

# Double edge redox-implications for the interaction between endogenous thiols and copper ions: In vitro studies

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## A B S T R A C T

The present study investigated the redox-consequences of the interaction between various endogenous thiols (RSH)-glutathione, cysteine, homocysteine,  $\gamma$ -glutamyl-cysteine, and cysteinyl-glycine- and  $\text{Cu}^{2+}$  ions, in terms of their free radical-scavenging, ascorbate-oxidizing and  $\text{O}_2^{\cdot-}$ -generating properties of the resulting mixtures. Upon a brief incubation (3–30 min) with  $\text{Cu}^{2+}$ , the free radical-scavenging properties (towards ABTS<sup>+</sup> and DPPH<sup>•</sup>) and thiol-titratable groups of the RSH added to the mixtures decreased significantly. Remarkably, both effects were only partial, even in the presence of a large molar  $\text{Cu}^{2+}$ -excess, and were unaffected despite increasing the incubation time. At equimolar concentrations, the RSH/ $\text{Cu}^{2+}$  mixtures led to the formation of (EPR paramagnetic) Cu(II)-complexes that were time-stable and ascorbate-reducible, but redox-inactive towards oxygen. In turn, at a slight molar thiol-excess (3:1), the mixtures resulted in the formation of time-stable Cu(I)-complexes (EPR silent) that were unreactive towards ascorbate and oxygen. The only exception was seen for the thiol, glutathione, whose mixture with  $\text{Cu}^{2+}$  mixture displayed a  $\text{O}_2^{\cdot-}$ -generating capacity (cytochrome *c*- and lucigenin-reduction). The data indicate that, depending on their molar ratio, the interaction between  $\text{Cu}^{2+}$  and the tested thiols would give place to mixtures containing either: (i) time-stable and ascorbate-reducible Cu(II)-complexes which display free radical-scavenging properties, or (ii) time-stable but redox-inactive towards oxygen Cu(I)-complexes. Among the latter, the only exception was that of glutathione.

### Keywords:

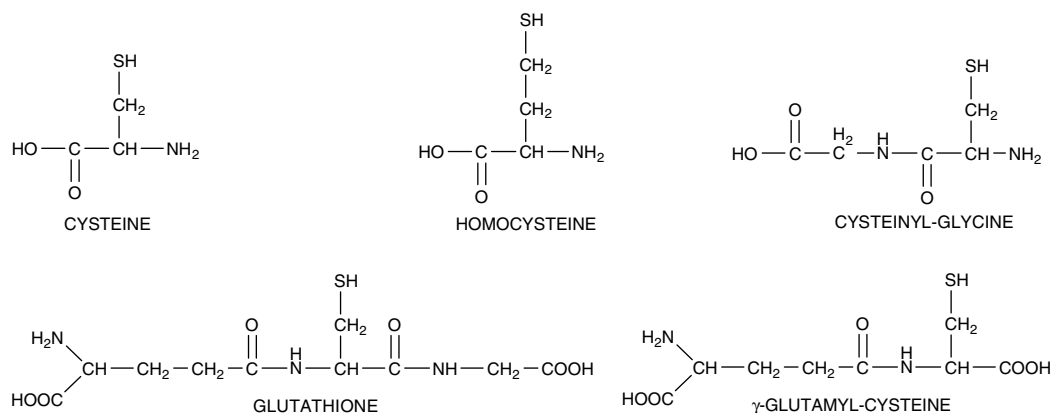
Endogenous thiols  
Glutathione  
Cysteine  
Homocysteine  
 $\gamma$ -Glutamyl-cysteine  
Cysteinyl-glycine  
Copper ions  
Copper complexes  
Redox implications  
ABTS<sup>+</sup>  
DPPH<sup>•</sup>  
DTNB  
Iodine titration  
EPR  
Ascorbic acid  
Cytochrome *c*  
Lucigenin

## 1. Introduction

Endogenous thiols contribute to the intracellular antioxidant defence system through the dynamic redox thiol equilibrium that exists between their reduced and oxidized form. The tripeptide glutathione (GSH), L- $\gamma$ -glutamyl-L-cysteinyl-glycine, represents the major low-molecular-mass non-protein thiol compound.<sup>1</sup> Other small molecular weight thiols which contribute to the intracellular thiol pool are: as precursors for the synthesis of GSH, the aminoacids homocysteine and cysteine, and the dipeptide  $\gamma$ -glutamyl-cysteine, and as first product of GSH degradation, the dipeptide cysteinyl-glycine<sup>1,2</sup> (Fig. 1). A common feature of these compounds is the presence of a sulfhydryl group in their structure. The latter endows these thiols with the capacity to scavenge free radicals, exerting their antioxidative properties.<sup>3</sup> On the other hand; however, the same moiety endows them with the ability

to reduce free transition metals (i.e., copper ions). Since the latter are capable of catalyzing the formation of superoxide from molecular oxygen, thiols should also be considered for their potential to contribute towards oxidative stress generation.<sup>4,5</sup> Several studies have addressed the effects that the interaction between endogenous thiols and copper ions may have on the oxidation of various biological targets. For instance, the co-occurrence of copper and thiols such as GSH,<sup>6,7</sup> homocysteine,<sup>8–10</sup> or cysteine<sup>10–14</sup> is recognized to promote, a greater degree of oxidative damage than that induced by copper ions alone. However, there are as well evidences indicating that an association between copper and thiols, such as the GSH,<sup>15–17</sup> or homocysteine,<sup>12,18,19</sup> can either ameliorate or even prevent the oxidative damage induced by the metal. Thus, taken as a whole, the evidences suggest that in the presence of copper ions, some thiols may have a metal-dependent double-edge redox behavior. Yet, in addition to the redox-implications (antioxidant vs pro-oxidant consequences) resulting from the association between copper ions and some thiols, the interaction between the latter species appears to be also of significance as a mechanism

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**Figure 1.** This picture resums the structures of the tested thiols.

by which incoming copper ions can be handled by the cell. The latter would be particularly important in the case of glutathione, cysteine, and homocysteine, since various studies point out not only to the formation of a complex between these thiols and copper,<sup>4,20–23</sup> but also indicate that, by ‘sequestering copper ions’ some of the resulting complexes could play a cell-protecting effect against the otherwise indiscriminate binding of the metal to essential macromolecules.<sup>15,16,24,25</sup>

In addition to its ability to form a complex with GSH, copper ions can also strongly bind cysteine forming a polymeric complex which bridges Cu(I) through a thiolate sulfur.<sup>22</sup> Likewise, homocysteine has been shown to interact with  $\text{Cu}^{2+}$  ions forming complexes which appear to exhibit some potential physiological actions.<sup>23</sup> Recently, we also observed that upon its reaction with copper, homocysteine generates complexes that, depending on the molar ratio of the interaction, could or not display redox-activity.<sup>26</sup>

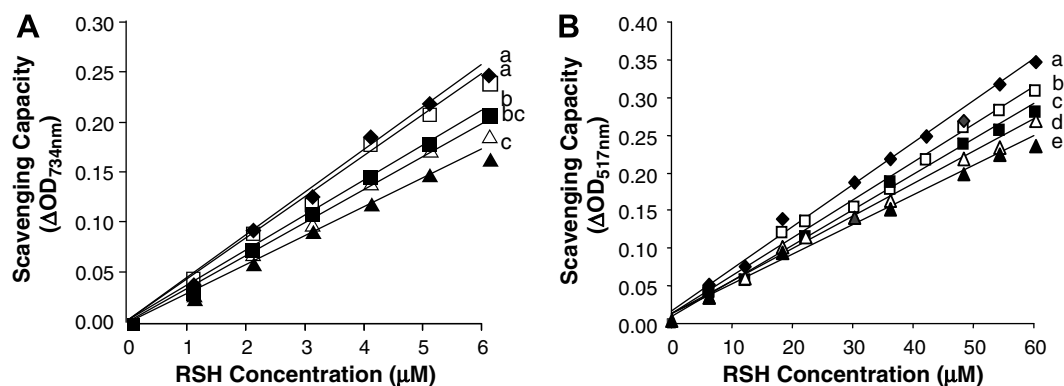
Considering that the above-referred complexes may underlie some of the, as yet controversial, antioxidant versus pro-oxidant actions associated with the thiol-copper interaction, in the present study we have addressed the redox-implications of such interaction. Towards that end, we investigated the ability of various endogenous thiols to either generate or to scavenge free radicals in the presence of molecular oxygen and copper ions.

## 2. Results

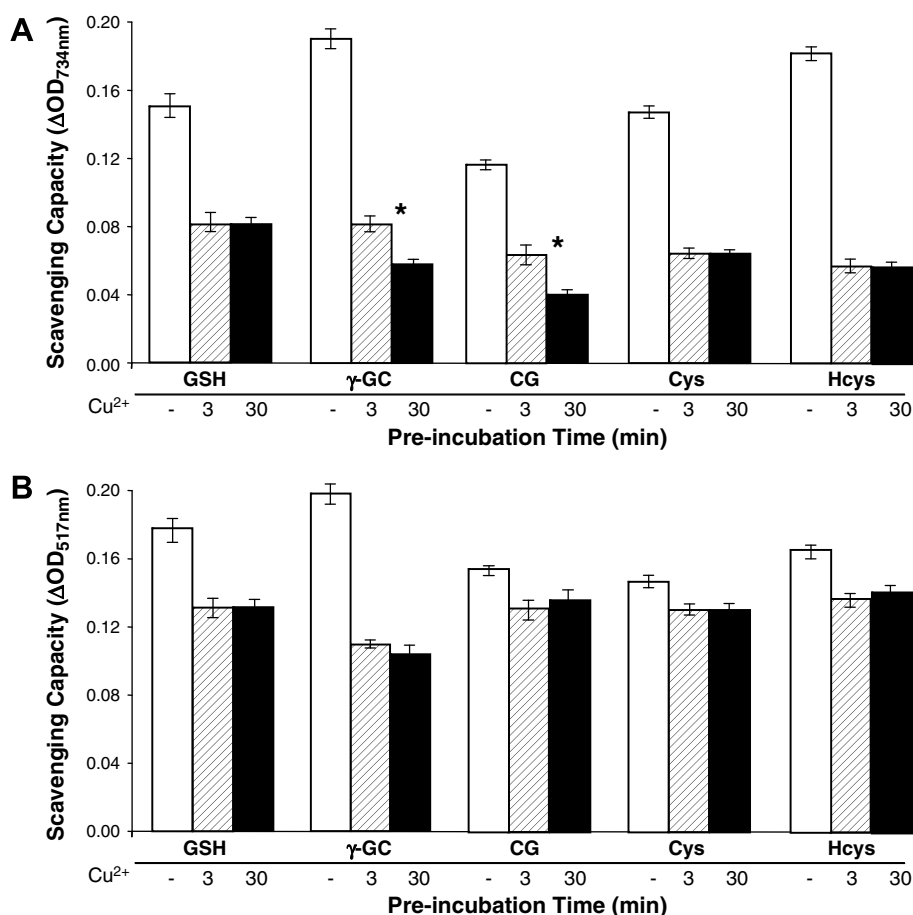
### 2.1. Free radical-scavenging capacity of the thiols: effects of $\text{Cu}^{2+}$ ions

The free radical-scavenging capacity of the thiols—glutathione, cysteine (Cys), homocysteine (Hcys),  $\gamma$ -glutamyl-cysteine ( $\gamma$ -GC), and cysteinyl-glycine (CG)—was evaluated using two different spectrophotometric assays: the bleaching of the ABTS radical cation and the decolorization of the DPPH radical. The ABTS<sup>•+</sup> assay represents an extremely simple and direct method to assess the free radical-scavenging properties of a variety of compounds.<sup>27</sup> Here, we have used such methodology to address the ability of the above thiols to scavenge ABTS<sup>•+</sup> radicals, both before and after their interaction with  $\text{Cu}^{2+}$  ions. Figure 2A shows that, in absence of added metals, the five tested thiols (RSH) bleach ABTS<sup>•+</sup> in a concentration dependent manner. They differ slightly from each other in their order of reactivity, as follows:  $\gamma$ -GC > Hcys > GSH > Cys > CG. When assessed through the DPPH-scavenging assay, such order was fully conserved, supporting the ranking order seen previously in their reaction with ABTS<sup>•+</sup> (Fig. 2B).

Figure 3A and B depicts the results from experiments addressing the effects of the interaction between these thiols and  $\text{Cu}^{2+}$  ions on the scavenging capacity (SC) of the former. The pre-incubation of each of the tested thiols with a fixed concentration of  $\text{Cu}^{2+}$  (molar



**Figure 2.** (A) Concentration-dependence of the bleaching of ABTS<sup>•+</sup> induced by various endogenous thiols. Increasing concentrations of RSH (1–6  $\mu\text{M}$ ) were added to a solution containing ABTS<sup>•+</sup>. The scavenging capacity ( $\Delta\text{OD}_{734\text{nm}}$ ) of the thiols was estimated as the difference between the OD at 734 nm at time zero and that seen 3 min after their addition (longer times, up to 30 min, gave identical results). ■ GSH ( $y = 0.036x - 0.001$ ,  $r^2 = 0.997$ )<sup>b</sup>; ◆  $\gamma$ -GC ( $y = 0.043x + 0.003$ ,  $r^2 = 0.991$ )<sup>a</sup>; ▲ CG ( $y = 0.029x + 0.002$ ,  $r^2 = 0.992$ )<sup>c</sup>; △ Cys ( $y = 0.034x + 0.001$ ,  $r^2 = 0.990$ )<sup>bc</sup>; □ Hcys ( $y = 0.041x + 0.005$ ,  $r^2 = 0.993$ )<sup>a</sup>. Curves with unlike superscript letters were significantly different ( $p < 0.05$ ). (B) Concentration-dependence of the bleaching of DPPH induced by various endogenous thiols. Increasing concentrations of RSH (6–60  $\mu\text{M}$ ) were added to a solution containing DPPH. The scavenging capacity ( $\Delta\text{OD}_{517\text{nm}}$ ) of the thiols was estimated as the difference between the OD at 517 nm at time zero and that seen 40 min after their addition. ■ GSH ( $y = 0.0047x + 0.009$ ,  $r^2 = 0.995$ )<sup>b</sup>; ◆  $\gamma$ -GC ( $y = 0.0056x + 0.0173$ ,  $r^2 = 0.991$ )<sup>b</sup>; ▲ CG ( $y = 0.0039x + 0.0138$ ,  $r^2 = 0.989$ )<sup>c</sup>; △ Cys ( $y = 0.0043x + 0.0127$ ,  $r^2 = 0.993$ )<sup>d</sup>; □ Hcys ( $y = 0.0050x + 0.0138$ ,  $r^2 = 0.989$ )<sup>e</sup>. Curves with unlike superscript letters were significantly different ( $p < 0.05$ ).



**Figure 3.** (A) Effects of copper ions on the ABTS<sup>+</sup>-bleaching capacity of endogenous thiols. The scavenging capacity (SC) of pre-incubated (▨: 3 min; ■: 30 min) mixtures of the thiols (4 μM) with Cu<sup>2+</sup> (12 μM) was assayed, and compared with the SC displayed by the thiols alone (□). SC values obtained at 3 and 30 min of pre-incubation were significantly different ( $p < 0.05$ ) only for the γ-GC/Cu<sup>2+</sup> and CG/Cu<sup>2+</sup> mixtures. In all cases, the SC values were identical when assayed 3 or 10 min after the addition of the mixtures to the ABTS<sup>+</sup>-containing solution. (B) Effects of copper ions on the DPPH radical-scavenging activity of endogenous thiols. The DPPH radical-scavenging activity of pre-incubated (▨: 3 min; ■: 30 min) mixtures of the thiols (36 μM) with Cu<sup>2+</sup> (108 μM) was assayed, and compared with the SC displayed by the thiols alone (□). In all cases, the OD<sub>517nm</sub> were registered 40 min after the addition of the mixtures to the DPPH-containing solution.

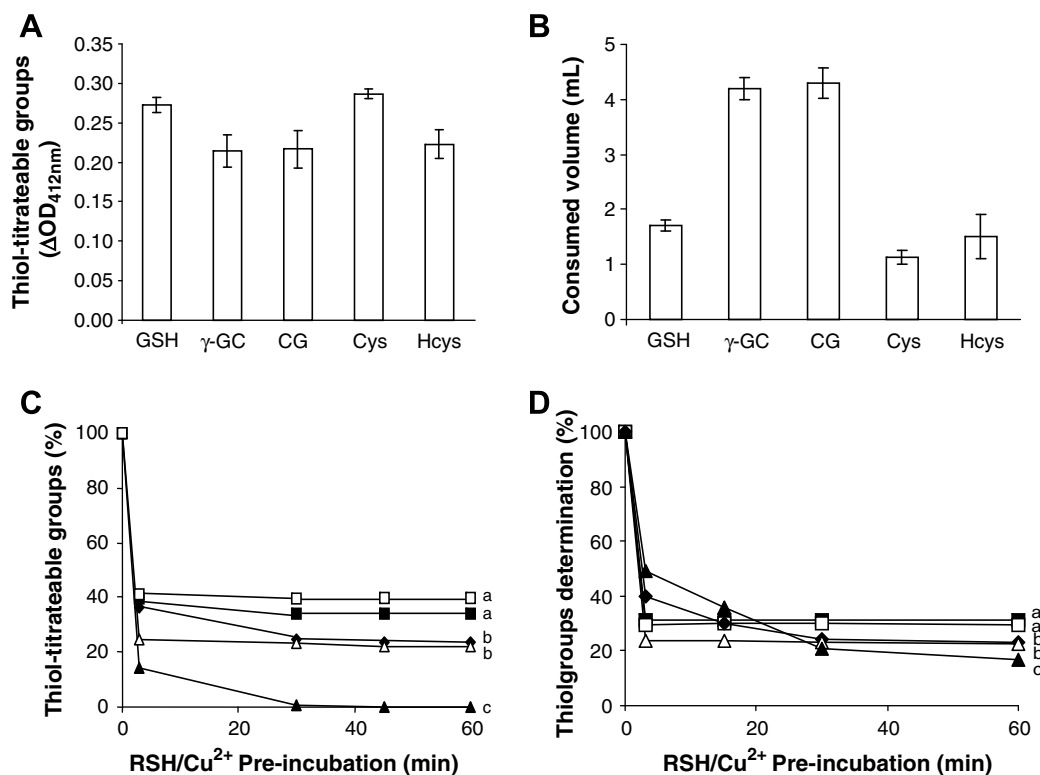
ratio RSH/Cu<sup>2+</sup> 1:3) led to a significant but only partial decrease of the SC seen in absence of the metal (Fig. 3A and B). Identical results were seen when Cu<sup>2+</sup> ions were added at a concentration 6-fold higher than that of the thiol (data not shown). Figure 3A and B show that the highest SC-decreasing effect of Cu<sup>2+</sup> ions was exerted on the γ-GC molecule, which dropped its initial SC by 70% (in the ABTS<sup>+</sup> assay) and by 54% (in the DPPH assay), respectively. As shown in Figure 3A, which depicts the results from using ABTS<sup>+</sup> as assay, the molecule least affected by the presence of copper ions was GSH (whose SC dropped by 45%); Figure 3B, reveals that when DPPH was used as assay for the SC, Cys was the least affected thiol (drop by 8%). Notably, for most of the thiols, the SC-decreasing effects of Cu<sup>2+</sup> did not differ (statistically) when these were pre-incubated with Cu<sup>2+</sup> ions during 30 min instead of 3 min (Fig. 3A and B); the only exceptions being the γ-GC and CG molecules, which upon extending the pre-incubation time underwent a further decrease in their SC (Fig. 3A). Prolonging further the pre-incubation time to 60 min resulted in no additional drop in the SC of none of the studied RSH/Cu<sup>2+</sup> mixtures (data not shown).

## 2.2. Thiols determination: effects of Cu<sup>2+</sup> ions

With the aim of getting information about the capacity of the thiols to reduce DTNB (referred as thiol-titratable groups-TTG) or an iodine solution, each one of them was evaluated. In both assays, the order of reactivity obtained was: Cys > GSH ≥ Hcys >

CG ≥ γ-GC (Fig. 4A and B). To assess whether the decrease in the SC resulting from the interaction between the tested RSH and Cu<sup>2+</sup> ions leads to a copper-dependent change in the determination of thiols present in the mixtures, the reactivity of the latter towards DTNB or iodine solution was measured. We found that the results from applying these two assays correlate closely. As seen in Figure 4 C and D, the pre-incubation of a fixed concentration of each thiol with copper ions (in a molar ratio RSH/Cu<sup>2+</sup> 1:3) led to a swift and marked decrease in their TTG and their capacity to reduce iodine. Identical results were obtained using a molar ratio RSH/Cu<sup>2+</sup> 1:6 (data not shown). While Hcys and GSH were the thiols least affected after their interaction with Cu<sup>2+</sup>, dropping its original TTG by 60% (Fig. 4C) and its reducing capacity toward iodine by 30% (Fig. 4D), CG was the most affected one, leading after 60 min of pre-incubation, in both assays. Extending further the pre-incubation time to 120 min resulted in no additional drop in the reducing capacity toward iodine of the CG/Cu<sup>2+</sup> mixtures (data not shown). In the case of the thiols GSH, Cys, and Hcys, the decreasing thiol determination effect of Cu<sup>2+</sup> was already maximal after 3 min of pre-incubation.

Table 1 compares, the percentage of remaining thiols (reducing capacity) in RSH/Cu<sup>2+</sup> mixtures pre-incubated during 30 min, with the percentage of SC against ABTS<sup>+</sup> and DPPH that remains in the same mixtures. In all cases, the percentage of remaining thiols by the mixtures were lower than those seen for their SC; the sole exception was for the Hcys/Cu<sup>2+</sup> mixture, whose percentage of



**Figure 4.** (A) Thiol-titratable groups of the different thiols. Each thiol (20  $\mu\text{M}$ ) was assayed for its reactivity towards DTNB. The  $\text{OD}_{412\text{nm}}$  was measured after the addition the pre-incubated samples to a solution containing the Ellman's reactive. Results are expressed as  $\Delta\text{OD}_{412\text{nm}}$  explained in Section 5. Curves with unlike subscript letters were significantly different ( $p < 0.005$ ). (B) Thiol groups determination by iodine titration. This method establishes the thiol (0.25 mM) concentration in a solution by a redox titration using iodine. At the end point of the titration, the volume consumed of each thiol was recorded. The result was expressed as the volume consumed of tested thiols. Curves with unlike subscript letters were significantly different ( $p < 0.005$ ). (C) Effects of copper ions on the thiol-titratable groups of the RSH/ $\text{Cu}^{2+}$  mixtures. Each thiol (20  $\mu\text{M}$ ) was pre-incubated in the presence of  $\text{Cu}^{2+}$  ions (60  $\mu\text{M}$ ) during various times (from 0, 3, 30, 45, or 60 min) and subsequently assayed for its reactivity towards DTNB. The  $\text{OD}_{412\text{nm}}$  was measured after the addition the pre-incubated samples to a solution containing the Ellman's reactive. Identical results were seen when the DTNB assay was run for 3 or 30 min. ■ GSH/ $\text{Cu}^{2+}$ ; ◆  $\gamma\text{-GC}/\text{Cu}^{2+}$ ; ▲ CG/ $\text{Cu}^{2+}$ ; △ Cys/ $\text{Cu}^{2+}$ ; □ Hcys/ $\text{Cu}^{2+}$ . Results are expressed as % of TTG as explained in Section 5. Curves with unlike subscript letters were significantly different ( $p < 0.005$ ). (D) Effects of copper ions on the thiol groups determination of the RSH/ $\text{Cu}^{2+}$  mixtures. Each thiol (0.25 mM) was pre-incubated in the presence of  $\text{Cu}^{2+}$  ions (0.75 mM) during various times (from 0, 3, 30, 45, or 60 min) and subsequently assayed for iodine titration. At the end point of the titration, the volume consumed of the mixture RSH/ $\text{Cu}^{2+}$  was recorded. ■ GSH/ $\text{Cu}^{2+}$ ; ◆  $\gamma\text{-GC}/\text{Cu}^{2+}$ ; ▲ CG/ $\text{Cu}^{2+}$ ; △ Cys/ $\text{Cu}^{2+}$ ; □ Hcys/ $\text{Cu}^{2+}$ . The result was expressed as the percentage of remained thiol as reducing agent, as explained in Section 5. Curves with unlike subscript letters were significantly different ( $p < 0.005$ ).

TTG was higher than that seen for its SC against  $\text{ABTS}^{\cdot+}$ . In the case of CG plus  $\text{Cu}^{2+}$ , the mixture still conserved about 21% of its reducing capacity toward iodine, despite undergoing a complete loss of reducing capacity toward DTNB. Moreover, such mixture maintained about 88% of its SC against  $\text{ABTS}^{\cdot+}$ , and about 33% of its SC against DPPH $\cdot$ .

### 2.3. EPR studies on the RSH/ $\text{Cu}^{2+}$ mixtures

Compared to a typical EPR spectrum of  $\text{Cu}^{2+}$  ions (Fig. 5A), mixtures of any of the tested thiols with  $\text{Cu}^{2+}$  (pre-incubated during

**Table 1**  
Comparison of the percentage of remaining thiols (reducing capacity) in RSH/ $\text{Cu}^{2+}$  mixtures pre-incubated during 30 min, with the percentage of SC against  $\text{ABTS}^{\cdot+}$  and DPPH $\cdot$  that remains in the same mixtures

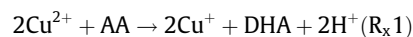
RSH/ $\text{Cu}^{2+}$	Reducing capacity (%)		Scavenging capacity (%)	
	DTNB	Iodine solution	$\text{ABTS}^{\cdot+}$	DPPH $\cdot$
GSH/ $\text{Cu}^{2+}$	36	31	55	76
$\gamma\text{-GC}/\text{Cu}^{2+}$	26	24	29	54
CG/ $\text{Cu}^{2+}$	0	20	33	88
Cys/ $\text{Cu}^{2+}$	24	23	44	92
Hcys/ $\text{Cu}^{2+}$	40	30	31	72

The percentage of scavenging capacity was estimated from multiplying by 100 the ratio of the SC shown by each thiol in the presence versus absence of  $\text{Cu}^{2+}$  ions (SC data from Figure 3A and B were used). The percentage of the reducing capacity toward DTNB and iodine solution corresponds to the data shown in Figure 4B and D, respectively.

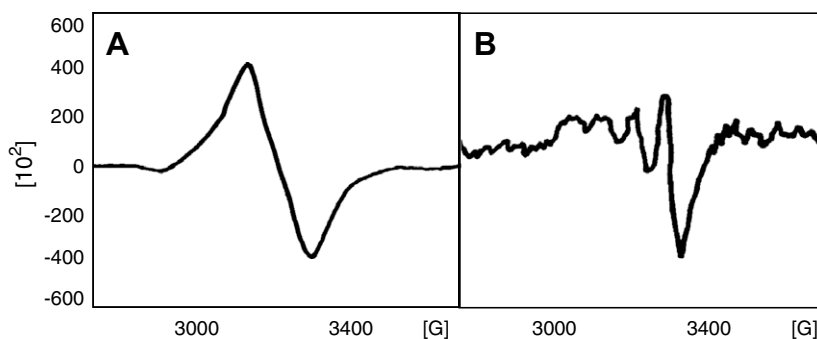
30 min at an equimolar ratio) resulted in the formation of EPR signals which are typically associated with the occurrence of paramagnetic  $\text{Cu(II)}$ -complexes.<sup>28</sup> The latter is exemplified in Figure 5B, which shows an EPR spectrum obtained after pre-incubating during 30 min a mixture of Hcys with  $\text{Cu}^{2+}$ .<sup>26</sup> This spectrum is representative of the spectra obtained when each of the tested thiols was pre-incubated with copper ions (in a molar ratio of 1:1). An spectrum identical to that shown in Figure 5B was observed when mixtures (1:1) of each of the tested thiols was pre-incubated with copper during 24 h instead of 30 min (not shown). An identical paramagnetic signal of  $\text{Cu(II)}$ -complexes was also seen when the thiols were pre-incubated in the presence of a copper molar excess (3:1). The paramagnetic spectra were no longer seen, however, when any of the thiols was pre-incubated in a 3:1 molar excess respect to  $\text{Cu}^{2+}$  (not shown).

### 2.4. Effects of thiols on $\text{Cu}^{2+}$ -dependent oxidation of ascorbate

$\text{Cu}^{2+}$  ions are capable of inducing the oxidation of ascorbic acid (AA), ( $\text{R}_x1$ ).<sup>29</sup>



In order to understand the redox properties of the  $\text{Cu(II)}$ -containing complexes, putatively formed during the above-referred pre-incubation, the effect of each thiol on the ability of  $\text{Cu}^{2+}$  to catalyze AA oxidation was studied. Table 2 shows the percentage of AA oxidation induced by both, a non-pre-incubated mixture of



**Figure 5.** EPR spectra of  $\text{Cu}^{2+}$  ions in the absence or in the presence of thiol. (A) Typical EPR spectrum of  $\text{Cu}^{2+}$  ions (5 mM). (B) Spectrum resulting from the incubation (during 30 min) of  $\text{Cu}^{2+}$  with Hcys (5 mM).

RSH/ $\text{Cu}^{2+}$  (assessed at time zero, namely, upon the direct addition of the mixture to an AA solution), and a similar mixture that had been pre-incubated during various times (up to 300 min). At equimolar concentrations of RSH and  $\text{Cu}^{2+}$  ions (1:1), the magnitude of AA oxidation was considerably higher when mixtures were pre-incubated; the highest AA-oxidizing activity of the thiols was observed with pre-incubated mixtures of  $\text{Cu}^{2+}$  plus the dipeptides  $\gamma$ -GC and CG. In the case of mixtures prepared with an excess of thiol (3:1), the addition of pre-incubated mixtures has, either a slightly stimulating (CG), a slightly inhibitory (GSH and Hcys), or simply no effect ( $\gamma$ -GC and Cys) on the extent of AA oxidation. The effect of these RSH/ $\text{Cu}^{2+}$  mixtures on AA oxidation was largely unaffected when the time of pre-incubation was extended from 3 to 300 min (Table 2).

### 2.5. Evaluation of a superoxide-generating activity in RSH/ $\text{Cu}^{2+}$ mixtures through the cytochrome c reduction assay

Based on the ability of  $\text{Cu}^{2+}$  ions to generate superoxide radicals during their interaction with oxygen, the ability of pre-incubated RSH/ $\text{Cu}^{2+}$  mixtures to either promote or inhibit  $\text{O}_2^{\cdot-}$ -dependent Cyt c reduction was evaluated.

Figure 6A and B depicts the effect of the addition of mixtures of the thiols GSH,  $\gamma$ -GC, CG, or Hcys with  $\text{Cu}^{2+}$  on the reduction of Cyt c. As shown in Figure 6A, all these thiols were effective in promoting the reduction of Cyt c when added directly (namely, without pre-incubation) with  $\text{Cu}^{2+}$ . These effects were markedly increased (by over 3-fold) when the concentration of the thiols was raised from 10 to 30  $\mu\text{M}$  (Fig. 6A). Yet, neither the equimolar nor the 3:1 molar ratio mixtures of these thiols with  $\text{Cu}^{2+}$  ions was able to promote Cyt c reduction when added as pre-incubated mixtures (Fig. 6B), excepting for GSH. In fact, the pre-incubated GSH/ $\text{Cu}^{2+}$

mixtures (3:1) were found to be highly effective in promoting the reduction of Cyt c; the addition of 250 U/mL of SOD to the assay prevented by over 90% such reduction (Fig. 6B).

### 2.6. Evaluation of a superoxide-generating activity in RSH/ $\text{Cu}^{2+}$ mixtures through a chemiluminescence assay

In addition to using the Cyt c reduction assay, the ability of pre-incubated RSH/ $\text{Cu}^{2+}$  mixtures to generate  $\text{O}_2^{\cdot-}$  was also evaluated using the chemiluminescence-based lucigenin reduction assay. GSH,  $\gamma$ -GC, CG, and Hcys, were effective in reducing lucigenin when added directly with  $\text{Cu}^{2+}$  (at equimolar concentrations; Fig. 7A). The lucigenin-reducing effect of these non pre-incubated mixtures was markedly increased when the concentration of the thiols tripled that of copper (Fig. 7A). On the other hand, when these thiols were pre-incubated with  $\text{Cu}^{2+}$  ions (whether at added at equimolar concentrations or as a 3:1 molar excess) no reducing effect of these mixtures on lucigenin was seen. An exception to the latter was seen when a  $\text{Cu}^{2+}$  plus an excess of GSH were pre-incubated and added to the chemiluminescent probe, as this mixture was able to reduce lucigenin in a reaction which was almost totally prevented by SOD (added at 250 U/well; Fig. 7B). Catalase had no effect on the ability of the pre-incubated GSH plus  $\text{Cu}^{2+}$  (3:1) mixture to reduce lucigenin (data not shown).

## 3. Discussion

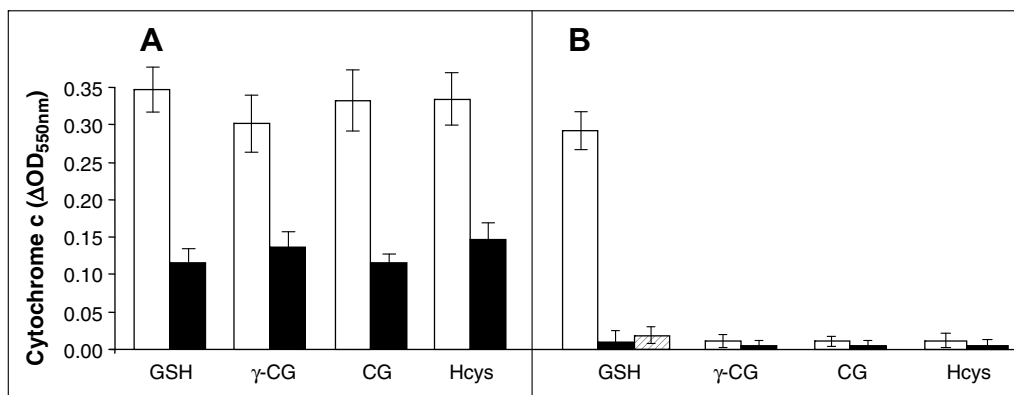
The present study addressed the ability of five endogenous thiols (GSH,  $\gamma$ -GC, CG, Cys, and Hcys) to scavenge  $\text{ABTS}^{\cdot+}$  and DPPH radicals, and evaluated the effect that the interaction between such thiols and  $\text{Cu}^{2+}$  ions has on the free radical-scavenging properties of the former. The results show that, although each of the tested thiols is able to react with  $\text{ABTS}^{\cdot+}$  and DPPH, these differ from each other in terms of the extent to which they scavenge these radicals.  $\gamma$ -GC, followed by Hcys showed the greatest scavenging capacity. The higher scavenging capacity of  $\gamma$ -GC relative to that of GSH suggests that binding of Gly to Cys would negatively affect the scavenging capacity of the tripeptide. In line with this interpretation, CG was found to exhibit a lower scavenging capacity than Cys. On the other hand, since  $\gamma$ -GC was more active than Cys, it would appear that the  $\gamma$ -binding of Glu to Cys acts favorably. The same reasoning applies for the greater scavenging capacity of GSH respect to that of CG. Finally, the slightly greater SC of Hcys relative to Cys would suggest that the presence of a methylene group on the structure of the former favors its ability to scavenge  $\text{ABTS}^{\cdot+}$  and DPPH. The scavenging capacity of each thiol might be directly related to the  $\text{pK}_a$  for the sulfhydryl group in the thiols ( $\text{pK}_a = \text{CG } 6.4$ ; Cys 8.3; GSH 8.83; Hcys 10;  $\text{pK}_a$  for  $\gamma$ -GC has not been reported).<sup>30-32</sup> At a pH of 7.4, those thiols which exhibit a higher  $\text{pK}_a$  value would be expected to exhibit a greater proportion of

**Table 2**

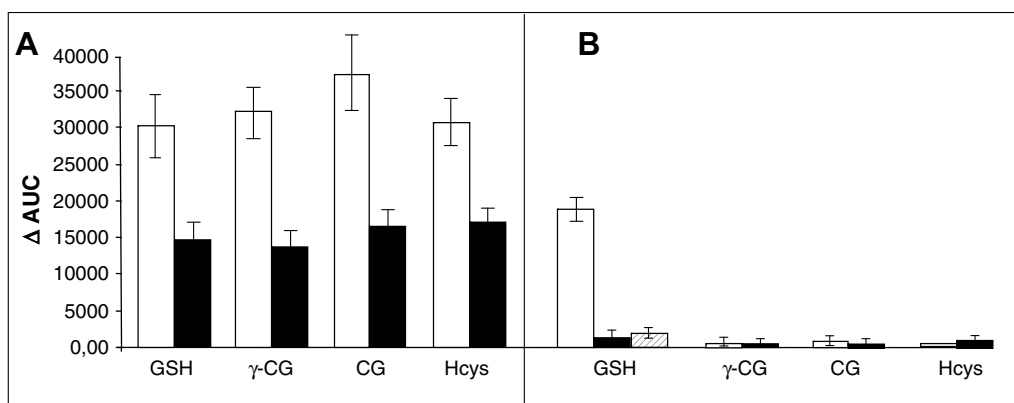
Percentage of AA oxidation induced by both, a non pre-incubated mixture of RSH/ $\text{Cu}^{2+}$  and a similar mixture that had been pre-incubated during various times

RSH/ $\text{Cu}^{2+}$	Pre-incubation time (min)					Molar ratio ( $\mu\text{M}$ )
	0	3	30	60	300	
	Oxidation of ascorbic acid (%)					
GSH/ $\text{Cu}^{2+}$	49 12	81 3	83 2	84 2	85 4	20:20 60:20
$\gamma$ -GC/ $\text{Cu}^{2+}$	58 8	81 4	87 4	89 6	97 6	20:20 60:20
CG/ $\text{Cu}^{2+}$	49 15	79 8	92 28	94 31	94 33	20:20 60:20
Cys/ $\text{Cu}^{2+}$	65 36	74 35	77 34	80 35	80 36	20:20 60:20
Hcys/ $\text{Cu}^{2+}$	37 8	81 1	84 1	86 1	85 3	20:20 60:20

The oxidation of ascorbic acid was induced by the addition of RSH/ $\text{Cu}^{2+}$  mixtures that were either non pre-incubated (time 0) or pre-incubated during a 3–300 min period. The mixtures were prepared as to provide final I, M ratios of either 20:20 or 60:20. The percentage of ascorbic acid oxidation was estimated as described in Section 5.



**Figure 6.** Superoxide-generating activity of RSH/Cu<sup>2+</sup> mixtures (cytochrome *c* assay). The effects of RSH on Cu<sup>2+</sup>-mediated (10 μM) superoxide-dependent cytochrome *c* reduction was tested by adding to a solution containing Cyt *c* (50 μM): non-pre-incubated (A) or pre-incubated (B) mixtures of the thiols with Cu<sup>2+</sup>. (A) Non-pre-incubated mixtures of the thiols 10 μM (■) or 30 μM (□) with Cu<sup>2+</sup>. The (B) Pre-incubated (30 min) mixtures of the thiols 10 μM (■) or 30 μM (□) with Cu<sup>2+</sup>, and the mixtures GSH/Cu<sup>2+</sup> in the presence of 250 U of SOD (▨).



**Figure 7.** Superoxide-generating activity of RSH/Cu<sup>2+</sup> mixtures (lucigenin assay). The effects of RSH on Cu<sup>2+</sup>-mediated (10 μM) superoxide-dependent lucigenin reduction was tested by a chemiluminescence assay. (A) The direct addition of each thiol (10 μM: ■ or 30 μM: □) and Cu<sup>2+</sup> (10 μM) to a solution containing lucigenin (15 μM); (B) depicts pre-incubated (30 min) mixtures of the thiols 10 μM (■) or 30 μM (□) with Cu<sup>2+</sup>, and the mixtures GSH/Cu<sup>2+</sup> in the presence of 250 U of SOD (▨).

their sulfhydryl group under a protonated form. Results indicate that those thiols in which the protonated form predominates (thiol) were found to be more reactive. According to the latter, it is suggested that the ability of these thiols to scavenge the ABTS<sup>•+</sup> and DPPH radicals would involve primarily a hydrogen atom transfer (HAT) mechanism.<sup>33</sup> However, it appears that together with the pK<sub>a</sub> value of the sulfhydryl moiety, the oxidation potential of the thiol might also be important to understand their reactivity. Hcys and GSH are known to have oxidation potentials that are more positive than that of Cys.<sup>34,35</sup> Thus, Cys, which was expected to be a better reducing agent, was found to be more effective than Hcys and GSH in reducing DTNB and iodine.

As stated previously, the scavenging capacity of all tested thiols decreased upon their prior incubation with Cu<sup>2+</sup> ions. In the ABTS<sup>•+</sup> assay, such decrease was only partial and meant conserving around 30–55% of the SC displayed by the thiols alone. However, when the effect of pre-incubating the thiols with Cu<sup>2+</sup> was assessed through the DPPH<sup>•</sup> assay, the mixtures were found to almost totally conserved (from 72% to 92%) their SC. The only exception was that for the mixture containing γ-GC, which retained about 54% of the original SC. From these results, it seems reasonable to assume that the differences in SC between both assays would be related to the mechanisms by which each type of free radical is scavenged. While the HAT and the SPLET (sequential proton loss electron transfer) mechanisms, both appears to be involved in the scavenging of DPPH radicals,<sup>33,36</sup> only the former mechanism is believed to be

involved in the scavenging of ABTS<sup>•+</sup> radicals.<sup>37</sup> Since the SC of the RSH plus Cu<sup>2+</sup> mixtures was higher when assessed in the DPPH<sup>•</sup> assay, we suggest that a SPLET-type would be the primary mechanism underlying the SC of such mixtures.

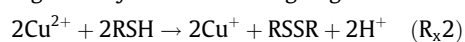
Although the current study does not allow us to unveil the exact mechanism underlying the partial conservation of the SC of the RSH/Cu<sup>2+</sup> mixtures, the results obtained are consistent with the possibility that the pre-incubation allows the formation of certain copper-containing complexes SC.<sup>21,26</sup> For instance, our observing that the decrease in the SC of the mixtures was not further incremented when the concentration of Cu<sup>2+</sup> ions was doubled, or when the time of pre-incubation was increased by 10-fold, is coherent with the interpretation that time-stable complexes, which are also stable to the presence of a large copper-excess are formed. Similarly, we observed that the RSH/Cu<sup>2+</sup> mixtures conserved part of their reducing capacity despite extending the time of pre-incubation of the mixtures and increasing the concentration of copper ions as to exceed by 6-fold that of the thiols (data not shown). Although the latter may suggest that the existence of thiols in the pre-incubated mixtures be relevant towards the maintenance of the SC by the postulated complexes, our results from Table 1 reveal that such relationship would be only weakly sustainable. For instance, data show that there is not correlation between reducing capacity and SC. For example, the mixtures of CG with Cu<sup>2+</sup> presents the highest SC toward DPPH<sup>•</sup>, without exhibiting any TTG. It suggests that the thiol groups present in most of the pre-incu-



bated RSH/Cu<sup>2+</sup> mixtures would not be related to their ABTS<sup>•+</sup>- and DPPH<sup>•</sup>-scavenging properties. Moreover, it is possible that under the assaying conditions, thiols groups are not always titrateable by DTNB (e.g., due to low accessibility or low reactivity), not discarding their absence in the mixtures, due to they still conserve reducing capacity toward iodine solution. Results seem to suggest that the capacity of the mixtures to induce the reduction of DTNB or iodine would be inversely proportional to the pK<sub>a</sub> of the free thiols. In fact, the mixtures containing the thiol CG exhibits the lowest pK<sub>a</sub> value and presents the lowest reducing capacity toward iodine, even such mixtures have not TTG. Mixtures containing Hcys show the highest pK<sub>a</sub> value were associated with one of the highest reducing capacity.

Support for the interpretation that the postulated complexes indeed are formed, stemmed also from the EPR data showing that a paramagnetic signal, typically associated with tetrahedral copper-containing complexes is formed.<sup>28</sup> Such a signal was observed when equimolar concentrations of the tested RSH plus Cu<sup>2+</sup> ions are mixed, or when the thiols are pre-incubated with an excess of the metal; the EPR signal thus observed, was found to disappear upon addition of a molar excess of thiol (3:1). The absence of a paramagnetic signal in the latter RSH/Cu<sup>2+</sup> mixtures is, certainly, no evidence for the formation of Cu(I)-containing complexes (since these are EPR silent). However, the fact that even after 24 h of pre-incubation, the 3:1 RSH/Cu<sup>2+</sup> mixtures showed no paramagnetic signal (neither that of the Cu(II)-complexes nor that of free Cu<sup>2+</sup> ions), could indicate that under thiol-excess conditions, time-stable Cu(I)-containing complexes are formed, as previously suggested by us<sup>21,26</sup> and other authors.<sup>15,20,23</sup>

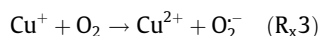
Consistent also with the EPR results showing the presence of Cu(II)-containing complexes in the equimolar RSH/Cu<sup>2+</sup> mixtures, we observed that such mixtures are able to promote ascorbic acid oxidation. Compared to the AA-oxidizing effect of the direct addition of RSH plus Cu<sup>2+</sup> (1:1), the pre-incubated 1:1 mixtures were found to be substantially more effective. Since the pre-incubation leads to the formation of Cu(II)-containing complexes, and considering that the magnitude of the oxidation induced by the 1:1 pre-incubated mixtures approached 100%, it would seem reasonable to suggest that the postulated Cu(II)-containing complexes fully conserve the AA-oxidizing properties displayed by Cu<sup>2+</sup> ions alone.<sup>29</sup> Presumably, when the thiols are added directly, the probability of forming such complexes is minimal. Under such conditions, the thiols would act mainly as Cu<sup>2+</sup>-reducing molecules (R<sub>x</sub>2), sparing thereby AA from undergoing oxidation.



The fact that the oxidation of AA induced by the 1:1 RSH/Cu<sup>2+</sup> mixtures did not increase despite increasing by a 100-fold the pre-incubation time (from 3 to 300 min), suggests that the Cu(II)-complexes contained in such mixtures form swiftly and remain stable along time. On the other hand, the probability that in the mixtures containing thiol-excess (namely, 3:1 RSH/Cu<sup>2+</sup>), Cu(I)-complexes, rather than Cu(II), are formed is coherent with the very limited capacity shown by such mixtures to induce AA oxidation. The fact that such oxidation was marginal at early times (e.g., 3 min) and remained constant along the 300 min of pre-incubation, is also coherent with the interpretation that time- and ascorbic acid-stable Cu(I)-complexes are being formed in the thiol-excess containing mixtures. Regarding the effect of the direct addition of the thiols and Cu<sup>2+</sup> ions (3:1) to a solution containing AA, the markedly low extent of AA oxidation could be attributed to the high AA-sparing capacity given by the molar excess of thiol (R<sub>x</sub>2).

Since the pre-incubations were conducted in an air-containing atmosphere, the apparent time-stability of the 1:1 and 3:1 RSH/Cu<sup>2+</sup> mixtures, inferred from the above-discussed EPR and AA oxidation data, could be construed as evidence that the corre-

sponding complexes are redox-inactive towards oxygen. In fact, the 1:1 and the 3:1 pre-incubated RSH/Cu<sup>2+</sup> mixtures, expected to form Cu(II)- and Cu(I)-complexes, respectively, totally failed to reduce Cyt c and lucigenin. These results reveal that neither of such complexes would be able to generate superoxide anions, and therefore suggest that they would lack redox-activity towards oxygen. In contrast, an important reduction of Cyt c and lucigenin was evident when the thiols and Cu<sup>2+</sup> ions were added directly, whether at equimolar concentrations (1:1) or under thiol-excess conditions (3:1). Under the latter conditions, the postulated complexes are unlikely to be formed, and therefore, the added thiols are left to act mainly as Cu<sup>2+</sup>-reducing molecules (R<sub>x</sub>2), generating Cu<sup>+</sup> which, in turn, would rapidly react with molecular oxygen to form superoxide anions (R<sub>x</sub>3).



Though all tested thiols could be expected to form Cu(I)-complexes when mixed with Cu<sup>2+</sup> (in a 3:1 molar ratio), none of these mixtures was able to reduce Cyt c or lucigenin. The only exception to the latter was that of the GSH plus Cu<sup>2+</sup> mixture. The interaction of the latter specie leads to the formation of a Cu(I)-glutathione complex, which would continually react with the molecular oxygen present in the pre-incubated solution, to generate superoxide anions.<sup>38</sup> Supporting the latter, the presence of 250 U SOD totally inhibited the capacity of Cu(I)-glutathione complex to reduce Cyt c and lucigenin. Since the effect of SOD was not altered by the co-addition of catalase, we suggest that the lost ability of the SOD-added pre-incubated GSH/Cu<sup>2+</sup> mixture to reduce both probes would not be attributable to an accumulation of hydrogen peroxide.

#### 4. Conclusions

The five endogenous thiols tested in the present study (GSH, γ-GC, CG, Cys, and Hcys) were found to display ABTS<sup>•+</sup>- and DPPH<sup>•</sup>-scavenging properties. When pre-incubated in the presence of an excess of copper, the scavenging capacity of the tested thiols was lost but to an extent that depended on both, the structure of the thiol and the nature of the applied assay. In general, the loss was only partial when assessed through the ABTS<sup>•+</sup> assay and almost null on the DPPH<sup>•</sup> assay. The mixtures resulting from pre-incubating RSH plus Cu<sup>2+</sup> excess (molar ratio 1:3) were found to (i) retain part of their TTG, being able to reduce DTNB and an iodine solution; and (ii) scavenge the ABTS<sup>•+</sup> and DPPH<sup>•</sup> free radicals; a SPLET mechanism is proposed to underlie the ability of the RSH/Cu<sup>2+</sup> mixtures to scavenge both type of radicals. EPR evidence supports the occurrence of time-stable Cu(II)-complexes in pre-incubated RSH/Cu<sup>2+</sup> mixtures, both at a 1:1 and 1:3 molar ratio. For all thiols, the equimolar mixtures were found to be ascorbate-reducible, but redox-inactive towards oxygen. In turn, in the presence of a molar excess of thiol (3:1), the mixtures appear to give place to time-stable Cu(I)-complexes which were unreactive towards ascorbate and oxygen. The only exception to the latter was that of the 3:1 GSH/Cu<sup>2+</sup> mixture, which displayed an O<sub>2</sub><sup>•-</sup>-generating capacity, as evidenced through their ability to reduce Cyt c as well as lucigenin. We conclude that depending on the molar ratio of the interaction, thiols and Cu<sup>2+</sup> ions react to form mixtures containing Cu(I)- or Cu(II)-complexes which, given their redox-activity, may promote either an antioxidant or a pro-oxidant action.

#### 5. Materials and methods

##### 5.1. Chemicals and reagents

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), cupric chloride (CuCl<sub>2</sub>·2H<sub>2</sub>O), cytochrome

c (Cyt c; bovine heart), reduced glutathione,  $\gamma$ -glutamyl-cysteine, cysteinyl-glycine, cysteine, homocysteine, L-ascorbic acid (AA), 2,2-diphenyl-1-picrylhydrazyl (DPPH $\cdot$ ), methanol, lucigenin (bis-N-methylacridinium nitrate), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), L(-) histidine (His), catalase (CAT; EC 1.11.1.6 from bovine liver), superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes) were all purchased from Sigma-Aldrich. 2,2'-Azobis (2-amidinopropane dihydrochloride) (AAPH) was obtained from Wako Pure Chemicals Industries Ltd, Osaka, Japan. Iodine and potassium iodide were obtained from Merck; and starch from J.T. Baker. Unless indicated otherwise, all solutions employed in this study were prepared in Chelex-100-treated sodium phosphate buffer (10 mM; pH 7.4), and major experiments were conducted at 22 °C. DPPH $\cdot$  and lucigenin assay were conducted at 30 °C.

## 5.2. Radical-scavenging assays

### 5.2.1. ABTS $^{\cdot+}$ bleaching assay

The stable chromo-active free radical ABTS $^{\cdot+}$  was generated by incubation (45 °C, 55 min) of ABTS (75  $\mu$ M) with the azo-derivative AAPH (2 mM). The latter compound (thermo-unstable) generates peroxy radicals which upon interaction with ABTS give place to a green and stable colored ABTS $^{\cdot+}$  solution (OD<sub>734nm</sub>). The OD<sub>734nm</sub> of the colored solution remained constant for at least 4 h (22 °C as working conditions).<sup>21</sup> Changes in the OD<sub>734nm</sub> resulting from the addition of each thiol alone (GSH,  $\gamma$ -GC, CG, Cys, or Hcys) at a concentration of 4  $\mu$ M, or a pre-incubated (during 3 or 30 min) mixture of each thiol with Cu $^{2+}$  (12  $\mu$ M), were monitored spectrophotometrically (Unicam Helios- $\alpha$  England). The addition of Cu $^{2+}$  or the copper chelator, histidine, to a cuvette containing ABTS $^{\cdot+}$  had no effect on the OD<sub>734nm</sub>.<sup>21</sup> The bleaching capacity was estimated and expressed as the difference in OD ( $\Delta$ OD<sub>734nm</sub>) of an ABTS $^{\cdot+}$  containing solution seen at time zero (initial OD value was 0.28) and 3 min after the addition of the thiols.

### 5.2.2. Antiradical activity against DPPH $\cdot$

The scavenging capacity against the stable free radical DPPH $\cdot$  was measured by a modified DPPH $\cdot$  method.<sup>39</sup> DPPH $\cdot$  is a stable organic nitrogen radical, which bears a deep purple color. The assay is based on the measurement of the reducing ability of the thiols toward DPPH $\cdot$ , and such ability can be evaluated by measuring the decrease of absorbance (OD<sub>517nm</sub>). The changes in color were monitored at 30 °C with a *Multi-Mode Microplate Reader (Synergy™ HT)* and results were expressed as changes in the optical density ( $\Delta$ OD). Each thiol (36  $\mu$ M) alone or pre-incubated (during 3 or 30 min) with Cu $^{2+}$  (108  $\mu$ M) were added to a solution containing DPPH $\cdot$  (30  $\mu$ M, prepared in methanol 80%). After 40 min of reaction the OD was registered. The solution of DPPH $\cdot$  was prepared daily and was kept in the dark before using.

## 5.3. Thiol content determination by reducing capacity assays

### 5.3.1. DTNB reduction (thiol-titratable groups-TTG)

Solutions containing any of the following thiols: GSH,  $\gamma$ -GC, CG, Cys, or Hcys (20  $\mu$ M), or mixtures of these with Cu $^{2+}$  ions (60  $\mu$ M), were pre-incubated during 0–60 min, and subsequently added to a cuvette containing a mixture of DTNB (0.3 mM) with histidine (1 mM). Histidine was included to prevent Cu $^{2+}$  from catalyzing the re-oxidation of the 5-thio-2-nitrobenzoic acid generated during the titration, and as such was found not to affect the ability of thiols/Cu $^{2+}$  to bleach ABTS $^{\cdot+}$ .<sup>26</sup> The OD<sub>412nm</sub> of the resulting solutions was read against blanks containing no thiol. Thiols alone results are expressed as  $\Delta$ OD<sub>412nm</sub>. Mixture results are expressed as the percentage of thiol-titratable groups (TTG). These were estimated from multiplying by 100 the ratio of DTNB-titratable thiols

assayed in the presence versus absence of Cu $^{2+}$  ions. The sole addition of Cu $^{2+}$  ions to the Ellman's reactive had no effect on the OD<sub>412nm</sub>.

### 5.3.2. Iodine reduction

The degree of oxidation of thiols group was analyzed by iodine titration. In the presence of starch an intensely blue compound with free iodine is formed. The color disappears suddenly when the last trace of iodine has been reduced. At the end point of the titration, the volume consumed of each thiol or of the mixture RSH/Cu $^{2+}$  was recorded.

An iodine stock solution 0.1 M was prepared by dissolving 25 mg of I<sub>2</sub> and 77 mg of KI in 1 mL of deionised water,<sup>40</sup> and was diluted 100 times. Starch was prepared by dissolving 300 mg in 50 mL of deionised water. The oxidation reaction was obtained by adding 1 mL of iodine diluted solution and 1 mL of starch solution to a final volume of 20 mL. A thiol solution 0.25 mM alone or pre-incubated with Cu $^{2+}$  0.75 mM (during 3–60 min) was added to the referred oxidation reaction solution. The result was expressed as the percentage of remained thiol as reducing agent. These were estimated from dividing by 100 the ratio of the volume consumed by the thiol assayed in the presence versus absence of Cu $^{2+}$  ions.

## 5.4. Electron paramagnetic resonance studies

To explore the possible formation of paramagnetic complexes between the tested thiols and Cu $^{2+}$  ions (5 mM), EPR-dependent signal was studied. 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; center field, 3180 gauss; amplitude modulation, 0.9 G; microwave power 25 mW; time constant 20 ms, time scan 40 s.

## 5.5. Copper-dependent ascorbate oxidation assay

Ascorbate oxidation was assessed by measuring the loss of OD<sub>265nm</sub> that follows the addition of Cu $^{2+}$  (20  $\mu$ M) to an ascorbate (50  $\mu$ M) solution. To assess the effect of the thiols on copper-induced ascorbate oxidation, mixtures of each thiol with Cu $^{2+}$  were pre-incubated during 3 and up to 300 min, and subsequently added to the ascorbate solution. The % of ascorbic acid oxidation was estimated multiplying by 100 the ratio of AA oxidation induced by copper ions in the presence and absence of thiols, as follows:

% AA oxidation

$$= \frac{(\text{OD of AA} - \text{OD of AA after addition of RSH/Cu}^{2+})}{(\text{OD of AA} - \text{OD of AA after addition of Cu}^{2+} 20 \mu\text{M})} \times 100$$

The OD values were registered 4 min after adding Cu $^{2+}$  ions alone or mixtures of RSH/Cu $^{2+}$  to a solution containing 50  $\mu$ M of AA. When added directly, the thiol was always added before the metal to the AA-containing solution. The sole addition of the thiols to an ascorbate-containing solution had no effect on the OD<sub>265nm</sub>.

## 5.6. Assays for superoxide radical detection

### 5.6.1. Cytochrome c reduction assay

The superoxide-dependent reduction of Cyt c was assessed by monitoring the increase in OD<sub>550nm</sub> (during 2 min) that follows the addition of mixtures of Cu $^{2+}$  (10  $\mu$ M) with each of the tested thiols (pre-incubated during 30 min) to a solution containing Cyt c (50  $\mu$ M).<sup>26</sup> Superoxide-dependent (SOD-inhibitable) reduction of Cyt c was assessed by adding 250 U/mL of SOD activity to the testing cuvette. Neither the thiols nor Cu $^{2+}$  ions, each added alone,



had an effect on Cyt c reduction, except for cysteine (Cys), which was excluded from these results.

### 5.6.2. Lucigenin chemiluminescence assay

The reduction of lucigenin induced by superoxide was monitored with a *Multi-Mode Microplate Reader (Synergy™ HT)* at 30 °C, after the addition of mixtures of Cu<sup>2+</sup> (10 μM) with each of the tested thiols (30 μM) to a solution containing lucigenin (15 μM). Counts were obtained every 22 s intervals and the results expressed as the area under curve (ΔAUC) for a counting period of 112 s.<sup>41</sup> The thiols, each added alone, had no significant effects on the chemiluminescence, excepting for Cys, which under the experimental conditions used in the present study, was able to induce lucigenin reduction at a significant rate. The latter made it not possible using this assay to reliably evaluate the capacity of Cys to generate superoxide.

### 5.7. Data expression and analysis

Data points in figures and values in tables represent the means of at least three independent experiments, each conducted in quadruplicate. The SD of such data is not included as these generally represented less than 10% of the means. When appropriate, data were processed by an analysis of variance (ANOVA), and statistical significance was evaluated using the Student's *t* test. Differences at *p* < 0.05 were considered to be significant. GraphPad Prism 4 was used as statistical software.

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### References and notes

- Dickinson, D. A.; Forman, H. J. *Biochem. Pharmacol.* **2002**, *64*, 1019.
- Meister, A. *Methods Enzymol.* **1995**, *251*, 3.
- Ghezzi, P.; Bonetto, V.; Fratelli, M. *Antioxid. Redox Signal.* **2005**, *7*, 964.
- Kachur, A. V.; Koch, C. J.; Biaglow, J. E. *Free Radic. Res.* **1998**, *28*, 259.
- White, A. R.; Barnham, K. J.; Huang, X.; Voltakis, I.; Beyreuther, K.; Masters, C. L.; Cherny, R. A.; Bush, A. L.; Cappai, R. *J. Biol. Inorg. Chem.* **2004**, *9*, 269.
- Prutz, W. A. *Biochem. J.* **1994**, *302*, 373.
- Spear, N.; Aust, S. D. *Arch. Biochem. Biophys.* **1995**, *317*, 142.
- Hultberg, B.; Andersson, A.; Isaksson, A. *Toxicology* **1997**, *123*, 33.
- White, A. R.; Huang, X.; Jobling, M. F.; Barrow, C. J.; Beyreuther, K.; Masters, C. L.; Bush, A. L.; Cappai, R. *J. Neurochem.* **2001**, *76*, 1509.
- Oikawa, S.; Murakami, K.; Kawanishi, S. *Oncogene* **2003**, *22*, 3530.
- Kachur, A. V.; Koch, C. J.; Biaglow, J. E. *Free Radic. Res.* **1999**, *31*, 23.
- Muñiz, P.; Saez, P.; Iradi, A.; Vina, J.; Oliva, M. R.; Saez, G. T. *Free Radic. Biol. Med.* **2001**, *30*, 354.
- Park, S.; Imlay, J. A. *J. Bacteriol.* **2003**, *185*, 1942.
- Patterson, R. A.; Lamb, D. J.; Leake, D. S. *Atherosclerosis* **2003**, *169*, 87.
- Freedman, J. H.; Ciriolo, M. R.; Peisach, J. *J. Biol. Chem.* **1989**, *264*, 5598.
- Steinebach, O. M.; Wolterbeek, H. T. *Toxicology* **1994**, *92*, 75.
- Jimenez, I.; Aracena, P.; Letelier, M. E.; Navarro, P.; Speisky, H. *Toxicol.* **2002**, *16*, 167.
- Halvorsen, B.; Brude, I.; Drevon, C. A.; Nysom, J.; Ose, L.; Christiansen, E. N.; Nenseter, M. S. *J. Lipid. Res.* **1996**, *37*, 1591.
- Lynch, S. M.; Frei, B. *Biochem. Biophys. Acta* **1997**, *1345*, 215.
- Corazza, A.; Harvey, I.; Sadler, P. J. *Eur. J. Biochem.* **1996**, *236*, 697.
- Jimenez, I.; Speisky, H. *J. Trace Elem. Med. Biol.* **2000**, *14*, 161.
- Rigo, A.; Corazza, A.; di Paolo, M. L.; Rossetto, M.; Ugolini, R.; Scarpa, M. *J. Inorg. Biochem.* **2004**, *98*, 1495.
- Apostolova, M. D.; Bontchev, P. R.; Ivanova, B. B.; Russell, W. R.; Mehandjiev, D. R.; Beattie, J. H.; Nachev, C. K. *J. Inorg. Biochem.* **2003**, *95*, 321.
- Llanos, R. M.; Mercer, J. F. *DNA Cell Biol.* **2002**, *21*, 259.
- Letelier, M. E.; Lepe, A. M.; Faundez, M.; Salazar, J.; Marin, R.; Aracena, P.; Speisky, H. *Chem. Biol. Interact.* **2005**, *151*, 71.
- Carrasco-Pozo, C.; Álvarez-Lueje, A.; Olea-Azar, C.; López-Alarcón, C.; Speisky, H. *Exp. Biol. Med.* **2006**, *231*, 1569.
- Campos, A. M.; Lissi, E. A. *Int. J. Chem. Kinet.* **1997**, *29*, 219.
- Daugherty, R. G.; Wasowicz, T.; Gibney, B. R.; DeRose, V. J. *Inorg. Chem.* **2002**, *41*, 2623.
- Scarpa, M.; Vianello, F.; Signor, L.; Zennaro, L.; Rigo, A. *Inorg. Chem.* **1996**, *35*, 5201.
- Del Bello, B.; Paolicchi, A.; Comporti, M.; Pompella, A.; Maellaro, E. *FASEB J.* **1999**, *13*, 69.
- Jacobsen, D. W.; Catanescu, O.; DiBello, P. M.; Barbato, J. C. *Clin. Chem. Lab. Med.* **2005**, *43*, 1076.
- Hu, T. M.; Chou, T. C. *AAPS J.* **2006**, *8*, E485.
- Prior, R.; Wu, X.; Schaich, K. J. *Agric. Food. Chem.* **2005**, *53*, 4290.
- Jacob, C.; Giles, G. I.; Giles, N. M.; Sies, H. *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 4742.
- Chen, Z.; Zheng, H.; Lu, C.; Zu, Y. *Langmuir* **2007**, *23*, 10816.
- Baclocchi, E.; Calcagni, A.; Lanzalunga, O. *J. Org. Chem.* **2008**, *22*, 8982.
- Tang, Y. Z.; Liu, Z. Q. *J. Am. Oil Chem. Soc.* **2007**, *84*, 1095.
- Speisky, H.; Gómez, M.; Carrasco-Pozo, C.; Pastene, E.; Lopez-Alarcón, C.; Olea-Azar, C. *Bioorg. Med. Chem.* **2008**, *16*, 6568.
- Brand-Williams, W.; Cuvelier, M. E.; Berset, C. *Food Sci. Technol.* **1995**, *28*, 25.
- Bravo-Osuna, I.; Teutonico, D.; Arpicco, S.; Vauthier, C.; Ponchel, G. *Int. J. Pharm.* **2007**, *340*, 173.
- Li, Y.; Zhu, H.; Kuppusamy, P.; Roubaud, V.; Zweier, J. L.; Trush, M. A. *J. Biol. Chem.* **1998**, *273*, 2015.