Generation of superoxide radicals by copper–glutathione complexes: Redox-consequences associated with their interaction with reduced glutathione

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ABSTRACT

The interaction between Cu^{2+} ions and GSH molecules leads to the swift formation of the physiologically occurring Cu(I)–$[GSH]_2$ complex. Recently, we reported that this complex is able to reduce molecular oxygen into superoxide in a reversible reaction. In the present study, by means of fluorescence, luminescence, EPR and NMR techniques, we investigated the superoxide-generating capacity of the Cu(I)–$[GSH]_2$ complex, demonstrated the occurrence and characterized the chemical nature of the oxidized complex which is formed upon removing of superoxide radicals from the former reaction, and addressed some of the redox consequences associated with the interaction between the Cu(I)–$[GSH]_2$ complex, its oxidized complex form, and an in-excess of GSH molecules. The interaction between Cu(I)–$[GSH]_2$ and added GSH molecules led to an substantial exacerbation of the ability of the former to generate superoxide anions. Removal of superoxide from a solution containing the Cu(I)–$[GSH]_2$ complex, by addition of Tempol, led to the formation and accumulation of Cu(II)–GSSG. Interaction between the latter complex and GSH molecules permitted the re-generation of the Cu(I)–$[GSH]_2$ complex and led to a concomitant recovery of its superoxide-generating capacity. Some of the potential redox and biological implications arising from these interactions are discussed.

1. Introduction

Copper ions adopt mostly two distinct oxidation states, Cu(I) and Cu(II), depending on the redox character of the molecules they interact with.[1–3] Such one-electron transfer property allows copper to play a role as cofactor of several enzymes involved in oxidation-reduction reactions.[4] Within cells, copper ions are likely to encounter a net reducing environment provided, largely, by the presence of reduced glutathione (GSH),[5,6] a tripeptide whose concentrations[7] can be estimated to exceed by, at least, two-fold those of copper.[4,8] Although GSH exerts important antioxidant functions, by acting as superoxide and hydroxyl radical-scavenger[9,10] and as cofactor of the glutathione peroxidases and transferases enzymes,[2] it is possible that the tripeptide exert also a pro-oxidant activity as result of its metal-reducing properties.[2] Regarding this latter aspect of GSH, its interaction with copper ions leads to the swift reduction of Cu^{2+} into Cu^{+} ions in a reaction which, in the presence of additional GSH molecules, results in the formation of the Cu(I)–$[GSH]_2$ complex (Rx. 1).

$$2Cu^{2+} + 6GSH \rightarrow 2Cu(I)\text{–}[GSH]_2 + GSSG \qquad (Rx.1)$$

The formation and occurrence of this complex has been documented both, in non-cellular systems[11–15] and within cells exposed to copper.[16–19] Although the biological function of the Cu(I)–$[GSH]_2$ complex has not been yet established, the complex is believed to play a role as Cu(I)-carrier to several copper-dependent proteins, including SOD,[13] ceruloplasmin[20] and metallothionein.[18,21] In addition to such potential biological function, the Cu(I)–$[GSH]_2$ complex has been postulated to serve as a mechanism to protect cells from undergoing the damage expected to arise from the ability of otherwise free copper ions to bind non-specifically to essential biomolecules[22] and/or to catalyze free radical generation.[23,24] According to various investigators,[11,25–27] sequestering of copper by GSH would stabilize Cu(I) ions under a form that renders the metal redox-inactive towards oxygen. Recently, however, work by our laboratory contended with such concept as it provided direct evidence that the Cu(I)–$[GSH]_2$ complex is able to react with molecular oxygen in a reaction which leads continuously to the formation of superoxide radicals.[3,28] To the extent to which the Cu(I)–$[GSH]_2$ complex occurs within cells, its ability to generate superoxide anions raises questions onto whether such radicals be involved in the yet-to-be established biological function of the complex, and onto whether the production of such radicals would underlie some of the cyto-deleterious effects of copper excess.

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In view of the potential biological and toxicological importance of the ability of the Cu(I)–$[GSH]_2$ complex to generate superoxide radicals, and taking into consideration the major role that GSH plays in both, forming the complex and scavenging superoxide radicals, in the present study we further characterized the superoxide-generating capacity and addressed the redox consequences associated with the interaction between the complex and GSH molecules.

2. Results and discussion

2.1. Capacity of the Cu(I)–$[GSH]_2$ complex to generate superoxide anions: concentration-dependence studies

To further characterize the recently reported ability of the Cu(I)–$[GSH]_2$ complex to generate superoxide anions,[28] we addressed the relationship between the concentration of this complex and the rate at which superoxide anions are generated. For such purpose, increasing concentrations of the complex were added to a fixed concentration of DHE (50 μM), a highly sensitive superoxide-oxidizable probe.[29,30] As shown in Figure 1, the ability of the Cu(I)–$[GSH]_2$ complex to oxidize DHE described a biphasic behaviour, as depicted by an ascending and a descending curve. Each of these phases depended on the range of concentrations at which the complex was tested. While DHE oxidation increased linearly as the concentrations of the complex increased from 1 to 8 μM, concentrations greater than 8 μM (and up to 25 μM) were associated with proportionally lower degrees of DHE oxidation. From these results, it would appear that beyond 8 μM, the rate at which the complex generates superoxide anions would start to decrease. As explained below, however, the latter interpretation is only apparent.

Eqs. 1 and 2 represent the rates at which superoxide anions react with DHE and with itself, respectively. From these equations, it can be inferred that while a lineal increase in the flux of superoxide anions would be expected to result in a proportionally linear increase in the rate DHE oxidation ($V_{DHE\text{-}Oxid}$; Eq. 1), the same increase in superoxide flux would be expected to result in a quadratic increase in the rate at which these radicals would autodismutate ($V_{Autodism}$; Eq. 2).

$$V_{DHE\text{-}Oxid} = k_1[O_2^{\cdot-}][DHE] \quad \text{(Eq.1)}$$

$k_1 = 2.6 \times 10^5\ M^{-1}\ s^{-1}$ (Zhao et al.)[30]

$$V_{Autodism} = k_2[O_2^{\cdot-}][O_2^{\cdot-}] \quad \text{(Eq.2)}$$

$k_2 = 2.4 \times 10^5\ M^{-1}\ s^{-1}$ (Bielski et al.)[31]

Under the experimental conditions used in our experiments (namely, 50 μM DHE), 8 μM appears to be a concentration of Cu(I)–$[GSH]_2$ at which the flux of superoxide radicals generated the complex encounter an equal chance to react with either DHE or with themselves. The latter is reflected as maximal degree of DHE oxidation in Figure 1. Beyond 8 μM, DHE oxidation begins to decline, presumably, because the greater flux of superoxide anions expected to be generated by the complex would start to favour the autodismutation of superoxide over its reaction with DHE. It should be noted that, as such, hydrogen peroxide is neither able to inhibit[28] nor to induce DHE oxidation.[30,32,33] The interpretation that a lineal increment in the flux of superoxide shall result in a quadratic increase in the rate of autodismutation stems not only from comparing Eqs. 1 and 2, but also from the following two experimental observations. Firstly, the incubation of increasing concentrations of the complex in a DHE-free medium was associated with the generation of correspondingly increasing concentrations of hydrogen peroxide (inset to Fig. 1). Secondly, we observed that incrementing DHE concentration, from 50 μM to 200 μM, more than doubled the magnitude at which the complex induced DHE oxidation (not shown). To confirm the contention that increasing concentrations of the complex indeed result in the generation of higher fluxes of superoxide, we pursued further experiments using lucigenin, instead of DHE, as a superoxide-reducible probe. As shown in Figure 2, increasing concentrations of the Cu(I)–$[GSH]_2$ complex resulted in linearly proportional increments in the amount of lucigenin susceptible to undergo superoxide-dependent reduction (e.g., SOD-inhibitable). Such increments are consistent with the fact that the rate constant for the reaction between superoxide and lucigenin ($k = 1 \times 10^8\ M^{-1}\ s^{-1}$; Afanas'ev et al.[34]) is around three orders magnitude higher than that between superoxide and DHE (Eq. 1). It must be pointed out, however, that unlike DHE, which undergoes direct oxidation by superoxide and is

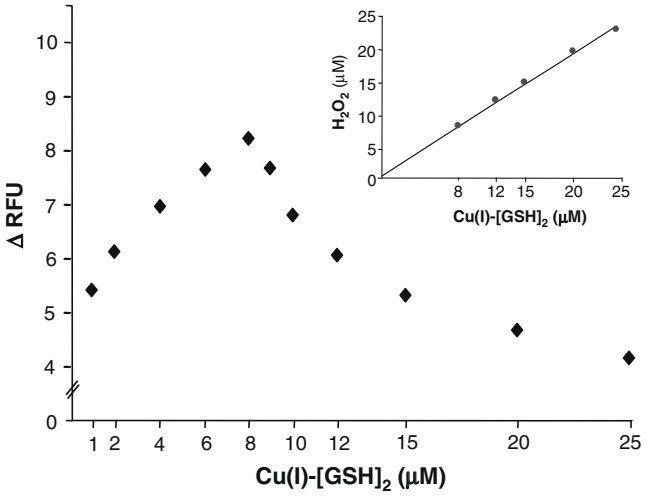

Figure 1. Dihydroethidium oxidation and hydrogen peroxide formation induced by increasing concentrations of the Cu(I)–$[GSH]_2$ complex. DHE (50 μM) was added to solutions containing increasing concentrations of the complex (1–25 μM). The increase in fluorescence due to the oxidation of DHE was registered 30 min after and the results expressed as the difference in relative fluorescence units (ΔRFU), as explained in Section 4. Inset to the figure shows the relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (8–25 μM) and the formation of hydrogen peroxide in the media.

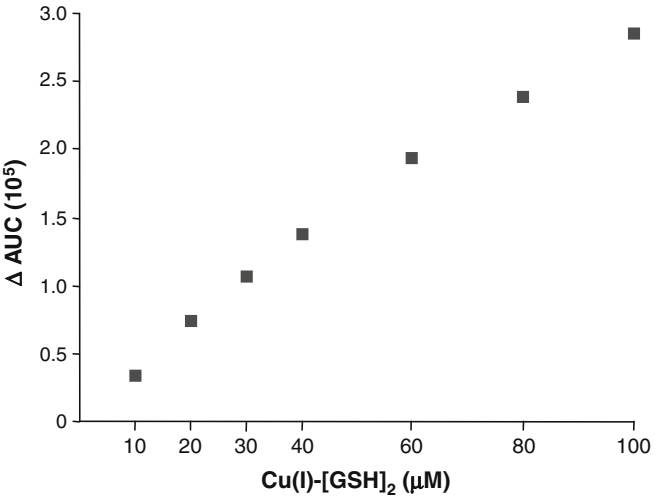

Figure 2. Reduction of lucigenin induced by increasing concentrations of Cu(I)–$[GSH]_2$. Lucigenin (15 μM) was added to solutions containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (10–100 μM) and the resulting chemiluminescence was monitored during 112 s. Results represent the difference in the area under the curve (ΔAUC) described by the chemiluminescence levels.

not subjected to artifactual redox-cycling,[35] lucigenin must be first reduced by superoxide into the non-quimioluminescent lucigenin cation radical; since the latter specie can reduce molecular oxygen into superoxide anion, it is recognized that lucigenin itself could artifactually generate more superoxide anions.[36]

2.2. Effects of added GSH on the ability of a preformed Cu(I)–$[GSH]_2$ complex to generate superoxide anions

In view of the recognized capacity of GSH to scavenge superoxide anions,[37] we investigated a possible effect of the tripeptide on the ability of the Cu(I)–$[GSH]_2$ complex to promote the DHE oxidation. For such purpose, increasing concentrations of GSH (from 25 to 750 μM) were added to a solution containing DHE (50 μM) and a fixed concentration of the complex (8 μM). As shown in Figure 3, the addition of GSH, within the 25–500 μM range, rather than inhibiting, enhanced the oxidation of DHE. Such effect of the tripeptide was susceptible to total inhibition upon addition of SOD (results not shown). The maximal effect of GSH was attained at 500 μM, and resulted in a five-fold increase in DHE oxidation. Although these results give no insight into the mechanism by which added GSH increases DHE oxidation, based on controls experiments carried out by us, which revealed the absence of an effect of GSH on DHE oxidation, and on similar observations previously reported by Fink et al.[33], we conclude that the effect of the tripeptide would not involve a direct oxidative action onto the DHE molecule. Nonetheless, our finding that SOD totally prevented the GSH-dependent increase in DHE oxidation (result not shown) indicates that, as seen before for the complex alone, the mechanism by which GSH induces its effect would also depend on the free occurrence of superoxide anions in the media. To investigate whether the interaction between GSH molecules and the Cu(I)–$[GSH]_2$ complex somehow involves an oxidative consumption of the tripeptide, the formation of oxidized glutathione was measured in a DHE-free system. As shown in the insert to Figure 3, the addition of GSH (25–500 μM) to a solution containing the complex (8 μM) was associated with a lineal increase in the concentrations of GSSG. A maximal effect of added GSH was attained at a 500 μM concentration.

All the experiments described above were conducted using a Cu(I)–$[GSH]_2$ complex which, as described in Materials and methods, was prepared by mixing directly Cu^{2+} ions and GSH in a 1:3 molar ratio (specifically, at 8 and 24 μM concentration each). As reported previously, molar ratios equal or greater than 1:3 are needed to secure the swift formation of the Cu(I)–$[GSH]_2$ complex.[12,13,28] Since in most biological systems, GSH concentrations largely surpass those of copper ions,[7] we conducted additional experiments to investigate whether the previously-seen increase in DHE oxidation induced by added GSH also manifests when the formation of the Cu(I)–$[GSH]_2$ complex occurs under a much larger molar GSH excess condition; the latter would represent complex-forming conditions which can be considered to resemble better those occurring within cells. To form the complex, a fixed concentration of Cu^{2+} (8 μM) was mixed with increasing concentrations of GSH, ranging from 24 to 1520 μM. Under these conditions, the concentration of Cu^{2+} is limiting, such that the concentration of complex expected to be formed will be—at least initially—equal to 8 μM, regardless of the concentration of added GSH. As shown in Figure 4, the oxidation of DHE induced by the complex was higher when the latter was formed by mixing 8 μM Cu^{2+} with GSH concentrations that were greater than 24 μM (namely, GSH concentrations which exceeded by at least 3-fold that of copper). A maximal increase in DHE oxidation was observed when the concentration of GSH molarly exceeded that of copper by around 60-fold. At a GSH concentration of 1520 μM (corresponding to a molar ratio of 1:190), the DHE oxidation-stimulating effect of GSH was not different from that attained with 504 μM. Inset to Figure 4 depicts the levels of GSSG generated in a DHE-free solution, by the reaction between Cu^{2+} and GSH, when these were mixed in molar ratios which ranged from 1:3 to 1:84. As shown, GSSG levels incremented as the molar excess of GSH was increased, suggesting that the increments in DHE oxidation would be closely associated with an increased oxidative consumption of the tripeptide. Since the maximal increase in DHE oxidation was totally inhibitable by SOD, it could

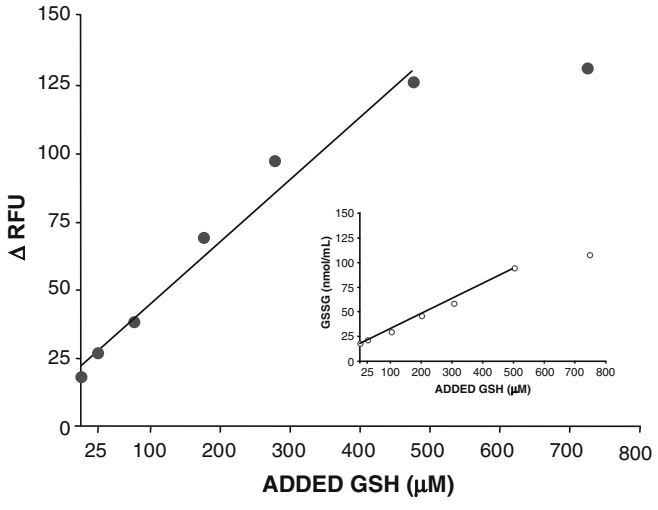

Figure 3. Effect of increasing concentrations of GSH on DHE oxidation and GSSG formation induced by Cu(I)–$[GSH]_2$. DHE (50 μM) was added to solutions containing increasing concentrations of GSH (25–750 μM) and a fixed concentration of Cu(I)–$[GSH]_2$ (8 μM). The increase in fluorescence resulting from DHE oxidation was registered 30 min after and the results expressed as the difference in relative fluorescence units (ΔRFU), as explained in Materials and methods. Insert to the figure shows the effects of adding increasing concentrations of GSH (25–750 μM) to a fixed concentration of Cu(I)–$[GSH]_2$ (8 μM) on the formation of oxidized glutathione. Results were expressed as nmol/mL of GSSG.

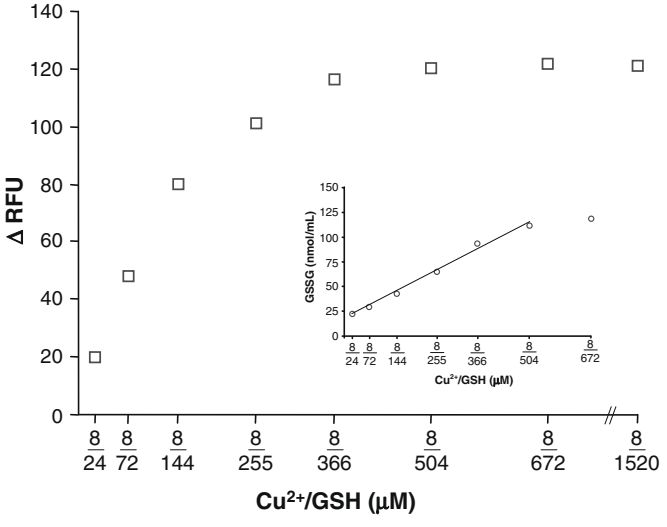

Figure 4. Effects of mixing a fixed concentration of Cu^{2+} with increasing concentrations of GSH on the oxidation of DHE. DHE (50 μM) was added to solutions containing a mixture of increasing concentrations of GSH (24–1520 μM) plus a fixed concentration of Cu^{2+} (8 μM). The increase in fluorescence resulting from the oxidation of DHE was registered 30 min after and the results expressed as the difference in relative fluorescence units (ΔRFU), as explained in Materials and methods. Insert to the figure depicts the effect of adding increasing concentrations of GSH (24–672 μM) to a fixed concentration of Cu^{2+} (8 μM) on the levels of GSSG, expressed as nmol/mL.

be assumed that the increments in DHE oxidation induced by the in-excess GSH results from conditions associated with a greater flux of superoxide anions. As described in Rx. 2, the net flux of superoxide depends on both, the rate at which these radicals are generated through the interaction between molecular oxygen and the Cu(I)–$[GSH]_2$ complex, and the rate at which they are consumed through their reaction with the 'oxidized form' of the former complex (referred to in Rx. 2 as Cu(II)–[Complex]).[28]

$$Cu(I)-[GSH]_2 + O_2 \rightleftarrows Cu(II)-[Complex] + O_2^{\cdot-} \quad (Rx.2)$$

As result of the superoxide-consuming reaction (Rx. 2), the Cu(I)–$[GSH]_2$ complex is regenerated. We suggest that in the presence of DHE, the superoxide anions generated in such reaction would be used partly in regenerating the complex, but also partly diverted towards their reaction with DHE. Thus, we consider that the five-fold increase in DHE oxidation induced by the in-excess GSH could be explained in terms that GSH molecules would 'spare superoxide anions' from being utilized in the regeneration of the complex. The latter implies that, rather than increasing the net flux of superoxide anions, the in-excess GSH would increase, by up to five-fold, the concentration of superoxide radicals available to react with DHE. Likewise the (reaction-reversing) effect of superoxide in Rx. 2, the tripeptide would act directly on the so-called 'oxidized form' of the Cu(I)–$[GSH]_2$ complex, reducing it, and assuring thereby that, at least, a similar flux of superoxide is maintained. Such contention is supported by the observation that the increments in DHE oxidation induced by in-excess GSH are closely paralleled by the pattern of increments in the formation of GSSG. Although GSH is also likely to be oxidized through a reaction with superoxide anions, the fact that DHE oxidation is actually enhanced by in-excess GSH suggests that the proposed superoxide-sparing effect of the tripeptide would occur at a rate that largely surpasses its ability to quench such radicals. Rate constant values reported for the reaction between superoxide and GSH range between 2.2×10^2 and $1.8 \times 10^5\ M^{-1}\ s^{-1}$ (Jones et al.).[37] Thus, the ability of GSH molecules to increase DHE oxidation could result from a reducing action of the tripeptide onto the 'oxidized form' of the Cu(I)–$[GSH]_2$ complex. The latter is schematically represented in Rx. 3.

$$Cu(II)-[Complex] + GSH \rightleftarrows Cu(I)-[GSH]_2 + GSSG \quad (Rx.3)$$

Although the exact chemical nature of the Cu(II)–[Complex] remains to be established, we recently observed that—under conditions that lead to the removal (through the addition of SOD) of the superoxide anions generated in Rx. 2—a Cu(II)-containing complex, whose EPR features were coincident with those of a preformed Cu(II)–GSSG complex (prepared as in Section 4), is accumulated.[28] In view of the latter, we decided to investigate whether, upon interacting with GSH, the Cu(II)–GSSG complex is susceptible to undergo reduction, regenerating the Cu(I)–$[GSH]_2$ complex.

2.3. EPR and NMR studies aimed to address a possible relationship between the Cu(II)–GSSG and the Cu(I)–$[GSH]_2$ complexes

To generate the Cu(II)–GSSG complex, Tempol was added to a solution containing the Cu(I)–$[GSH]_2$ complex. We took advantage of the capacity of Tempol to function as a superoxide-spin trap agent,[38] allowing us to evidence the occurrence of the latter radicals in the media, and as SOD-mimetic agent,[39,40] permitting an effective removal of the superoxide radicals generated by the Cu(I)–$[GSH]_2$. Upon its interaction with Tempol, the Cu(I)–$[GSH]_2$ complex (EPR silent as such) gave place to a paramagnetic spectrum (Fig. 5 A) which, excepting for the signal associated with the presence of Tempol in the mixture, was found to be identical to that of a preformed Cu(II)–GSSG complex (Fig. 5B). To assess a possible relationship between the Tempol-generated Cu(II)–GSSG complex and the Cu(I)–$[GSH]_2$ complex, we investigated the effect of the addition of GSH to a solution containing the former. As shown in Figure 5C, the addition of GSH resulted in the swift disappearance of the EPR signal shown in Figure 5A. The result obtained with Tempol is consistent with our previous observation[28] that upon removal of superoxide anions from Rx. 2 leads to the accumulation of Cu(II)–GSSG (Rx 4), and indirectly suggest that the Cu(II)–[Complex] could correspond to the Cu(II)–GSSG complex. The last referred experiments conducted with GSH, reveal that the latter complex would be susceptible to undergo reduction into an EPR silent specie. To gain some possible insights into the chemical nature of the EPR silent compound, we pursued NMR

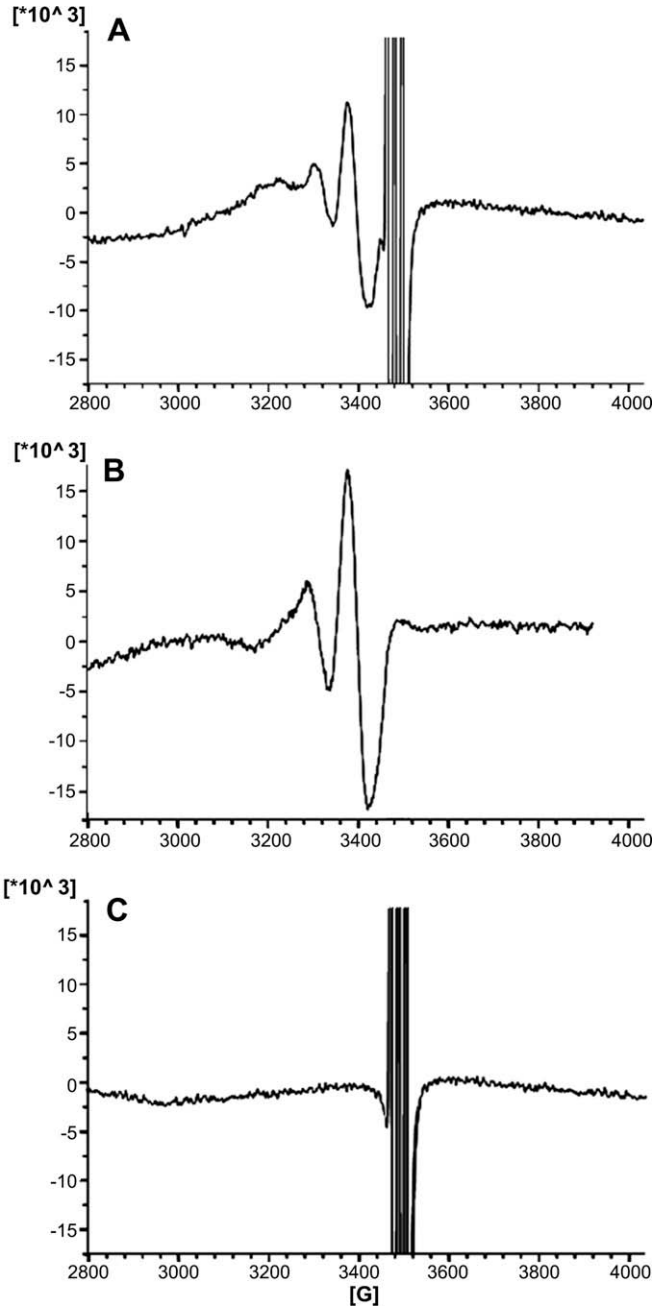

Figure 5. EPR spectra of Tempol-treated and GSH-added Cu(I)–$[GSH]_2$ complex. (A) Spectrum obtained 2 h after the addition of Tempol (4 mM) to a solution containing the Cu(I)–$[GSH]_2$ complex (5 mM). (B) Spectrum of a preformed Cu(II)–GSSG complex (5 mM). (C) Spectrum resulting from the addition of GSH (15 mM) to a solution containing the Tempol-treated Cu(I)–$[GSH]_2$ complex (arising from in A).

studies. For comparative purposes, a typical NMR spectrum of the Cu(I)–$[GSH]_2$ complex is depicted in Figure 6A. This spectrum differs from that of GSSG (depicted in Fig. 6D) in terms of the shape of several of its peaks, but coincides with the peaks of the spectrum reported previously by Ciriolo et al.[13] for the Cu(I)-glutathione complex. The spectrum of Cu(I)–$[GSH]_2$ also differs from that of GSH (Fig. 7D) since the former shows non-equivalence between the protons of the beta-CH_2 moiety of cysteine. The addition of Tempol to the Cu(I)–$[GSH]_2$ complex led to the formation of NMR signals (Fig. 6B) which are coincident with those depicted by a preformed Cu(II)–GSSG complex (Fig. 6C). Interestingly, an identical result was observed in the absence of Tempol, when a solution containing the Cu(I)–$[GSH]_2$ complex was incubated at 37 °C during 24 h (not shown); presumably, such a condition prompts spontaneous superoxide autodismutation. Thus, taken together, the latter results provide additional evidence to support the initially EPR-based contention that superoxide removal from Rx. 2 indeed leads to the formation, and to the accumulation, of Cu(II)–GSSG (Rx. 4). Likewise seen through the EPR experiments, in the presence of an excess of added GSH, the Tempol-treated Cu(I)–$[GSH]_2$ complex mixture was found to exhibit an NMR spectrum (Fig. 7B) which is very similar to that of Cu(I)–$[GSH]_2$ (Fig. 7C). Thus, is seems likely that the effect of GSH seen in Figure 7B be due to a direct reducing action of the tripeptide on the Cu(II)–GSSG complex (Rx. 5).

$$\text{Cu(II)}-[\text{Complex}] + 2O_2^{\cdot-} \rightarrow \text{Cu(II)}-\text{GSSG} + H_2O_2 + O_2 \qquad \text{(Rx.4)}$$

$$2\text{Cu(II)}-\text{GSSG} + 6\text{GSH} \rightarrow 2\text{Cu(I)}-[\text{GSH}]_2 + 3\text{GSSG} \qquad \text{(Rx.5)}$$

To the extent to which GSH is indeed involved in the reductive regeneration of the Cu(I)–$[GSH]_2$ complex, the tripeptide would be sparing superoxide anions from being oxidatively used in such reaction (reversal to Rx. 2). An implication of the latter would be that, in the presence of DHE, those superoxide anions spared by GSH would became available to react with this probe, explaining thereby the increment in DHE oxidation induced by the in-excess GSH. Further support to the contention that the EPR and NMR-based evidence that GSH is able to reduce the Cu(II)–GSSG into the Cu(I)–$[GSH]_2$ complex was obtained through experiments in which we assessed the ability of GSH to induce DHE oxidation when added to a preformed Cu(II)–GSSG complex. As shown in Figure 8, the addition of increasing concentrations of GSH to a fixed concentration of Cu(II)–GSSG (8 μM) led to a GSH-dependent increment in the extent of DHE oxidation. The maximal effect of GSH resulted in an almost 12-fold increase in DHE oxidation. Interestingly, in a DHE-free system, increasing concentrations of GSH added to the Cu(II)–GSSG complex were linearly associated with increasing concentrations of GSSG in the media. The latter result reveals, not only that GSH is oxidatively consumed during its interaction with Cu(II)–GSSG, but also, that superoxide anions are generated in a reaction that is coupled to the reduction of the latter complex.

3. Conclusions

The present study confirms the recently proposed capacity of the Cu(I)–$[GSH]_2$ complex to generate superoxide anions (Rx. 2).[28] It demonstrates that such ability of the complex is concentration-dependent and that, in the absence of superoxide interceptors, a significant part of the superoxide radicals undergo autodismutation, leading to the accumulation of hydrogen peroxide in the media. A potential implication of the latter is that, when redox-active metal ions are present, the co-occurrence of hydrogen peroxide

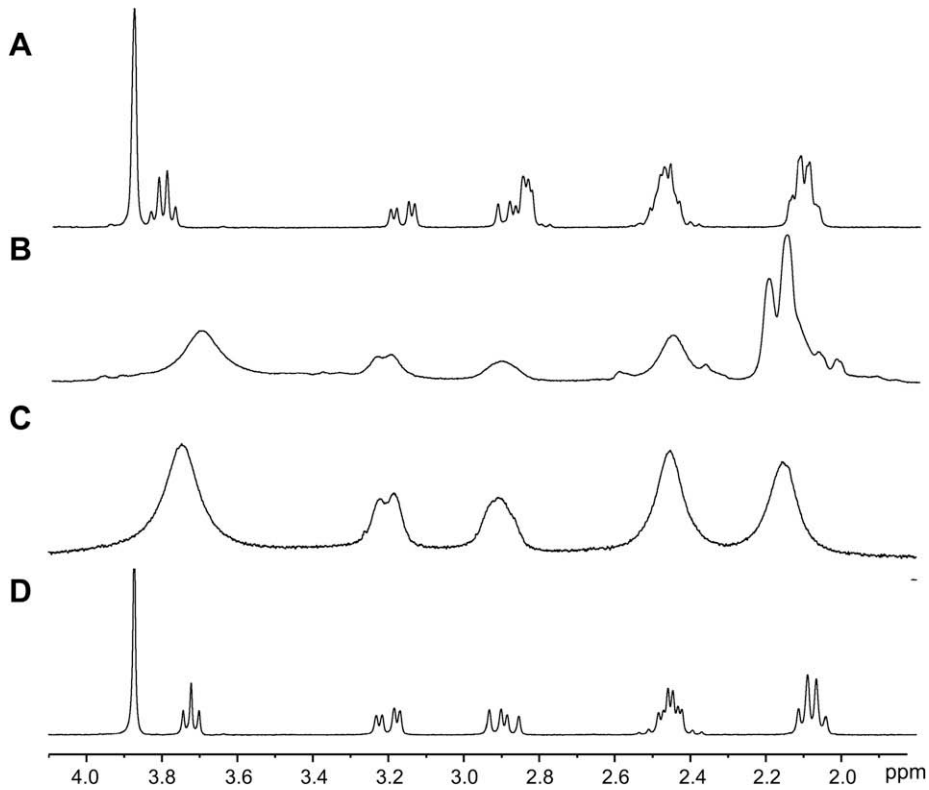

Figure 6. Effect of Tempol on the ^{1}H NMR spectra of the Cu(I)–$[GSH]_2$ complex. (A) Spectrum of the Cu(I)–$[GSH]_2$ complex (3 mM). (B) Same as in A, but obtained 24 h after addition of Tempol (6 mM). (C) Spectrum of a preformed Cu(II)–GSSG complex (3 mM). (D) Spectrum of GSSG (3 mM). Line assignment for spectrum D: 3.87 ppm, Gly-CH_2; 3.72 ppm, α-CH of Glu; 3.20 and 2.89 ppm, β-CH_2 of Cys; 2.45 and 2.08 ppm, γ-CH_2 and β-CH_2, respectively, of Glu. For spectra A, B and C, the signal assignment was similar to that of spectrum D.

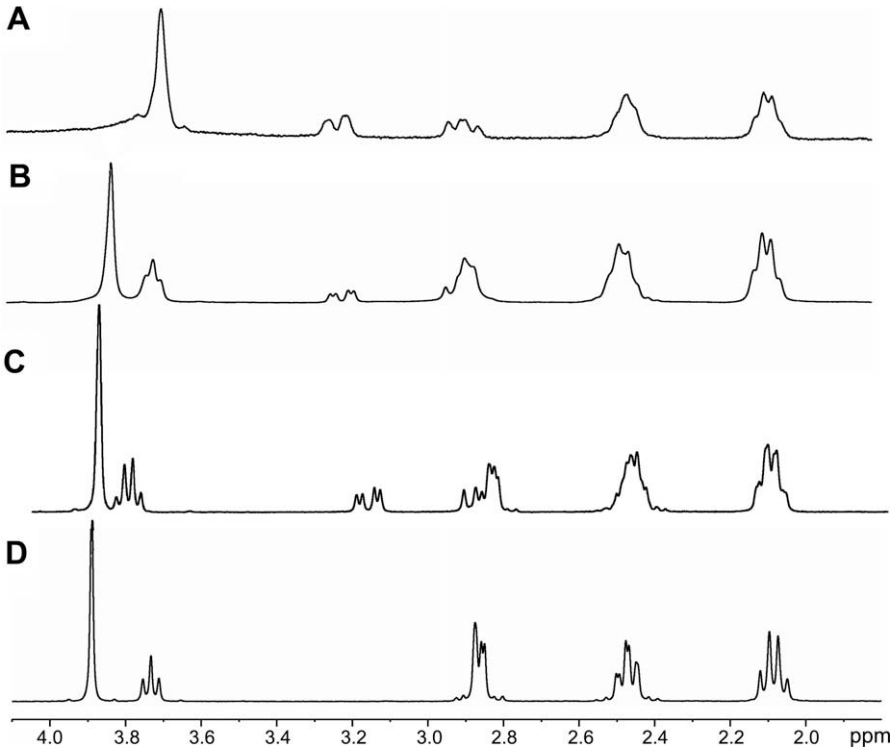

Figure 7. ^{1}H NMR spectra of Tempol-treated and GSH-added Cu(I)–$[GSH]_2$ complex. (A) Spectrum of Cu(I)–$[GSH]_2$ (3 mM) obtained 24 h after addition of Tempol (6 mM). (B) Same as in A, but obtained immediately after addition of GSH (9 mM). (C) Spectrum of Cu(I)–$[GSH]_2$ complex (3 mM). (D) Spectrum of GSH (3 mM). Line assignment for spectrum D: 3.88 ppm, Gly-CH_2; 3.73 ppm, α-CH of Glu; 2.87 ppm, β-CH_2 of Cys; 2.47 and 2.08 ppm, γ-CH_2 and β-CH_2, respectively, of Glu. Spectra A and C are identical to those shown in Figure 6, as B and A, respectively, but are included here to allow a direct comparison between the four spectra.

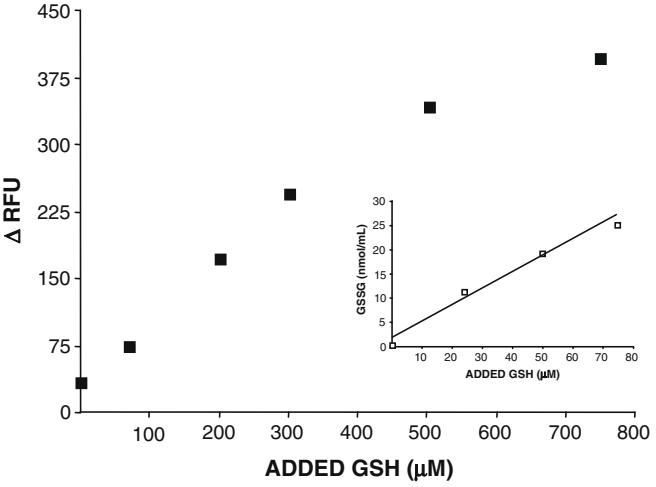

Figure 8. Effect of increasing concentrations of GSH on DHE oxidation and GSSG formation induced by the Cu(II)–GSSG complex. DHE (50 μM) was added to solutions containing increasing concentrations of GSH (75–750 μM) and a fixed concentration of Cu(II)–GSSG (8 μM). The increase in fluorescence resulting from DHE oxidation was registered 30 min after and the results expressed as the difference in relative fluorescence units (ΔRFU), as explained in Materials and methods. Insert to the figure shows the effects of adding increasing concentrations of GSH (25–75 μM) to a fixed concentration of Cu(II)–GSSG (8 μM) on the formation of oxidized glutathione. Results were expressed as nmol/mL of GSSG.

and superoxide anions can easily lead to the formation of hydroxyl radicals, the single most reactive specie in biological systems.[41] Our studies also reveal that in the presence of a molar GSH excess, the capacity of the Cu(I)–$[GSH]_2$ complex to generate superoxide anions could be extended beyond the initial concentration of the complex. Such ability of the tripeptide appears to involve a (superoxide-sparing) reductive regeneration of Cu(I)–$[GSH]_2$ from a putative 'oxidized form' (Cu(II)–[Complex]) of this complex. Removal of superoxide anions from a solution containing the Cu(I)–$[GSH]_2$ complex, by the addition of superoxide interceptors, led to the formation and accumulation of Cu(II)–GSSG, a complex which—as such—is unable to generate superoxide radicals. Interestingly, GSH was found to reduce Cu(II)–GSSG in a reaction which was associated with the generation of superoxide anions. Thus, GSH appears to be able not only to exacerbate superoxide formation through the Cu(I)–$[GSH]_2$ complex (as in Rx. 3 followed by Rx. 2), but to also facilitate the generation of such radicals through a reducing action onto Cu(II)–GSSG (as in Rx. 5 followed by Rx. 2).

4. Materials and methods

4.1. Chemicals and reagents

Cupric chloride ($CuCl_2{\cdot}2H_2O$), deuterium oxide (HOD), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (EC 1.6.4.2 from baker's yeast), bis-*N*-methylacridinium nitrate (lucigenin), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes), hydrogen peroxide and DMSO were all purchased from Sigma–Aldrich. Dihydroethidium (DHE) and 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl (Tempol) were purchased from Calbiochem. Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188) was obtained from Invitrogen-Molecular Probes (Molecular Probes, Inc., OR, USA).

All aqueous solutions were prepared in Chelex-100-treated sodium phosphate buffer (120 mM; pH 7.4).

4.2. Preparation of copper–glutathione complexes

The Cu(I)–$[GSH]_2$ complex was prepared as previously described,[28] by mixing $CuCl_2$ and GSH in a 1:3 molar ratio, respectively. Whenever referring to a given concentration of such complex, it should be understood that it reflects the concentration of copper used in its preparation. The Cu(II)–GSSG complex (referred in the text as preformed), was prepared by direct mixing of $CuCl_2$ and GSSG in a 1:1 molar ratio.[42,43] Unless indicated otherwise, both complexes were prepared always freshly and used—at the most—within 30 min.

4.3. Dihydroethidium oxidation assay

The oxidation of DHE was monitored fluorimetrically in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). Excitation and emission wavelengths were 470 nm and 590 nm, respectively. Freshly prepared DHE, dissolved in DMSO, was added (50 μM) to wells containing the copper–glutathione complexes. Incubations were carried out at 30 °C and readings of fluorescence were obtained after 30 min. When employed (as referred in the text), SOD was added to the wells at 100 U/mL. Results were expressed as ΔRFU (delta relative fluorescence units) and represent the difference in RFU which results from subtracting the fluorescence of DHE alone from that of mixtures of DHE plus the complexes.

4.4. Lucigenin chemiluminescence assay

The reduction of lucigenin was monitored in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). The assays were run at 30 °C, and initiated after the addition of increasing concentrations of the Cu(I)–$[GSH]_2$ complex (10–100 μM) to a solution containing lucigenin (15 μM). Count readings were registered every 22 s intervals and the results expressed as ΔAUC (delta area under curve) for a counting period of 112 s. Controls were run using SOD (250 U/mL) or GSH (300 μM) alone.

4.5. Determination of hydrogen peroxide

Hydrogen peroxide was assessed using the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)-horseradish peroxidase Kit. The formation of resorufin, a red-fluorescence oxidation product which, in the presence of peroxidase, is formed during the interaction between hydrogen peroxide and Amplex Red, was monitored fluorimetrically in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). Excitation and emission wavelengths were 530 nm and 590 nm, respectively. Amplex Red reagent and horseradish peroxidase were added to wells containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (8–25 μM) and readings were carried out at 30 °C. Results were expressed as concentration of H_2O_2 (μM) using a standard curve of hydrogen peroxide. Controls were carried out using Cu^{2+} (25 μM) or GSH (75 μM).

4.6. Determination of oxidized glutathione

GSSG formation was assessed as described by Tietze[44], employing the NADPH/glutathione reductase assay. The decay in $OD_{340\ nm}$ was monitored at 30° C in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). Freshly prepared NADPH (200 μM) and glutathione reductase (2 U/mL) were added to wells containing the copper–glutathione complexes. Results were estimated from a standard curve of GSSG and expressed as nmol of GSSG/mL. Controls were carried out using Cu^{2+} (8 μM) or GSH (1520 μM).

4.7. Electron paramagnetic resonance (EPR) studies

The presence or absence of paramagnetic signals was assessed by EPR in solutions containing the Cu(I)–$[GSH]_2$ complex (5 mM) treated with Tempol (4 mM) during 2 h at 37 °C, or in the same Tempol-treated complex added GSH (15 mM) 1 min before assessment. For comparative purposes, the EPR spectrum of a solution containing a preformed Cu(II)–GSSG complex (5 mM) was assessed. Spectra were recorded in a Bruker ECS 106 spectrometer, using an X band (9.85 GHz), a rectangular cavity and 50 kHz field modulation at 22 °C, under the following conditions: frequency, 9.79 GHz; centre field, 3180 G; amplitude modulation, 0.9 G; microwave power 25 mW; time constant 20 ms, time scan 40 s.

4.8. Nuclear magnetic resonance (NMR) studies

^1H NMR spectra of solutions (prepared in 50% deuterated, sodium phosphate buffer, 120 mM, pH 7.4) containing the Cu(I)–$[GSH]_2$ complex (3 mM) treated with Tempol (6 mM) during 2 h at 37 °C, or in the same Tempol-treated complex added GSH (9 mM) 1 min before assessment, were recorded at 300 MHz, using a Bruker AVANCE DRX 300 spectrometer. The spectra were recorded with solvent suppression of the water proton resonance. The resonance at 4.7 ppm, due to residual solvent HOD, was used as internal reference. 64 decays were accumulated on a spectral width of 5 kHz, using a time domain of 16 k data points. Spectra were acquired at 298 K.

4.9. Data expression and analysis

Data points represent the means of at least 3 independent experiments, each conducted in triplicate. The SD of such data was not included as these generally represented less than 10% of the means. When evaluated, statistical significance of the difference between points was assessed using the Student's *t* test. Differences at $p < 0.05$ were considered significant. GRAPHPAD PRISM 4 was used as statistical software.

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