Possible mechanisms underlying copper-induced damage in biological membranes leading to cellular toxicity

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Abstract

It is generally accepted that copper toxicity is a consequence of the generation of reactive oxygen species (ROS) by copper ions via Fenton or Haber–Weiss reactions. Copper ions display high affinity for thiol and amino groups occurring in proteins. Thus, specialized proteins containing clusters of these groups transport and store copper ions, hampering their potential toxicity. This mechanism, however, may be overwhelmed under copper overloading conditions, in which copper ions may bind to thiol groups occurring in proteins non-related to copper metabolism. In this study, we propose that indiscriminate copper binding may lead to damaging consequences to protein structure, modifying their biological functions. Therefore, we treated liver subcellular membrane fractions, including microsomes, with Cu^{2+} ions either alone or in the presence of ascorbate (Cu^{2+}/ascorbate); we then assayed both copper-binding to membranes, and microsomal cytochrome P450 oxidative system and GSH-transferase activities. All assayed sub-cellular membrane fractions treated with Cu^{2+} alone displayed Cu^{2+}-binding, which was significantly increased in the presence of Zn^{2+}, Hg^{2+}, Cd^{2+}, Ag^{+1} and As^{3+}. Treatment of microsomes with Cu^{2+} in the μM range decreased the microsomal thiol content; in the presence of ascorbate, Cu^{2+} added in the nM concentrations range induced a significant microsomal lipoperoxidation; noteworthy, increasing Cu^{2+} concentration to $\geq 50\,\mu M$ led to non-detectable lipoperoxidation levels. On the other hand, μM Cu^{2+} led to the inhibition of the enzymatic activities tested to the same extent in either presence or absence of ascorbate. We discuss the possible significance of indiscriminate copper binding to thiol proteins as a possible mechanism underlying copper-induced toxicity.

Keywords: Copper toxicity; Cu^{2+}/ascorbate; Thiols; Biological membranes; Copper-binding to proteins

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1. Introduction

The unique electron structure of copper allows the direct interaction with spin-restricted molecular oxygen, thus enabling copper to participate as a protein cofactor in fundamental redox reactions. A wide range of enzymes exploit copper chemistry to catalyze such type of reactions; these include cytochrome oxidase, superoxide dismutase, dopamine β-hydroxylase, lysyl oxidase and ceruloplasmin. Thus, copper ions are essential in cellular respiration, antioxidant defence, neurotransmitter function, connective tissue biosynthesis and cellular iron metabolism. Proteins take advantage of the redox nature of copper to achieve facile electron transfer reactions and to bind reactive intermediates and avoid their reactivity [1]. Nevertheless, the chemical properties that make copper biologically useful are also potentially toxic.

Taking to consideration the occurrence of 0.1–10 mg of copper per 100 g of wet tissue or biological fluid in human body, copper-binding to specialized proteins and some free aminoacids is recognized as an important mechanism for prevention of oxidative damage. Under oxidant stress, however, the accumulation of reactive oxygen species (ROS) would initiate the oxidative damage of many biological targets, such as single macromolecules, including lipoproteins, DNA, or thiol-containing enzymes, and more complex biological systems, including membranes, organelles, and intact cells. In these conditions, bound copper ions may be released from proteins and become redox-active. It has been suggested that oxidative damage to biological molecules is a determinant factor in a number of diseases, such as cardiovascular disease, cancer, Parkinson's disease, inflammation and rheumatoid arthritis [2]. Binding of copper ions is also important in the prevention of L-ascorbate oxidation, which is extremely accelerated by free copper ions [3]. Several hypotheses have been proposed to explain the mechanisms of copper-induced cellular toxicity. Copper toxicity would be the consequence, at least in part, of Fenton or Haber–Weiss reactions, in which copper ions catalyze the formation of ROS such as hydroxyl ($^\bullet$OH) and superoxide anion radicals ($O_2^{\bullet -}$) [4].

Copper-binding is not a completely safe mechanism preventing its redox cycling. It has been reported that related protein–metal complexes act as catalytic centers for the generation of reactive oxygen species involved in the oxidative damage present both in Alzheimer's and Prion disease [5,6]. Copper-binding to thiol clusters occurring in proteins, nonetheless, appears to be an efficient mechanism for hampering copper redox toxicity in the intracellular environment under physiological conditions.

Metallothionein is the major intracellular copper-binding protein in the liver; its major feature is a high capacity to bind copper (7–10 g atoms/mol) in virtue of its unusual amino acid composition: cysteine residues account for about 30% of the protein sequence, all of which are involved in the binding of metals [7–9]. In addition, metallothionein may play a significant and direct role as an intracellular antioxidant since it exhibits the highest known affinity towards $^\bullet$OH and electrophilic species [10]. Moreover, a wide range of chemicals with pro-oxidant properties (e.g., terbutyl-hydroperoxide, carbon tetrachloride, adriamycin, phorone, and diethyl maleate) and conditions that generate free radicals, such as γ or ultraviolet radiation, have also been reported to be effective inducers of metallothionein [11,12]. The ability of metallothionein to bind copper and to maintain its antioxidant properties intact appears intimately related to the hepatocellular availability of GSH [13,14]. In addition, Cu^+-GSH has also been shown to act efficiently in the delivery of Cu^+ to copper–zinc superoxide dismutase [8,15].

In this work, we measured the ability of copper ions per se to bind indiscriminately to different subcellular membrane fractions. We also measured the ability of copper, in the absence or presence of ascorbate, to inhibit the microsomal enzymatic activities of cytochrome P450 oxidative system and GSH-transferase. Thiol groups of these enzymes are involved in their catalytic activities; thus, its modification may alter their enzymatic activities [16–20]. We found that all subcellular membrane fractions assayed bound Cu^{2+}. Micromolar Cu^{2+} concentrations decreased the microsomal thiol content, in the absence or presence of ascorbate. Unexpectedly, while lipoperoxidation induced by Cu^{2+} in the presence of ascorbate was significant with nM Cu^{2+} concentrations, it was almost undetectable at $\geq 50\,\mu M\ Cu^{2+}$. Moreover, cytochrome P450 system and GSH-transferase activities were inhibited to a similar extent by Cu^{2+} at μM concentrations either in absence or presence of ascorbate.

Microsomes, the in vitro system used in this work to evaluate the ability of Cu^{2+}/ascorbate to induce lipoperoxidation, allowed us to discriminate pro-oxidant and protein-binding effects elicited by copper ions. Our data suggest that copper binding to thiol-containing proteins other than the specialized copper-binding proteins may lead to conformational changes altering their biological function. This indiscriminate copper binding to proteins may represent an additional mechanism underlying copper-induced toxicity. This phenomenon might be especially relevant in diseases featured by copper overload, such as Wilson's disease or under conditions leading to copper ions release from storage proteins, such as ischemia–reperfusion injury.

2. Materials and methods

2.1. Reagents

[64Cu]-CuSO$_4$ (specific activity 0.157 μCi/μg) was obtained from the Chilean Commission of Nuclear Energy (Santiago, Chile). Ascorbic acid, thiobarbituric acid (TBA); potassium dihydrogen phosphate (KH$_2$PO$_4$); copper sulfate (CuSO$_4$·5H$_2$O) were obtained from Merck Chile. NADP; glucose-6-phosphate dehydrogenase; glucose-6-phosphate (G-6-P); 5,5′-dithio-bis (2-nitrobenzoic) acid (Ellman's reagent, DTNB); *p*-nitroanisole (PNA), *p*-nitrophenol (PNP), 1-chloro-2,4-dinitrobenzene, Folin-Ciocalteu's reagent; BSA; GSH; *N*-ethyl-maleimide (NEM); tri-ethylene-tetra-amine-tetrahydrochloride (TTM); etilen-diami-ne-tetra-acetic acid (EDTA); iminodiacetic acid sodium form in polystyrene matrix (CHELEX-100) were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals were p.a. grade. All these compounds were prepared in buffer solution previously treated with CHELEX-100.

2.2. Animals

Adult male Sprague Dawley rats (200–250 g), derived from a stock maintained at the University of Chile, were used. They were allowed free access to pelleted food, maintained with controlled temperature (22 °C) and photoperiod (lights on from 07:00 to 19:00 h). All animals' procedures were performed using protocols approved by the Institutional Ethical Committee of the Chemical and Pharmaceutical Sciences School, University of Chile.

2.3. Membrane preparations

Animals were fasted for 15 h with water ad libitum, and sacrificed by decapitation. Four volumes of 25 mL 0.9% w/v NaCl were used to perfuse the livers in situ; then, they were excised and placed on ice (4 °C). All homogenization and fractionation procedures were performed at 4 °C using either a Suprafuge 22 Heraeus centrifuge or an XL-90 Beckman ultracentrifuge. Liver tissue devoid of connective and vascular tissue was homogenized with five volumes of 0.154 M KCl, with eight strokes in a Dounce Wheaton B homogenizer. Homogenate was centrifuged at $2000 \times g$ for 10 min and the sediment obtained was collected (P-2000) and the supernatant was centrifuged at $20,000 \times g$ for 20 min. The sediment obtained was also collected (P-20,000) and the supernatant was further centrifuged at $105,000 \times g$ for 60 min; the sediment obtained (P-105,000) was also collected. All collected sediments were stored at -80 °C until use. With this differential centrifugation procedure P-2000 contains a mixture of cell debris, nuclei and plasma membrane, P-20,000 is enriched in mitochondria and P-105,000 corresponds to microsomes. Total protein content was measured according to Lowry et al. [21] using BSA as standard.

2.4. Binding of copper to sub-cellular membrane fractions

P-2000, P-20,000 sub-cellular membrane fractions or microsomes (1 mg/mL total protein) were incubated with 100 μM [64Cu]-CuSO$_4$ in 50 mM phosphate buffer, pH 7.4, for 10 min at 37 °C with constant agitation. In the case of microsomes, different [64Cu]-CuSO$_4$ concentrations (5–100 μM), incubation times (2–30 min), and protein concentrations (0.25–1 mg/mL) were used. In some cases, microsomes were preincubated with 10 μM [64Cu]-CuSO$_4$ for 10 min at 37 °C and further incubated with 0.1–100 μM of either ZnSO$_4$, HgCl$_2$, AgNO$_3$, As$_2$(SO$_4$)$_3$, or CdCl$_2$, or with the chelating agents EDTA or TTM (1 mM) for an additional 10 min at 37 °C. In other cases, microsomes (1 mg/mL) were preincubated with a mixture of 10 μM [64Cu]-CuSO$_4$ and 1 mM either EDTA or TTM in 50 mM phosphate buffer, pH 7.4. After incubations,

mixtures were filtered through CF-C Whatman filters (0.25 mesh), which were washed with 50 mM phosphate buffer, pH 7.4 until a constant radioactivity was achieved. Radioactivity measurement was carried out on a Packard PR spectrometer model 1600 at room temperature.

2.5. Lipoperoxidation

The extent of microsomal lipoperoxidation following Cu^{2+}/ascorbate pre-incubation was estimated by determining TBARS concentration. Mixtures (1 mL final volume) contained 1 mg/mL microsomal protein, $CuSO_4$ (ranging from 0.5 nM to 100 μM), 1 mM sodium ascorbate, 4 mM $MgCl_2$, in 50 mM phosphate buffer, pH 7.4. Blanks contained all the reagents but microsomal protein. Blanks and samples were incubated for 20 min at 37 °C with constant agitation. Afterwards, 250 μL of 0.24 M TCA (4 °C) were added and all mixtures were centrifuged at $10,000 \times g$ during 10 min and 4 °C using a Suprafuge 22 Heraeus. Then, mixtures of 500 μL of the supernatants and 500 μL of 35 mM TBA were incubated at 50 °C for 1 h. At the end of this period, the absorbance at 532 nm of samples was measured in a UV3 Unicam UV–vis spectrophotometer, using their respective blanks as reference. Results are expressed in nmols of TBARS conjugated/min/mg of microsomal protein using the extinction coefficient 156 mM^{-1} cm^{-1} of malondialdehyde as reference described by Engineer and Shridhar [22].

2.6. Microsomal thiol content

Microsomes (1 mg/mL total protein) were incubated with 25 nM, 10 or 50 μM $CuSO_4$, either in the absence or presence of 1 mM NEM for 30 min at 37 °C. Afterwards, microsomal thiol content was titrated with DTNB, before and after Cu^{2+} or Cu^{2+}/ascorbate preincubations, as described by Jiménez et al. [23]. Thiol concentration was estimated by the equimolar apparition of 5-thio-2-nitrobenzoic acid ($\varepsilon_{410} = 13,600 \, M^{-1} \, cm^{-1}$).

2.7. Oxygen consumption

Oxygen consumption extent was polarographically determined during 5 min (continuous) with a Clark electrode N°5331 (Yellows Springs instrument) in a Gilson 5/6 oxygraph.

2.8. Cytochrome P450 oxidative system enzymatic activity assay

This activity was determined assaying *O*-demethylation of *p*-nitroanisole (PNA), as described in Letelier et al. [24]. The reaction mixture (1 mL final volume) contained 3 mg/mL microsomal protein, 1 mM PNA, 4 mM $MgCl_2$; 1 mM G-6-P; 10 mM NADP and 5 U/mL of G-6-P-dehydrogenase in 50 mM phosphate buffer, pH 7.4, in the absence or presence of 25 nM or 50 μM Cu^{2+}, or 25 nM Cu^{2+}/1 mM ascorbate or 50 μM Cu^{2+}/1 mM ascorbate. Blanks contained all reagents except for G-6-P dehydrogenase. Blanks and samples were incubated for 30 min at 37 °C. Addition of 0.5 mL of 10% w/v TCA was used to stop the enzymatic reaction. Mixtures were centrifuged for 10 min at $10,000 \times g$; 0.5 mL aliquots from the supernatants were alkalinized with 0.75 mL of 1 M NaOH and the absorbance of the samples was determined at 410 nm in a UV3 Unicam UV–vis spectrophotometer. PNP formed in the enzymatic reaction was quantified using a PNP standard solution.

2.9. GSH-transferase enzymatic activity assay

This activity was determined by assaying 1-chloro-2,4-dinitrobenzene conjugation with GSH, as described by Habig et al. [25]. The reaction mixture contained 0.1 mg/mL microsomal protein, 1 mM 1-chloro-2,4-dinitrobenzene, and 4 mM GSH in 100 mM phosphate buffer, pH 6.5, in the absence or presence of 25 nM or 50 μM Cu^{2+}, or 25 nM Cu^{2+}/1 mM ascorbate or 50 μM Cu^{2+}/1 mM ascorbate. Conjugated product apparition was recorded continuously for 3 min at 25 °C, at 340 nm ($\varepsilon_{340} = 9.6 \times 10^{-3} \, M^{-1} \, cm^{-1}$) in a UV3 Unicam UV–vis spectrophotometer.

2.10. Statistical analysis

The results are presented as mean ± S.D. of at least four independent experiments. Groups of test data (mean ± S.D.) were compared using Student's *t*-test for paired observations. Values were considered to differ significantly at the level of $p < 0.05$. Analyses were performed using Origin 5.0.

3. Results

3.1. Copper binding to sub-cellular membranes

We first tested potential copper binding to biological membranes by incubating microsomes with radiolabelled copper under different conditions of ion concentration, incubation time, and protein concentration, as described in Section 2. Following [^{64}Cu]-CuSO$_4$ incubation of microsomes for 10 min, a linear non-saturable concentration-dependent binding of copper to microsomes was observed at all the concentrations tested (Fig. 1A). Copper binding to microsomes followed a single-exponential increase with incubation time, displaying a binding half-time of 1.9 ± 0.02 min and reaching a maximum copper binding at 10 min (Fig. 1B). Additionally, copper binding displayed a hyperbolic increase with microsomal protein concentrations, reaching a maximum of 1.9 ± 0.06 nat-mole/mg protein with no further increase with concentrations above 0.5 mg/mL (Fig. 1C). In order to assess whether copper binding to microsomes was specific to these type of membranes, we incubated P-2000 or P-20,000 sub-cellular membrane fractions (1 mg/mL total protein) with 100 μM [^{64}Cu]-CuSO$_4$ for 10 min at 37 °C, as described in Section 2. Copper binding to these membrane fractions was indeed observed, reaching binding values of 5.2 ± 0.25 and 5.1 ± 0.31 nat-mole/mg proteins for P-2000 and P-20,000 fractions, respectively (Fig. 2).

We also tested the stability of copper binding to microsomes by further incubating radiolabelled copper-treated samples with the following metal ions: Cu^{+2} (cold), Zn^{+2}, Hg^{+2}, Ag^{+1}, As^{+3}, or Cd^{+2} as detailed in Section 2. As shown in Fig. 3, although we used 10-fold higher concentration (100 μM) of each metal ion than initial copper ion concentration, none of them

were able to displace copper already bound to the microsomes; on the contrary, addition of these metal ions increased the amount of copper bound to microsomes to a range between 5 and 9 nat-mole/mg protein, approximately. In this regard, Zn^{2+} and Hg^{2+} were the most powerful enhancers of copper binding to microsomes, while Cd^{2+} was the weakest (Fig. 3); noteworthy, non-labelled copper ions were not able to modify
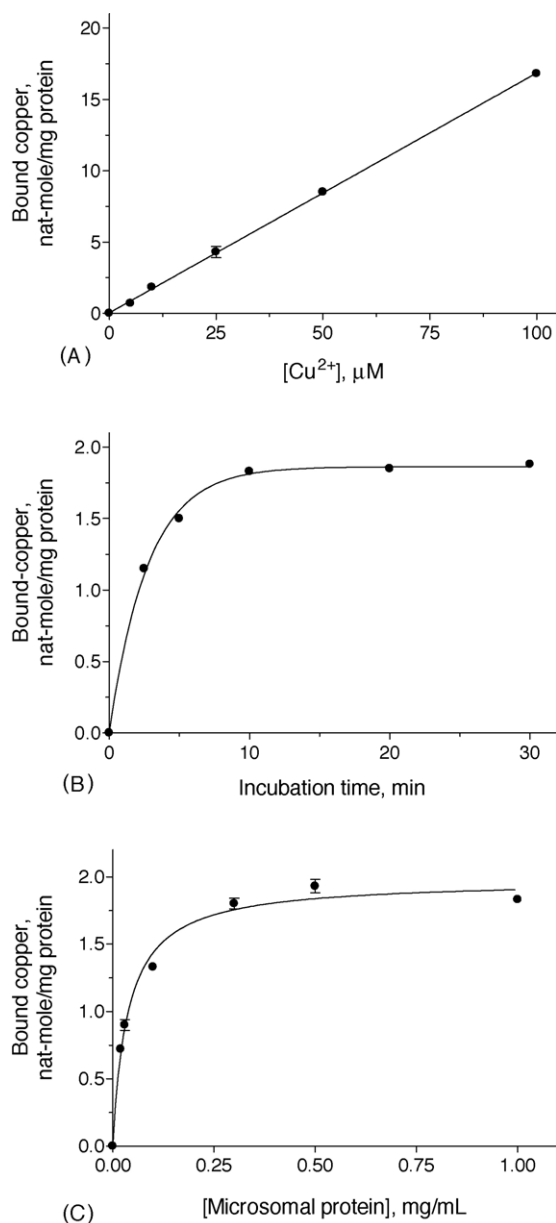

Fig. 1. Copper binding to microsomal membranes. Microsomes were incubated, at 37 °C, with different concentrations (5–100 μM) of [^{64}Cu]-CuSO$_4$ (A), or with 10 μM [^{64}Cu]-CuSO$_4$ at different times (B) or different protein concentrations (C), and Cu^{2+}-binding was assayed as detailed in Section 2. (A) Dose dependent curve of Cu^{2+} binding to microsomes (1 mg/mL total protein and 10 min incubation). (B) Time-dependence of Cu^{2+} binding to microsomes (1 mg/mL total protein). (C) Protein concentration dependence curve of Cu^{2+} binding to microsomes (10 min incubation). All data correspond to the mean of at least four independent experiments ± S.D.
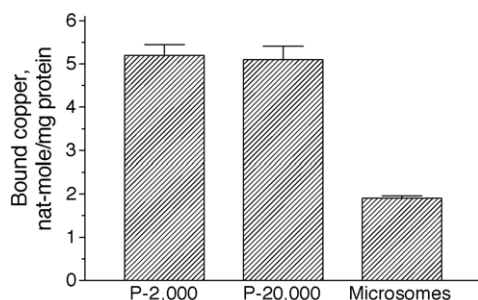
Fig. 2. Copper binding to sub-cellular membrane fractions. Different membrane fractions were obtained as described in Section 2. Fractions (1 mg/mL total protein) were incubated with 100 μM [^{64}Cu]-CuSO$_4$ for 10 min at 37 °C, and Cu^{2+}-binding was assayed as detailed in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.

the amount of copper initially bound to microsomes. We further tested the effect of Zn^{2+} and Hg^{2+} as enhancers of copper binding to microsomes performing a dose-dependent study, as detailed in Section 2. Fig. 4 shows that these ions behaved as copper-binding enhancers in a dose-dependent manner at all the concentrations used (0.1–100 μM); both ions displayed enhancing effects in a similar extent. We further studied the stability of copper bound to microsomal membranes, by incubating microsomes with a mixture of radiolabelled-copper and the chelating agents EDTA or TTM in a ratio of 1:100 (Cu^{2+}/chelating agent), as detailed in Section 2. Fig. 5A shows that while the
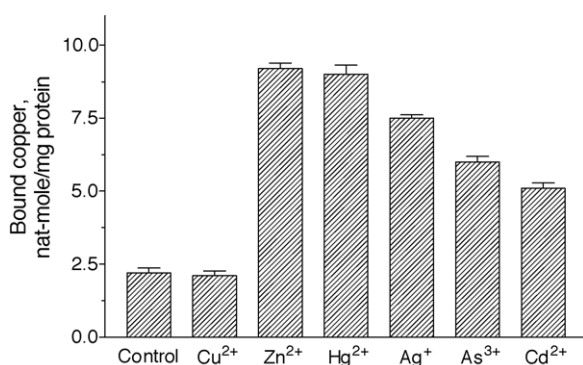

Fig. 3. Copper binding to microsomes. Effect of different metal ions. Microsomes (1 mg/mL total protein) were incubated with 10 μM [^{64}Cu]-CuSO$_4$ for 10 min at 37 °C. Afterwards, microsomes were further incubated with 100 μM Cu^{2+} (cold) and the described metal ions, and Cu^{2+}-binding was assayed as detailed in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.
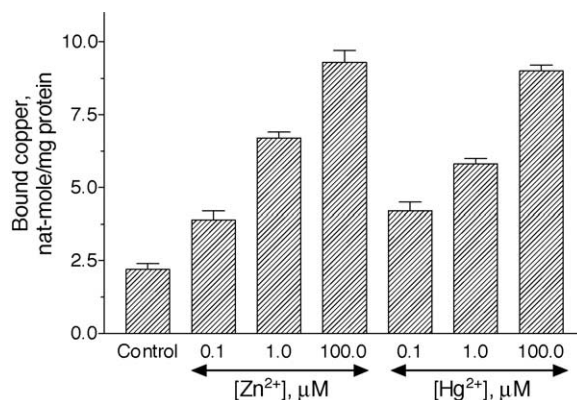

Fig. 4. Copper binding to microsomes. Effect of Zn^{2+} and Hg^{2+}. Microsomes (1 mg/mL total protein) were incubated with 10 μM [^{64}Cu]-CuSO$_4$ for 10 min at 37 °C. Afterwards, microsomes were further incubated with 0.1, 10 and 100 μM ZnCl$_2$ or HgCl$_2$, and Cu^{2+}-binding was assayed as detailed in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.

[^{64}Cu]-Cu^{2+}/TTM mixture induced copper binding to microsomes to a lesser extent than using the radiolabelled copper alone (0.4 ± 0.06 nat-mole/mg protein); noteworthy, the [^{64}Cu]-Cu^{2+}/EDTA mixture abolished copper binding. On the other hand, Fig. 5B depicts that further incubating radiolabelled-copper-treated microsomes with either of these chelating agents induced less copper binding to microsomes to a similar extent (approximately 50%).

3.2. Lipoperoxidation induced by Cu^{2+}/ascorbate system

We obtained maximum copper binding to microsomes at 10 min using μM concentrations of copper ions. In order to address the potential oxidative damage induced by copper ions, we used the Cu^{2+}/ascorbate system as a source of ROS generation driven by copper. We assayed microsomal lipid peroxidation under these conditions, in the presence of ascorbate, as described in Section 2. As shown in Fig. 6, lipoperoxidation was induced following incubation of microsomes with Cu^{2+} between 0.5 nM and 10 μM in the presence of 1 mM ascorbate; between 0.5 and 100 nM, however, lipoperoxidation remained constant and only displayed a dose-dependent behaviour between 1 and 10 μM. Unexpectedly, following treatment of microsomes with higher Cu^{2+} concentrations (in the
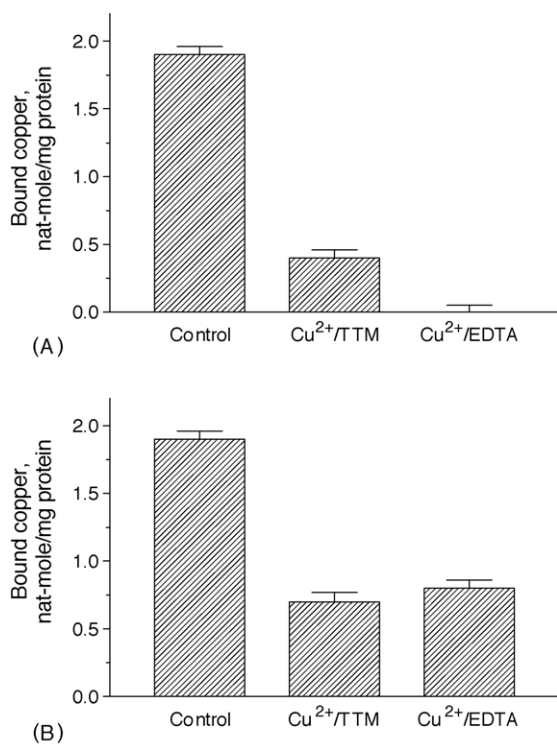
(A)

(B)

Fig. 5. Copper binding to microsomes. Effect of TTM and EDTA. Microsomes (1 mg/mL total protein) were incubated either with a mixture of 10 μM [64Cu]-CuSO₄ plus 1 mM TTM or EDTA for 10 min at 37 °C (A) or 10 μM [64Cu]-CuSO₄ for 10 min at 37 °C and further incubated with plus 1 mM TTM or EDTA for and additional 10 min at 37 °C (B). Afterwards Cu^{2+}-binding was assayed as detailed in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.

presence of ascorbate) lipoperoxidation decreased to non-detectable levels at 100 μM.

3.3. Oxygen consumption

With the purpose to investigate the decrease of microsomal lipoperoxidation observed after microsomes treatment with 50 and 100 μM Cu^{2+} concentrations, in the presence of 1 mM ascorbate, we assayed oxygen consumption using a polarographic technique (see Section 2). As depicted in Fig. 7, oxygen consumption was induced by either Cu^{2+}/ascorbate in the presence or absence of microsomes (traces b and c, respectively); remarkably, oxygen consumption was 95% less in the presence than in the absence of microsomes (see slopes in Fig. 7).
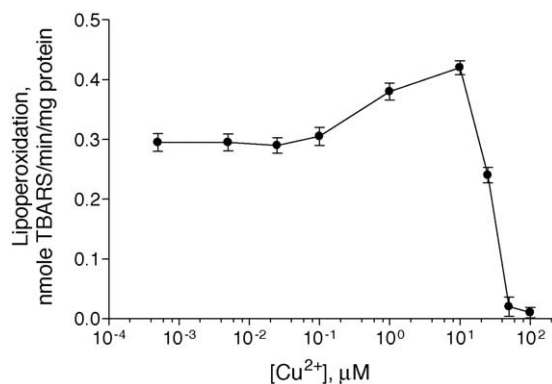

Fig. 6. Microsomal lipoperoxidation induced by the Cu^{2+}/ascorbate system. Microsomes (1 mg/mL total protein) were incubated with different CuSO₄ concentrations (in the range of 0.5 nM to 100 μM), in the presence of 1 mM ascorbate, for 30 min at 37 °C. Induced lipoperoxidation was assayed as described in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.
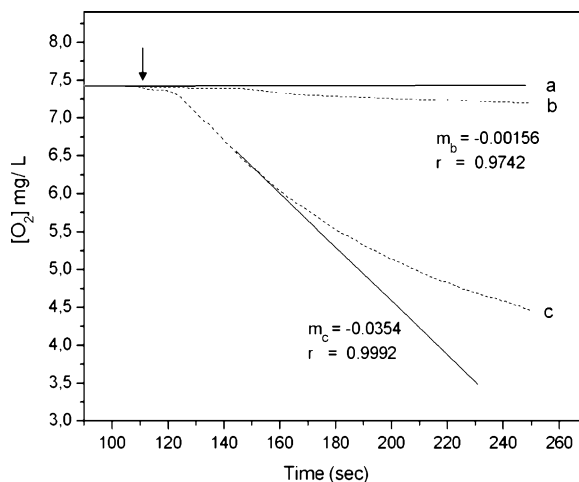

Fig. 7. Oxygen consumption induced by the Cu^{2+}/ascorbate system. Effect of microsomes. Oxygen consumption of a solution containing a mixture of 50 μM CuSO₄ plus 1 mM ascorbate, in the the presence (trace b) or absence (trace c) of microsomes (1 mg/mL total protein), was polarographically measured as detailed in Section 2. The arrow shows the addition of 1 mM ascorbate for traces b and c. Trace a corresponds to the baseline of oxygen consumption (50 μM CuSO₄ in the absence of ascorbate). Linear regression analyses (shown as continuous traces) were performed for the initial rates of oxygen consumption traces b and c, and the respective slopes (m_b and m_c) and regression coefficients (r) obtained are presented in the figure. A representative experiment of at least four independent determinations is shown.
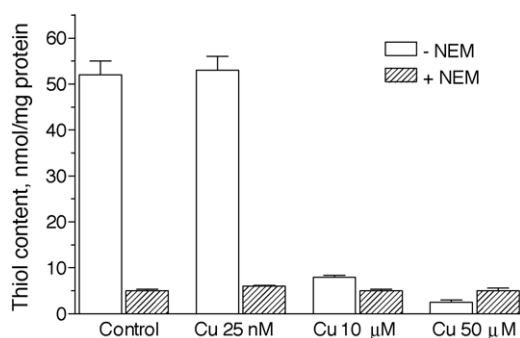
Fig. 8. Effect of Cu^{2+} on microsomal thiol content. Microsomes were incubated with different Cu^{2+} concentrations (25 nM, 10 or 50 μM), in the absence or presence of 1 mM NEM for 30 min at 37 °C, and the thiol content was titrated as described in Section 2. Data correspond to the mean of at least four independent experiments ± S.D.

3.4. Microsomal thiol content

To address the potential relevance of thiol groups in copper binding to microsomes, we performed a "titration" of the microsomal thiol content before and after Cu^{2+} incubation, and in the absence or presence of NEM, as detailed in Section 2. As shown in Fig. 8, microsomal thiol content was significantly decreased by 1 mM NEM (88%) or 10 and 50 μM Cu^{2+} (84 and 93%, respectively), while it was not modified by 25 nM Cu^{2+}. On the other hand, mixtures of 1 mM NEM with 25 nM, 10 or 50 μM Cu^{2+} promoted a decrease of microsomal thiol content to the same extent to that observed with NEM alone.

3.5. Microsomal enzymatic activities

In order to address the potential damaging effect of copper-binding to microsomal proteins, we assayed the activities of both cytochrome P450 oxidative system and GSH-transferase, which display key cysteine residues for their catalytic activities (see Section 2). As shown in Table 1, incubation of microsomes with 25 nM Cu^{2+}, either in the presence or absence of ascorbate, did not modify these activities. When microsomes were incubated with 50 μM Cu^{2+}, however, inhibition of both cytochrome P450 oxidative system and GSH-transferase activities was observed (77 and 49%, respectively); presence of ascorbate did not modify these values significantly.

4. Discussion

4.1. Copper binding to microsomal proteins

Copper-binding proteins are involved in both its transport and metabolism. Metallothionein is recognized as the major cellular copper-binding protein in the liver, in virtue of its high cysteine residues content (30%), all of which are involved in metal ions binding [1,7]. Albumin and ceruloplasmin, proteins accounting for the transport and distribution of copper in circulating plasma, are also characterized by their high content of cysteine [26,27]. In addition, a relation between copper metabolism and the intracellular availability of

Table 1
Enzymatic activities (effect of Cu^{2+} and Cu^{2+}/ascorbate)

Condition[a]	Cytochrome P450 oxidative system activity[b]	GSH-transferase activity[c]
Control	7.90 ± 0.34	115.1 ± 0.17
25 nM Cu^{2+}	7.87 ± 0.29	114.9 ± 0.16
50 μM Cu^{2+}	$1.82 \pm 0.11^*$	$58.9 \pm 0.59^*$
25 nM Cu^{2+}/1 mM ascorbate	7.88 ± 0.31	114.8 ± 0.17
50 μM Cu^{2+}/1 mM ascorbate	$1.71 \pm 0.13^*$	$46.8 \pm 1.10^*$

[a] Microsomal enzymatic activities were assayed in the presence of each described reagent.

[b] Cytochrome P450 oxidative system activity was assayed as O-demethylation of p-nitroanisole, as described in Section 2. Data, shown as nmol of p-nitrophenol produced/min/mg protein, correspond to the mean of at least four independent determinations ± S.D.

[c] GSH-transferase activity was assayed as the conjugation of 1-chloro-2,4-dinitrobenzene, as detailed in Section 2. Data, shown as nmol of conjugate formed/min/mg protein, correspond to the mean of at least four independent determinations ± S.D.

* $p < 0.05$ compared to control condition.

glutathione has been defined [28]. Aside of cysteine residues occurring in specialized copper-binding proteins, these residues are also present in the majority of globular proteins; thus, it is possible that copper ions may indiscriminately bind to these proteins through their thiol groups. Copper toxicity is thought to be the result of Fenton and Haber–Weiss reactions, in which copper ions catalyze the formation of ROS, leading to oxidative stress-related damage. Indiscriminate binding of copper ions to proteins different from the specialized copper-binding proteins, may also have damaging consequences to their biological activities. Therefore, we explored both copper binding to microsomal proteins and its effects on microsomal enzymatic activities; we studied this phenomenon in the absence of ascorbate to assess the effects of copper binding alone, and in the presence of this agent to evaluate the additional effect of copper ion-catalyzed Fenton and Haber–Weiss reactions.

In this work, we showed that copper ions binds avidly to microsomal membranes: 1 mg of microsomal protein displayed copper-binding capability in the range of 5–100 μM Cu^{2+} concentrations, without displaying saturation of binding sites (see Fig. 1A). This suggests that proteins resident in this fraction display substantial copper-binding sites, even when there is no occurrence of specialized copper-binding proteins or copper-containing metalloproteins; this avid copper binding property of microsomal membranes is also supported by its time-dependence (half-time of 2 min, approximately; Fig. 1B). It is important to note that any possible electrostatic interaction between copper ions and membrane phospholipids and phosphoinositides is likely to be interrupted by the binding assay conditions used in this study, thus, copper binding to microsomes is likely to be due to the occurrence of proteins. However, increasing protein concentration did not promote binding of all copper ions present in the incubation medium, supporting the idea of a limited number of copper binding sites (Fig. 1C). Our results also demonstrate that indiscriminate copper-binding is not an exclusive feature of microsomal proteins, since other sub-cellular membrane fractions exhibited the capability of copper binding (Fig. 2). The higher copper-binding capability shown by other sub-cellular membrane fractions compared to microsomes is likely to be reflecting the different nature of proteins resident in each fraction.

4.2. Stability of copper binding to microsomal proteins

Specialized copper-binding proteins are able to bind other metal ions as well in virtue of the occurrence of thiol clusters in their aminoacid sequences, such as Zn^{2+}, Cd^{2+}, Ag^+ and Hg^{2+}. Indeed, this is the case of metallothionein, which makes possible to experimentally perform competition assays between these metal ions, and is the basis to assay metallothionein binding capacity [29,30]. In an effort to understand whether copper binding to microsomal proteins could be the result of its interaction with thiol clusters such as those of metallothionein, we studied the stability of copperbound to microsomes when exposed to different metal ions (see Figs. 3 and 4). Surprisingly, a 10-fold excess of non-radiolabelled copper ions did not displace copper already bound to microsomes, suggesting a strong copper binding property. Furthermore, all the other metal ions tested (also in 10-fold excess) increased the amount of copper bound to this fraction. This may be the result of changes in protein conformation elicited by these metal ions, which may expose new copper binding sites. Specialized thiol clusters in metallothionein are able to bind metal ions with different affinity: both Hg^{2+} and Ag^+ are capable to displace bound Cu^{2+}, while the latter displaces Zn^{2+} and Cd^{2+} from this protein [29,30]; this is not the case with copper-binding to microsomes (even at lower concentrations of Hg^{2+} or Zn^{2+}), strongly suggesting that this phenomenon relies upon structures not based on thiol clusters per se. These observations further support the idea of indiscriminate copper binding to proteins. Interestingly, $ZnSO_4$ is used in the treatment of oral intoxication with copper, producing gastrointestinal side effects. If indiscriminate copper binding to proteins is damaging, the increasing of this phenomenon elicited by Zn^{2+} observed in our experiments may explain these side effects.

Finally, we studied the stability of copper binding to microsomal proteins using the chelating agents TTM and EDTA (see Fig. 5), which are recognized Cu^{2+}-sequestering agents [31]. Copper ions in a mixture with both chelating agents (in a 100-fold excess) elicited a lower level of copper binding compared to copper ions alone; this observation suggests that only free copper ions are capable of interacting with proteins resident in microsomes. The different extents of prevention in copper binding are in agreement to the different affinities

for copper ions displayed by these agents (EDTA higher than TTM) [31]. Additionally, both chelating agents displaced copper binding to this fraction to the same extent, suggesting that this phenomenon is not irreversible.

4.3. Copper-induced oxidative effects in microsomes: a comparison with indiscriminate copper binding

In the presence of ascorbate, copper ions are thought to catalyze ROS generation via Fenton and Haber–Weiss reactions. Thus, we assayed lipoperoxidation induced by copper ions (in a range of 0.5 nM to 100 μM) in the presence of 1 mM ascorbate (see Fig. 6). Although low copper ions concentrations (5–100 nM) elicited an increase of lipoperoxidation, it remained constant throughout all this range; significantly, there is no copper binding to microsomal proteins noticeable in this range of Cu^{2+} concentrations (not shown). These data may be explained by different substrates for ROS generated by this system: we propose that one likely explanation is that certain microsomal lipids are very sensitive to ROS-induced oxidation and account for the observed induced lipoperoxidation at 5 nM Cu^{2+}, but at higher ROS generation (i.e. between 5 and 100 nM Cu^{2+}), microsomal proteins highly susceptible to ROS-induced oxidation may be the substrate for the additional ROS generated. This is supported by the higher accessibility of oxidizing groups occurring in lipids compared to those in proteins. Intermediate Cu^{2+} concentrations (100 nM to 10 μM) elicited an increase in induced lipoperoxidation in a dose-dependent manner, which would reflect a higher ROS generation inducing higher lipid oxidation without significant competition with protein oxidation. Interestingly, the extent of the increase in lipoperoxidation (25%) does not significantly reflect the increase in Cu^{2+} concentrations (1000-fold); we suggest that copper binding to microsomal proteins (which is detectable at 5 μM Cu^{2+}) may decrease the available copper ions to undergo Fenton or Haber–Weiss reactions. Noteworthy, higher Cu^{2+} concentrations (10–100 μM) did not further increase lipoperoxidation; moreover, this phenomenon decreased with Cu^{2+} concentrations to basal levels. This observation may be explained also by the indiscriminate copper binding to microsomal proteins, which at 50 μM completely prevents Cu^{2+}/ascorbate

oxidative damage to lipids. Altogether, our data supports the idea that Cu^{2+} μM binds to microsomal proteins, even in the presence of ascorbate. This idea is in agreement with our studies in oxygen consumption by the Cu^{2+}/ascorbate system in the absence or presence of microsomes (see Fig. 7). In this case, 50 μM Cu^{2+}/1 mM ascorbate caused a significant increase in oxygen consumption, demonstrating that it is indeed a ROS-generating system; in the presence of microsomes, however, this consumption was prevented almost entirely (95%), showing that most likely microsomal proteins act as a Cu^{2+} sink, preventing any significant Fenton and Haber–Weiss reactions.

But, which are the copper-binding sites in proteins? We explored this point with Cu^{2+} concentrations in the 50–100 μM range, which displayed the most noticeable copper binding levels. Copper ions most likely will bind to thiol-displaying cysteine residues in proteins; thus, we "titrated" microsomal thiol content at different Cu^{2+} concentrations ranging from 25 nM to 50 μM (see Fig. 8). Micromolar Cu^{2+} concentrations indeed decreased microsomal thiol content to a similar extent that the decrease promoted by NEM, a specific S-alkylating agent, while nM Cu^{2+} concentrations did not. In other words, at Cu^{2+} concentrations of noticeable binding to microsomal proteins correlate with a decrease in the thiol content. Furthermore, the addition of a mixture of Cu^{2+} plus NEM did not further decrease the thiol content, showing that most likely the thiol groups reactive to NEM are the same than those capable of binding Cu^{2+}. Moreover, lipoperoxidation of microsomes previously treated with NEM was higher than that of untreated microsomes, only at μM—but not at nM—Cu^{2+} concentrations in the presence of ascorbate (not shown). Altogether, our data strongly suggest that copper binding to microsomal proteins is indeed through thiol groups.

Up to this point, we have shown that copper binds indiscriminately to microsomal proteins most likely through their thiol groups, and that this binding prevents copper-induced oxidative damage when in the presence of ascorbate. Does indiscriminate copper binding have damaging effects? To answer this question, we assayed enzymatic activities known to be sensitive to thiol group modification/oxidation and occurring in microsomes (see Table 1). This is the case of cytochrome P450 oxidative system and GSH-transferase; cytochrome P450 monooxygenase

isoenzymes (constituents of the cytochrome P450 ox-idative system) display 1 thiol group in the active site, which is involved in the substrate binding [32], while GSH-transferase display 1 thiol group involved in the formation of a catalytically active dimeric form [17–20]. If there is a significant oxidation of thiol groups, the cytochrome P450 monooxygenase activity will be decreased, while that of GSH-transferase will be increased. On the other hand, if there is a copper binding event in these thiol groups, both enzymatic activities will be decreased. Our data show that only μM—but not nM—Cu^{2+} concentrations elicited a decrease in both enzymatic activities. Interestingly, μM Cu^{2+} concentrations promoted these effects to the same extent, regardless of the presence of ascorbate. Thus, the only possible explanation is that the inhibition of these enzymatic activities is due to copper binding to these proteins and that Cu^{2+}-induced oxidative effects (in the presence of ascorbate) are not determinant in the effects shown. Additionally, these data show that indiscriminate copper binding has damaging effects on microsomal protein function.

4.4. Copper-related toxicity: Cu^{2+}-induced oxidative effects versus indiscriminate Cu^{2+} binding

This work shows for the first time experimental data supporting the idea that copper-related toxicity in microsomal protein systems may be due to indiscriminate copper binding to proteins rather than the generally accepted ROS-generation hypothesis. This statement is supported by the evidence showing that only microsomal lipids—but not proteins—are susceptible to oxidative damage induced by the Cu^{2+}/ascorbate system. On the other hand, damage to microsomal proteins can be explained solely by the Cu^{2+}-binding effect. Furthermore, the latter overrides lipoperoxidation induced by μM Cu^{2+} concentration in the presence of ascorbate. We then propose that, under copper overload conditions, toxicity would arise mainly from indiscriminate copper binding to proteins other than the specialized-copper handling proteins. This would be especially relevant in hereditary diseases such as Wilson disease, or acquired diseases, such as Indian childhood cirrhosis or idiopathic copper toxicosis, in which there is an accumulation of this ion in the liver [33]. Indiscriminate copper binding to proteins would also be rele-vant in pathologies involving the release of copper ions from the specialized copper-handling proteins (such as metallothionein and ceruloplasmin), which takes place under oxidative stress conditions. We are currently investigating these copper effects on other enzymes occurring in hepatic microsomes.

Indiscriminate copper binding such as the one shown in this study may be the underlying mechanism distinguishing copper toxicity from iron toxicity, since iron does not appear to display such indiscriminate binding to proteins [34]. Similar to copper, however, iron in the presence of a reducing agent induces oxidative stress in several systems, including our in vitro liver microsomal fraction. Moreover, it has been shown that chronic copper exposure of the human hepatoblastoma HepG2 cells does not generate oxidative stress per se, but a decrease in the glutathione content [14], most likely through this indiscriminate binding phenomenon. Thus, in light of the data presented in our study, we propose a re-evaluation of the mechanisms underlying copper-induced toxicity in biological systems.

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