered to date include: (i) some highly active and moderately active mimics such as iron porphyrins are hampered by their cytotoxicity profiles [11], (ii) poor stability of manganese salen mimics in the presence of endogenous and exogenous components (EDTA) [18], (iii) destruction of metal porphyrins by the hydrogen peroxide generated [11], and (iv) the ability of mimetics to act as epoxidation catalysts [19].

Recently, we observed that a number of aquated transition metal ions and their complexes with the common chelator EDTA exhibit considerable SOD and catalase mimic activities [20]. This led us to examine the SOD and catalase-like activities of other commonly used chelators and their metal chelates. In a model of multiple sclerosis (MS), the calcium ion chelator ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) protects against the nitric oxide-induced calcium influx into nerve cells resulting in cell death [21]. Second, low concentrations of EGTA have been shown to be effective at resolubilizing amyloid plaques from Alzheimer's disease (AD) patients, post mortem brain samples [22].

Here we present the first report that EGTA has a secondary beneficial role whereupon binding excess copper ions (elevated levels in MS and AD) it affords significant SOD activity preventing further generation of $ONOO^-$. The related chelator ethylenebis(hydroxyphenylglycine) (EHPG) is used as an imaging contrast agent for magnetic resonance imaging and as a transferrin mimic to study manganese transport [23,24]. It also exhibited SOD activity when complexed to Cu(II) and Mn(II) ions. No pro-oxidant activities were observed for either chelator under the conditions of the study.

On comparison to compounds that have been developed as SOD mimics, these results demonstrate that commonly used chelators provide new avenues for the development of SOD mimics. They exhibit activities in line with some of the best SOD mimics shown in the literature. In addition, they have the advantage of already being used and hence much is known about their side effects and toxicity in biological systems. In this study we will demonstrate their efficacy as SOD mimics when complexed to various metal ions and compare their respective activities to other established SOD mimics.

Materials and methods

Chemicals. All chemicals were of reagent grade and were purchased from Sigma–Aldrich. Bovine erythrocyte SOD and bovine buttermilk xanthine oxidase Grade IV from the same source were used. All glassware was acid washed to remove transition metal ions and HPLC grade water was used throughout.

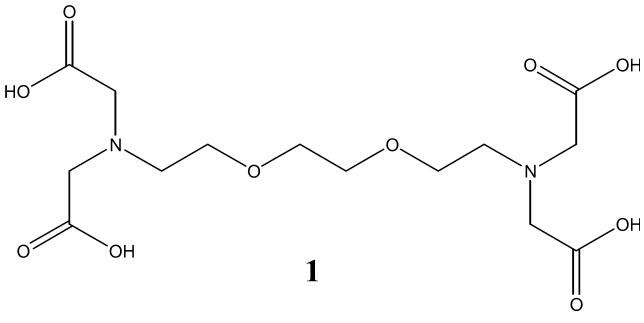
Spectroscopic studies of cupric and ferric ion complexation. The complexation characteristics of the metal ion-chelator complexes were studied using spectrophotometric titrations. Timed studies demonstrated that in aqueous solution complexation was instantaneous. Aliquots (0.1 molar equivalents) of a 1.4 mM $FeCl_3$ solution were added to an aqueous solution of 0.28 mM EHPG.

The metal-ligand binding ratio was investigated using Job's method. Solutions of 7 mM $CuCl_2$ and 7 mM chelator were prepared with increasing mole ratios of EBMT:Cu(II) from 1:0 to 0:1. The absorbance was noted at the λ maxima of EGTA-2Cu(II) of 689 nm and EHPG-Cu(II) of 645 nm.

SOD activity. The SOD activities of aqueous solutions of Fe(III), Cu(II), and Mn(II) ions and their EGTA and EHPG complexes were assessed using a modified NBT assay with xanthine oxidase (XO) as the source of $O_2^{\cdot-}$. All reagents were obtained from Sigma–Aldrich Chemical and assays were run in 3 mL solution. Results are given as units of SOD activity per milligram of compound calculated from comparison to bovine erythrocyte SOD. IC_{50} values are given for comparison to those of other chelators.

Results and discussion

We have studied the complexation profiles of EGTA and EHPG (Fig. 1) using time dependent studies, spectrophotometric titrations and Job's method of continued variances. Time dependent studies and spectrophotometric titrations gave initial metal to chelator ratios



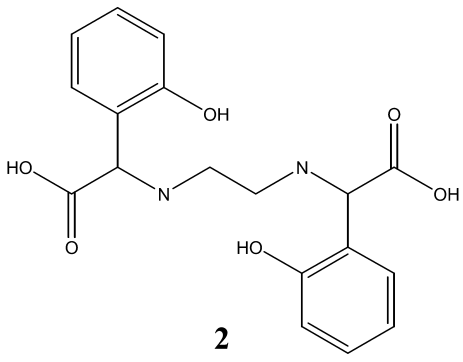


Fig. 1. Structures of chelators EGTA (**1**) and EHPG (**2**).

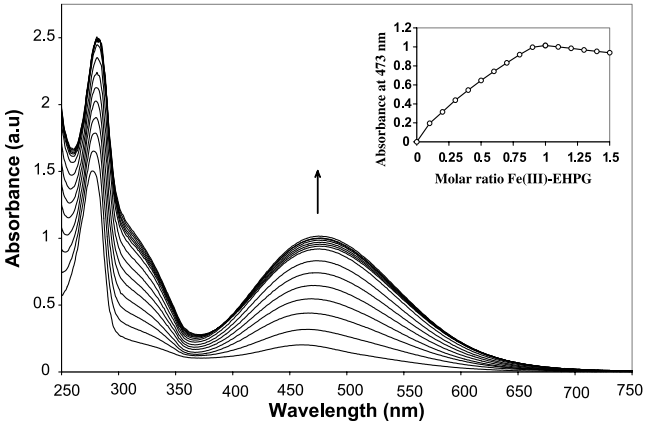


Fig. 2. Spectrophotometric titration for additions of a solution of Fe(III) to EHPG. Additions of 0.1 M equivalents of aquated Fe(III) were added to a 0.28 mM solution of EHPG.

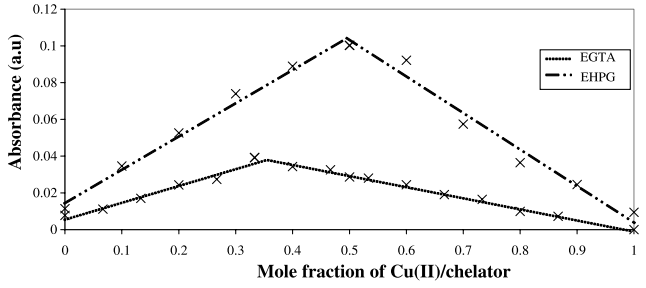


Fig. 3. Job plots for EGTA (wavelength 689 nm) and EHPG (wavelength 645 nm) with Cu(II) demonstrating 2:1 and 1:1 complexation, respectively.

for Fe(III) ion and Cu(II) ion complexation. Fig. 2 illustrates a typical spectrophotometric titration showing maximal complexation at the Fe(III):EHPG ratio of 1:1. Metal-binding characteristics of each chelator to Cu(II) were analysed by Job's method (Fig. 3). The plot shows the formation of 2:1 complexes for Cu(II) with EGTA and 1:1 complexes for Cu(II) with EHPG in line with previous solid state studies [24,25]. Crystallographic analyses have ascertained that EGTA can form both 1:1 and 1:2 chelator to metal complexes whereas EHPG predominantly forms 1:1 complexes. The stability constants for these are given in Table 1. Throughout this study, both 2:1 and 1:1 EGTA metal complexes were used and 1:1 EHPG metal complexes were employed to assess the anti- and pro-oxidant activities.

Previously, we reported superoxide dismutase (SOD) and catalase activities of Cu(II), Mn(II), and Fe(III) complexes of EDTA [20]. The inhibition profile of bovine erythrocyte SOD on the NBT assay was reported previously and identical conditions are used here. The calibration graph was used in preference to IC_{50} determinations, however IC_{50} values have been given for comparative purposes.

Table 1
SOD activities and stability constants for metal ions and their EGTA and EHPG complexes[a]

Compound	Metal complex	Activity (U/mg)[a]	K_1
EGTA	Cu(II)	314	17.71[b]
EGTA	Mn(II)	981	12.28[b]
EGTA	Fe(III)	8.76	20.5[c]
EGTA	2Cu(II)	413	—
EGTA	Cu(II)Zn(II)	255	—
EHPG	Cu(II)	409	>15[d]
EHPG	Mn(II)	879	NA
EHPG	Fe(III)	0	33.91[e]
EDTA	Cu(II)	261[f]	18.8[b]
Cu(II)		35,583[f]	—
CuZn-SOD		3730[g]	—

[a] Results are given as units activity per milligrams of compound (comparable to bovine erythrocyte SOD).
[b] Ref. [26].
[c] Ref. [27].
[d] Ref. [28].
[e] Ref. [29].
[f] Ref. [20].
[g] Figure based on bovine erythrocyte SOD from Sigma.

In the current study, we found considerable SOD mimic activities for the commonly used chelators EGTA and EHPG (Fig. 4 and Table 1). Mn(II)-EGTA exhibited the best activity where 0.77 μM was equivalent to 1 U of bovine erythrocyte SOD (from calibration curve [20]), closely followed by the Mn(II) complex of EHPG where 1.206 μM was equivalent to 1 U SOD. This is consistent with previous studies on Mn(II) complexes of EDTA which showed activities much greater than those exhibited by either the Cu(II) or Fe(III) complexes. EGTA-2Cu(II), EGTA-Cu(II), and EGTA-Cu(II)Zn(II) exhibited activities where 1.58, 2.36, and 2.54 μM, respectively, were equivalent to 1 U SOD. In the case of the EHPG chelator the Cu(II) complex exhibited 1 U SOD activity at 1.21 μM. Neither

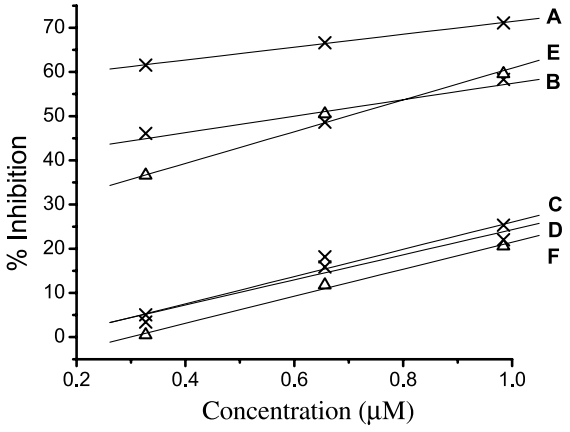


Fig. 4. SOD activity profiles of (A) EGTA-Mn(II), (B) EGTA-2Cu(II), (C) EGTA-Cu(II), (D) EGTA-Cu(II)Zn(II), (E) EHPG-Mn(II), and (F) EHPG-Cu(II) as assessed by the NBT assay.

Table 2
IC_{50} values for a range of SOD mimics

SOD mimic	IC_{50} (μM)	Reference
Mn(II)-EGTA	0.19	This work
Mn(II)-EHPG	0.69	This work
2Cu(II)-EGTA	0.60	This work
Manganese salen C12	0.32	[30]
Cu(II)-Ac-HisValHis-NH_2	0.16	[32]
Mn(III)TE-2-PyP^{5+}	0.045	[11]
Fe(III)TE-2-$PyP(OH)^{4+}$	0.026	[11]
1/2[{Mn(III)BVDME}$_2$]	0.047	[17]
CuZn-SOD	0.0028	[33]

chelators exhibited any significant activity when complexed to Fe(III).

Cupric and Mn(II) complexes of both chelators exhibited maximum activities commensurate with a number of synthetic SOD mimics developed for therapeutic purposes. Table 2 gives the IC_{50} values for a range of SOD mimics (the optimum values for each class are listed). Although many of these activities are competitive, there are many problems associated with them. The activity of the manganese salen C12 is reduced by 50% in the presence of the chelator EDTA [30] and the manganese salen EUK 8 has also been shown to interact with DNA [31].

The more active porphyrin complexes (Fe(III)TE-2-$PyP(OH)^{4+}$ and Mn(III)TE-2-PyP^{5+}) have been shown to undergo oxidative degradation by H_2O_2 and would therefore be of little use as SOD mimics which dismutate $O_2^{\bullet-}$ to form H_2O_2. Those which are not reported to be oxidatively degraded by H_2O_2 demonstrate activities of 20 and 0.16 μM for the Mn(III) and Fe(III) porphyrins, respectively; however, the Fe-porphyrins have also been shown to be toxic to *Escherichia coli*. [11]. The manganese biliverdin complex 1/2[{Mn(III)BVDME}$_2$] shows excellent activity and has also been reported to be resistant to attack to H_2O_2 and facilitate the growth of SOD-deficient *E. coli* [17].

In addition, the chelators EGTA and EHPG also have the advantage that as they have been used for other uses much of the toxicity screening and long-term effects in biological systems have already been demonstrated. Serious consideration should be given to these compounds as SOD mimics for the treatment of conditions associated with superoxide overproduction. They exhibit metal binding stability constants which should not result in the removal of metals from metalloproteins and conversely prevent demetallation of the mimic by endogenous chelators. They also have the advantage that in conditions where there is an excess of metal ions, they can be used to bind the metal ions and direct scavenging to these areas more prone to oxidative stress.

In summary, the common chelators EGTA and EHPG: (i) bind deleterious redox-active metal ions, (ii) detoxify RONS, and (iii) have potential therapeutic applications in many inflammatory conditions. In contrast to many reported SOD mimics they are readily available and have been used in many biological systems.

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