Copper modifies liver microsomal UDP-glucuronyltransferase activity through different and opposite mechanisms

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Abstract

Treatment of hepatic microsomes with Fe^{3+}/ascorbate activates UDP-glucuronyltransferase (UGT), a phenomenon totally prevented and reversed by reducing agents. At μM concentrations, iron and copper ions catalyze the formation of ROS through Fenton and/or Haber–Weiss reactions. Unlike iron ions, indiscriminate binding of copper ions to thiol groups of proteins different from the specialized copper-binding proteins may occur. Thus, we hypothesize that incubation of hepatic microsomes with the Cu^{2+}/ascorbate system will lead to both UGT oxidative activation and Cu^{2+}-binding induced inhibition, simultaneously. We studied the effects of Cu^{2+} alone and in the presence of ascorbate on rat liver microsomal UGT activity. Our results show that the effects of both copper alone and in the presence of ascorbate were copper ion concentration- and incubation time-dependent. At very low Cu^{2+} (25 nM), this ion did not modify UGT activity. In the presence of ascorbate, however, UGT activity was increased. At higher copper concentrations (10 and 50 μM), this ion led to UGT activity inhibition. In the presence of ascorbate, 10 μM Cu^{2+} activated UGT at short incubation periods but inhibited this enzyme at longer incubation times; 50 μM Cu^{2+} only inhibited UGT activity. Thiol reducing agent 2,4-dithiothreitol prevented and reversed UGT activation while EDTA prevented both, UGT activation and inhibition. Our results are consistent with a model in which Cu^{2+}-induced oxidation of UGT leads to the activation of the enzyme, while Cu^{2+}-binding leads to its inhibition. We discuss physiological and pathological implications of these findings.

Keywords: UGT-inhibition/activation; UGT-Cu; UGT-Cu/ascorbate

1. Introduction

UDP-glucuronyltransferase isoenzymes (UGT, EC 2.4.1.17) comprise a large gene super family classified into two major gene families, UGT1 and UGT2, based in the extent of nucleotide sequence identity of the isoforms. Nascent UGT isoforms have a 20 amino acid signal peptide, cleaved during protein maturation [1]. These enzymes catalyze the conjugation of glucuronic acid with different substrates, including many structurally different compounds, such as phenols, carboxylic acids, aliphatic and aromatic alcohols, certain aromatic amines, and physiological molecules, including bile acids, sex hormones, and serotonin [1,2].

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The major portion of mature UGT molecules, including the binding sites for UDP-glucuronic acid (UDPGA) and the aglycone substrates seem to be located within the endoplasmic reticulum cistern. There is a single 17 amino acid membrane-spanning segment and a 26 amino acid cytoplasm tail at the C-terminal end of the molecule. hUGT1A1 contains a total of 11 cysteine residues, one of which is within the signal peptide (amino acid 18) that is cleaved during synthesis of the enzyme. Seven cysteine residues (Cys127, Cys156, Cys177, Cys186, Cys223, Cys280 and Cys383) are present within the major segment of the enzyme that seems to be located within the endoplasmic reticulum cistern. Cys509, Cys510 and Cys517 are located within the C-terminal cytoplasm tail. Sequence alignment revealed that the ten-cysteine residues that are present in mature hUGT1A1 are highly conserved in all human UGT1A isoforms. Furthermore, most of these cysteine residues are conserved across species (rats, mice and Rhesus monkeys) hinting their potential importance within the protein. Substitution of the three-cysteine residues within the C-terminal cytosolic tail had minimal effect on basal UGT1A1 activity, but prevented UGT1A1 activation by its physiological activator UDP-GlcNAc, which stimulates the import of the donor substrate UDPGA [1,3].

Since UGT catalytic domain is oriented to the endoplasmic reticulum lumen, there is a lipid physical constraint for the access of UDPGA from the cytosol to the enzyme active site. In fact, various methods and conditions that perturb the lipid phase of the microsomal membrane increase UGT activity. These methods include sonication and treatment with phospholipases, bilirubin, organic solvents, detergents and lipid peroxidation-inducing agents [4–7]. A compartmentation-based hypothesis has been widely accepted in order to explain this activation phenomenon. The destruction of the vesicular structure of microsomes, which facilitates the passage of UDPGA through holes in the microsomal membranes to the enzyme active site, result in an increase of UGT activity, supporting a complex model of compartmentation. However, it has also been reported that treatment of microsomes with elevated concentrations of detergents or for extensive lengths of time, abolish microsomal UGT activity [4,8–10].

Treatment of microsomes with μM Fe^{3+} concentration in the presence of ascorbate causes oxidative effects on endoplasmic reticulum proteins, including UGT; such treatment also activates the enzyme. The reducing agents such as DTT, GSH, or cysteine prevented and reversed totally the UGT oxidative activation, indicating that only redox changes on the UGT seem to be involved in its activation [11]. Iron ions as well as copper ions, at μM concentrations catalyze the formation of ROS through Haber–Weiss and/or Fenton reactions. Thus, Cu^{2+}/ascorbate can cause UGT oxidative activation similar to that described for Fe^{3+}/ascorbate. However, μM copper concentrations mainly cause binding of copper ions to microsomal protein [12]. Lawrence et al. [13] reported that copper ions Cu^{1+} and Cu^{2+} inhibited morphine glucuronidation. Authors postulated that copper ions could be acting directly on the enzyme to mediate this inhibition, possibly via the formation of covalent bonds with suitable free residues on the enzyme molecule. Alternatively, they proposed that copper ions could act as allosteric inhibitors, by attaching to a specific site on the enzyme. In this case, authors used total liver homogenates and mM copper ions concentrations to assay UGT activity. These copper ions concentrations are higher than those hepatic concentrations described in copper-associated diseases. Because Cu^{1+} and Cu^{2+} inhibited differentially the formation of morphine 3-glucuronide and morphine 6-glucuronide, more than one isoenzyme could have been involved in the morphine glucuronidation. Thus, the UGT inhibitory effects of copper ions seem to affect to all UGT isoforms, probably because all of them are thiol proteins. Since the substrate specificity of UGT isoenzymes is very broad and they can metabolize in distinct extension several substrates, agents capable to modify their catalytic activity may exert differential effects on them. Because the hepatic endoplasmic reticulum is the principal subcellular organelle where UGT isoforms are localized, the studies of UGT activity modifiers in a liver microsomal preparation may result in an approximation of what *in vivo* could occur.

The mechanisms of copper toxicosis are not still clear. Since it affects mainly liver function, it may represent, among other possibilities, a risk in the drug biotransformation. Noteworthy phase II enzymes act on some toxins directly, while others must first be activated by the phase I enzymes. Patients with Gilbert and Crigler-Najjar's syndromes are characterized by a chronically elevated serum bilirubin level, an endogenous compound biotransformed by UGT and eliminated as diglucuronide [14]. The risk in drug biotransformation is especially high in these patients. Ideally, phase I and phase II detoxification mechanisms work synergistically. If phase I detoxification is highly active and phase II detoxification is lethargic, the individual is referred to as a "pathological detoxifier," a condition which increases sensitivities to environmental poisons. The intermediates of phase I are accumulated when glucuronidation is low and re-enter in the phase I cycle. Several of these intermediates are extremely toxic products, i.e.

metabolites of car exhausts, food contaminants and pesticides, since the cytochrome P450 system can convert such substances into carcinogenic compounds [15–17]. A deleterious effect of copper toxicosis on UGT activity may thus have a relevant impact in the delicate balance between phases I and II biotransformation of drugs.

Data presented in this work indicate that endogenous compounds and xenobiotics could reach toxic concentrations if copper ions inhibit the UGT activity. Thus, in order to investigate the possible effects of high copper concentrations on UGT activity exploring the changes on its liver microsomal activity provoked by 25 nM, 10 μM and 50 μM of Cu^{2+} alone and in the presence of ascorbate. To select these copper concentrations, we considered a previous study in which, we demonstrated that indiscriminate binding of copper could take place only at high μM copper concentrations and only oxidative effects at nM copper concentrations [12]. Our results showed that 25 nM Cu^{2+} alone did not alter microsomal UGT activity, but in the presence of ascorbate, it provoked only UGT oxidative activation. Interestingly, only UGT inhibition was observed with 10 μM Cu^{2+} concentration; but, 10 μM Cu^{2+}/ascorbate either activated or inhibited microsomal UGT activity depending of incubation time assayed. On the other hand, 50 μM Cu^{2+} alone and in the presence of ascorbate only inhibited the UGT microsomal enzymatic activity. Moreover, DTT prevented and reversed the UGT activation and EDTA prevented both the activation and the inhibition of the UGT activity, so indicating that copper ions provoked both phenomena. Thus, our results seem to indicate that at means two different mechanisms may be involved in the changes that Cu^{2+}/ascorbate cause on UGT: (1) oxidation of its thiol groups may account for UGT activation; (2) the binding of copper ions to these groups, may explain UGT inhibition. Because these phenomena may occur simultaneously, the final microsomal UGT activity would be the balance of both processes. The pharmacokinetic, pharmacodynamic and toxicological implications of these results are discussed.

2. Materials and methods

2.1. Chemicals

Cysteine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4-dithiothreitol (DTT), glutathione (GSH), *p*-nitrophenol (PNP), UDP-glucuronic acid (ammonium salt) (UDPGA), ethylen-diamine tetra-acetic acid (EDTA), iminodiacetic acid sodium form in polystyrene matrix (CHELEX-100) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloracetic acid (TCA), ascorbate (sodium salt) and $CuSO_4 \times 5\ H_2O$ were purchased from Merck Co. Chile. 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 Faculty of Chemical and Pharmaceutical Sciences, University of Chile.

2.3. Microsomal preparation

Animals were fasted for 15 h with water *ad libitum* and sacrificed by decapitation. Livers were perfused *in situ* with 4 volumes of 25 ml 0.9% (w/v) NaCl, excised, and placed on ice. All homogenization and fractionation procedures were performed at 4 °C and all centrifugations were performed using either a Suprafuge 22 Heraeus centrifuge or an XL-90 Beckmann ultracentrifuge. Liver tissue (9–11 g wet weight), 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. Homogenates were centrifuged at 9000 × *g* for 15 min, and sediments were discarded. Then, supernatants were centrifuged at 105,000 × *g* for 60 min. Sediments (microsomes, enriched in endoplasmic reticulum) were stored at −80 °C until use. Protein determinations were performed according to Lowry et al. [18].

2.4. Microsomes treated with Triton X-100

Rat liver microsomes were treated with 0.1% (v/v) of Triton X-100 for five min at 20 °C; then, microsomes suspension were centrifuged at 105,000 × *g* for 60 min in a XL-90 Beckmann ultracentrifuge. Pellets were used to estimate protein and UGT remaining activity in microsomal membrane and, supernatants, to assess protein and UGT activity solubilisation.

2.5. Copper and ascorbate concentrations assayed

$CuSO_4$ 25 nM, 10 and 50 μM alone and in the presence of 1 mM sodium ascorbate were assayed. In all

assays, microsomes were preincubated with Cu^{2+} or Cu^{2+}/ascorbate during 15 min to 37 °C before to determine UGT activity; the only exceptions were those in which we evaluate the microsomes preincubation time with Cu^{2+} and Cu^{2+}/ascorbate.

2.6. UGT activity

p-Nitro phenol (PNP) conjugation was studied essentially as described in Letelier et al. [11]. Activity was assayed determining the remaining PNP after 15 min incubation at the following conditions: 0.5 mM PNP; 2 mM UDPGA, 100 mM Tris–HCl, pH 8.5, 4 mM $MgCl_2$ and 1 mg of microsomal protein/ml. Control samples were performed in absence of UDPGA. Trichloroacetic acid (5% final concentration) was used to stop reactions; samples were then centrifuged at 10,000 × *g* for 10 min in a Suprafuge 22 Heraeus centrifuge and NaOH was added to the mixture in order to achieve a 0.5 M final concentration. Remaining PNP was determined at 410 nm using control samples of known PNP initial concentration as standards. Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

2.7. Microsomal thiol content

Microsomal thiols were titrated with DTNB, as described by Letelier et al. [12]. Microsomal thiol concentration was estimated by the equimolar apparition of 5-thio-2-nitrobenzoic-acid ($e_{410} = 13{,}600\ M^{-1}\ cm^{-1}$).

2.8. Statistical analysis

Data groups (means ± S.E.M.) were compared using Student's *t*-test for paired observations. Statistical significance and regression analyses were performed using the Origin 7.0 Software. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of Cu^{2+} and Cu^{2+}/ascorbate on UGT activity

We first tested the effects of preincubation time at 37 °C of microsomes alone (control values), in the presence of Cu^{2+} and in the presence of Cu^{2+}/ascorbate on UGT activity. These results are shown in Figs. 1 (Cu^{2+}) and 2 (Cu^{2+}/ascorbate). UGT activity control values increased with preincubation time.

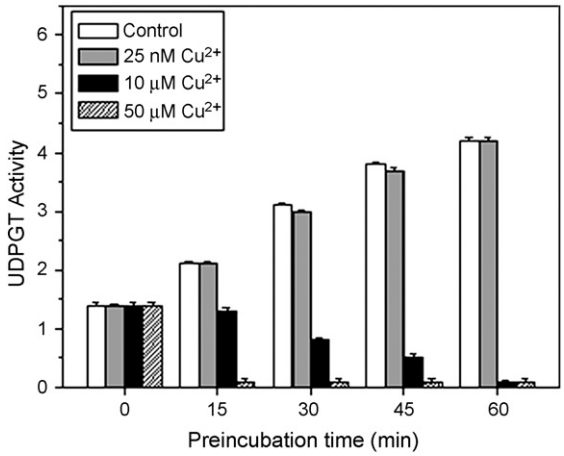

Fig. 1. UGT activity: effect of preincubation time of microsomes with Cu^{2+}. Microsomes were preincubated without (control) or with Cu^{2+} alone before to determine the UGT activity as described in Section 2. UGT activities values are expressed as nmoles of conjugate/min/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments).

UGT activity remained unchanged following treatment of microsomes with 25 nM Cu^{2+} at all the preincubation times assayed. After preincubation of microsomes with 10 µM Cu^{2+}, however, a robust UGT inhibition was observed; this phenomenon was preincubation time-dependent until total loss of enzymatic activity, at 60 min. A similar effect was observed with 50 µM Cu^{2+}, but UGT activity was abolished at the minimum preincubation time assayed (15 min).

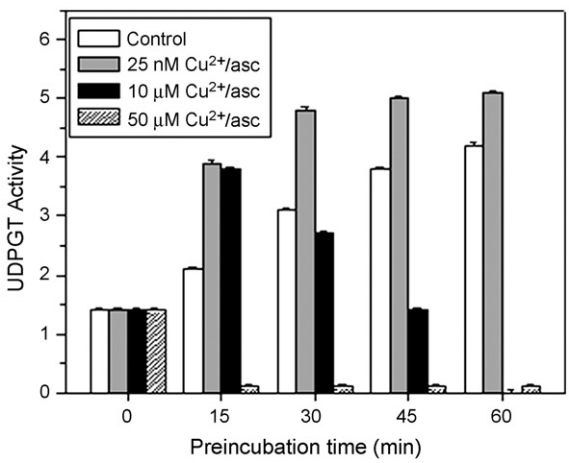

Fig. 2. UGT activity: effect of preincubation time of microsomes with Cu^{2+}/ascorbate. Microsomes were preincubated without (control) or with Cu^{2+}/ascorbate before to determine the UGT activity as described in Section 2. UGT activities values are expressed as nmoles of conjugate/min/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments).

Preincubation of microsomes with 25 nM Cu^{2+}/ascorbate induced UGT activation in a preincubation time-dependent manner; the extension of this phenomenon ranged between ~1.9 fold (microsomes preincubated during 15 min) and ~1.2 fold (microsomes preincubated during 60 min). Treatment of microsomes with 10 μM Cu^{2+}/ascorbate for 15 min increased UGT activity; longer preincubation times, however, caused a strong inhibition of this enzymatic activity until its total loss at 60 min. Finally, preincubation of microsomes with 50 μM Cu^{2+}/ascorbate for 15 min was sufficient to completely abolish UGT activity, similar to what was observed with 50 μM copper ions in the absence of ascorbate.

3.2. *Effects of copper ions on UGT activity in microsomes treated with Triton X-100*

It is known that the microsomal proteins conformation can be altered by treatment with Triton X-100, resulting in changes of their biological functions. In fact, UGT activity from microsomes treated with this detergent was ~3 fold higher than the control activity (Fig. 3). This effect was not due to solubilisation of the enzyme as UGT activity remained completely associated to the membrane fraction following detergent treatment, as assessed by centrifugation at 105,000 × *g* and screening of pellet and supernatant for UGT activity (data no shown).

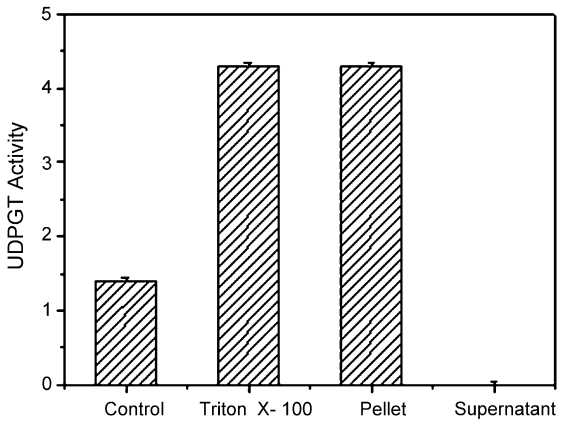

Fig. 3. Microsomal UGT activity in the presence of 0.1% (v/v) Triton X-100. Control: microsomes not treated with Triton X-100; Triton X-100: microsomes treated with Triton X-100; pellet and supernatant: fractions of microsomes treated with Triton X-100 and then centrifuged to 105,000 × *g* according to Section 2. UGT activity values are expressed as nmoles of conjugate/min/mg of microsomal protein. All values represent the mean ± S.E.M. of at least four independent experiments.

The conformational changes of microsomal protein may induce different susceptibility of the amino acid residues such as cysteinyl groups; these groups can be oxidized or/and bind copper ions. UGT isoenzymes are thiol proteins [1,3]. Thus, we assayed the UGT activity of microsomes treated with Triton X-100 in the presence of Cu^{2+} alone (Fig. 4A) and Cu^{2+}/ascorbate (Fig. 4B). When these microsomes were treated with 25 nM Cu^{2+} the UGT activity control value was not modified although microsomes were preincubated during 30 min with 25 nM Cu^{2+} before to determinate the enzymatic activity (Fig. 4A: T1 and T2). However, UGT activity was inhibited 30% when microsomes treated

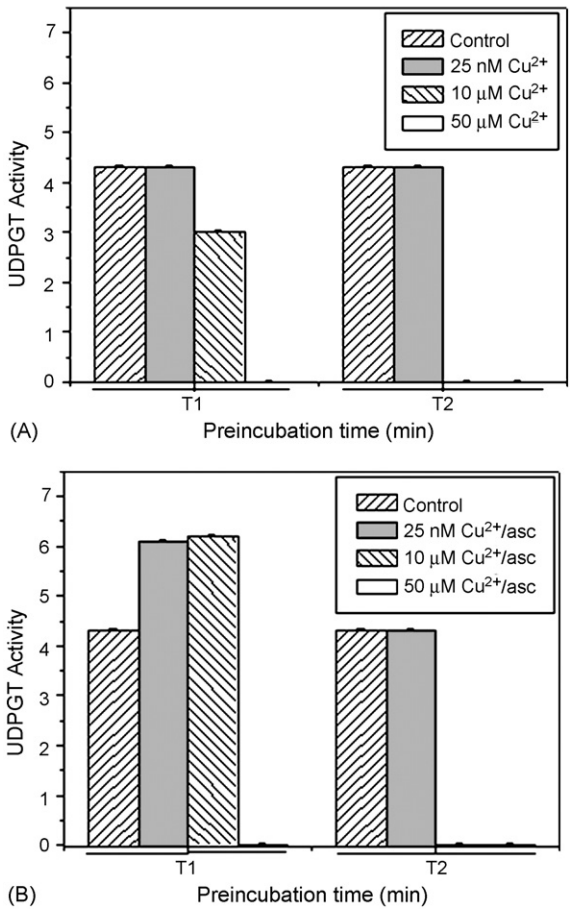

Fig. 4. UGT activity of microsomes treated with 0.1% (v/v) Triton X-100. Effect of Cu^{2+} (A) and Cu^{2+}/ascorbate (B). Microsomes were treated with Triton X-100, under conditions detailed in Section 2. T1: microsomal UGT activity was determined immediately after to add Cu^{2+} or Cu^{2+}/ascorbate according to Section 2. T2: microsomes preincubated for 30 min with Cu^{2+} or Cu^{2+}/ascorbate before to determine UGT activity. Values represent the UGT activity expressed as nmoles of conjugate/min/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments).

with Triton X-100 were tested with 10 μM Cu^{2+} alone; moreover, the preincubation of these microsomes with 10 μM Cu^{2+} abolished the enzymatic activity (Fig. 4A: T1 and T2). Likewise, treatment of these microsomes with 50 μM Cu^{2+} (0 and 30 min before to measure the enzymatic activity), abolished the UGT activity (Fig. 4A: T1 and T2).

On the other hand, microsomes treated with Triton X-100 tested in the presence of 25 nM Cu^{2+}/ascorbate increased in 40% the UGT activity (Fig. 4B: T1); when microsomes were preincubated during 30 min with 25 nM Cu^{2+}/ascorbate before assaying the enzymatic activity, however, UGT activity remained unchanged (Fig. 4B: T2). Triton X-100-solubilised microsomes treated with 10 μM Cu^{2+}/ascorbate displayed increased UGT activity ~40% (Fig. 4B: T1); preincubation of these microsomes for 30 min with 10 μM Cu^{2+}/ascorbate prior to determination of UGT activity, abolished this enzymatic activity (Fig. 4B: T2). Preincubation of microsomes treated with Triton X-100 with 50 μM Cu^{2+}/ascorbate for 0 and 30 min before assaying UGT activity abolished this enzymatic activity (Fig. 4B: T1 and T2).

3.3. Microsomal thiol content

To address the potential relevance of thiol groups of UGT in its enzymatic activity, we measured the microsomal thiol content in the presence of Cu^{2+} alone (Fig. 5) and Cu^{2+}/ascorbate (Fig. 6). To evaluate microsomal membrane conformational changes we performed these experiments in native microsomes (Figs. 5A and 6A) and microsomes pre-treated with 0.1% (v/v) Triton X-100 (Figs. 5B and 6B). Microsomal thiol content of samples without preincubation (T1) or preincubated during 30 min (T2) with Cu^{2+} alone or Cu^{2+}/ascorbate, are shown in Fig. 5A. In the condition T1, native microsomes treated with 25 nM, 10 μM and 50 μM Cu^{2+} alone did not modify microsomal thiol content. In the condition T2, thiol content of native microsomes was not modified by 25 nM Cu^{2+}, but 10 and 50 μM Cu^{2+} decreased it in the same extension (~56%). Pre-treatment of microsomes with 0.1% (v/v) Triton X-100, according to Section 2, increased the thiol content (~13%). Treatment of these microsomes for 0 and 30 min with 25 nM Cu^{2+} led to a decrease (~10%) in microsomal thiol content (Fig. 5B). In the condition T1, 10 and 50 μM Cu^{2+} caused a decrease of ~10% and ~55% in thiol content, respectively. As shown in Fig. 5B, further decreases of microsomal thiol content was observed when these microsomes were preincubated for 30 min with 10 and 50 μM Cu^{2+} (~78 and ~85%, respectively).

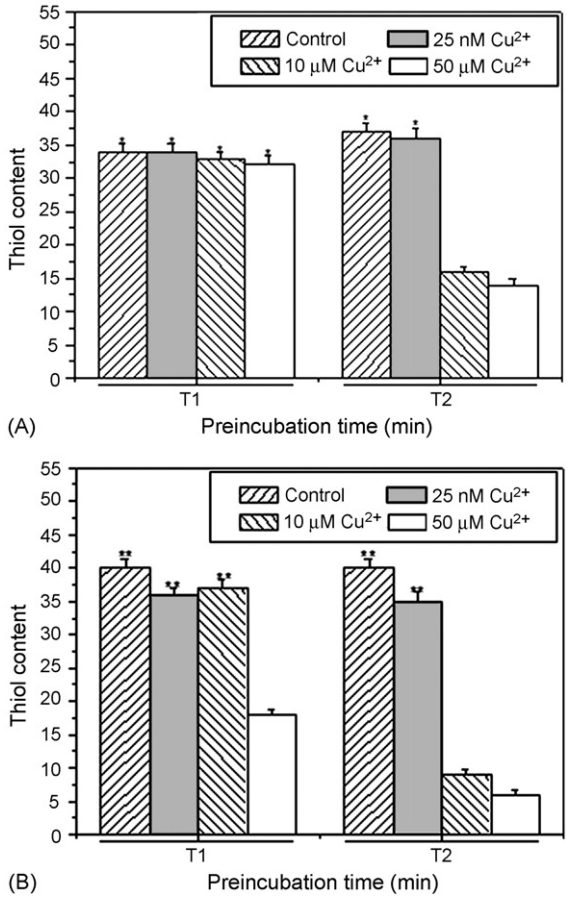

Fig. 5. Effect of Cu^{2+} on microsomal thiol content. (A) Native microsomes. (B) Microsomes treated with Triton X-100, under conditions detailed in Section 2. T1: microsomal UGT activity was determined immediately after to add Cu^{2+} or Cu^{2+}/ascorbate according to Section 2. T2: microsomes preincubated during 30 min with Cu^{2+} before to determine thiol content according to Section 2. Thiol content values are expressed as nmoles of thiol/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments). *Values not significantly different to control values ($p > 0.05$); **values significantly different to control values ($p < 0.05$).

As shown in Fig. 6A, in the condition T1, 25 nM Cu^{2+}/ascorbate did not modify thiol content of native microsomes while in the condition T2 a decrease of ~20% was observed. Likewise, 10 μM Cu^{2+}/ascorbate and 50 μM Cu^{2+}/ascorbate decreased the thiol content of native microsomes in ~15 and ~18%, respectively (Fig. 6A). In the condition T2, however, these values were increased to ~62% (10 μM Cu^{2+}/ascorbate) and ~70% (50 μM Cu^{2+}/ascorbate). As illustrated in Fig. 6B, thiol content of microsomes treated with Triton X-100 following 25 nM Cu^{2+}/ascorbate treatment did not modify thiol content; preincubation of these microsomes for 30 min with 25 nM Cu^{2+}/ascorbate, however,

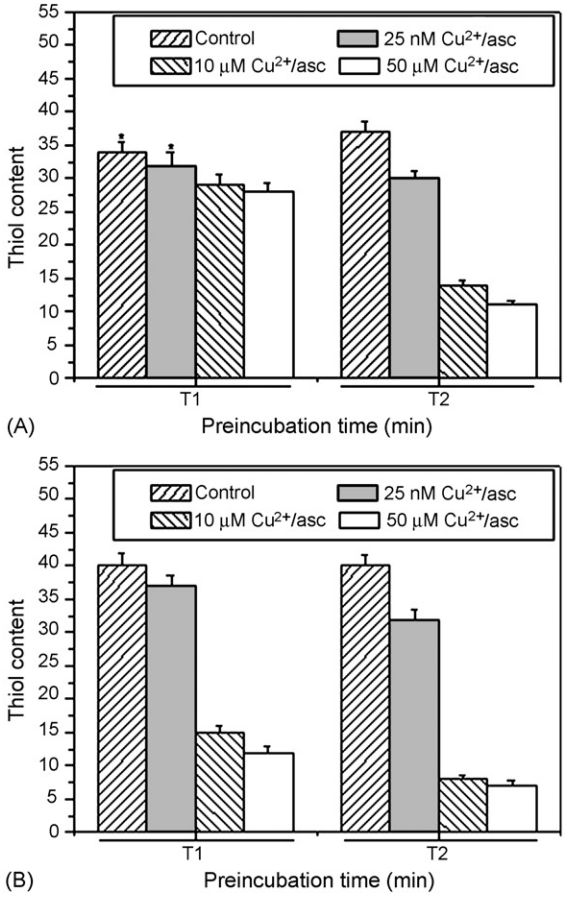

Fig. 6. Effect of Cu^{2+}/ascorbate on microsomal thiol content. (A) Native microsomes. (B) Microsomes treated with Triton X-100, under conditions detailed in Section 2. T1: microsomal UGT activity was determined immediately after to add Cu^{2+} or Cu^{2+}/ascorbate according to Section 2. T2: microsomes preincubated for 30 min with Cu^{2+}/ascorbate before to determine thiol content according to Section 2. Thiol content values are expressed as nmoles of thiol/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments). *Values not significantly different to control values ($p > 0.05$).

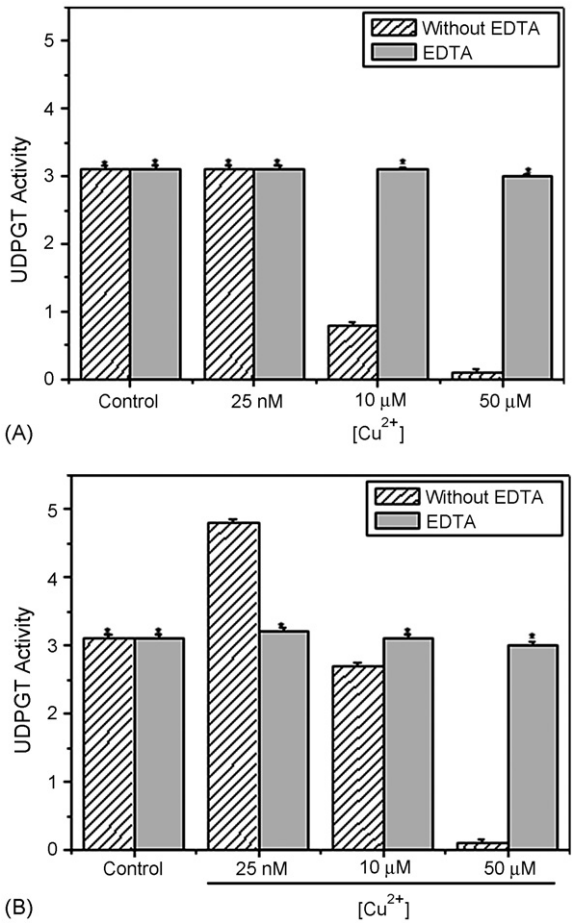

Fig. 7. UGT activity in the presence of Cu^{2+} and Cu^{2+}/ascorbate. Effect of EDTA. [EDTA]: 1 mM. (A) Microsomes preincubated during 30 min with a mixture of Cu^{2+}/EDTA before to determine the UGT activity. (B) Microsomes preincubated during 30 min with a mixture of Cu^{2+}/ascorbate/EDTA before to determine the UGT activity according to Section 2. Control: microsomes preincubated for 30 min with or without EDTA before to determine the UGT activity. Values represent the UGT activity expressed as nmoles of conjugate/min/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments). *Values not significantly different to control values ($p > 0.05$).

decreased thiol content in ~20%. In the condition T1, the microsomal thiol content was decreased ~63 and ~70% by 10 μM Cu^{2+}/ascorbate and 50 μM Cu^{2+}/ascorbate, respectively, and in the condition T2, these values were decreased to ~80% (10 μM Cu^{2+}/ascorbate) and ~83% (50 μM Cu^{2+}/ascorbate).

3.4. EDTA and UGT activity in the presence of Cu^{2+} and Cu^{2+}/ascorbate

If Cu^{2+} is exerting direct effects, the presence of chelating agents such as EDTA will prevent them. Thus, we assayed the effect of Cu^{2+} (Fig. 7A) and Cu^{2+}/ascorbate (Fig. 7B) on the UGT activity in the presence of EDTA. This chelating agent did not modify by itself the UGT activity of control value and that measured in the presence of 25 nM Cu^{2+}. However, EDTA abolished the inhibitory effects on UGT activity induced by 10 and 50 μM Cu^{2+} (Fig. 7A). Moreover, EDTA abolished the UGT activation induced by 25 nM Cu^{2+}/ascorbate and the UGT inhibition induced by 10 μM/ascorbate and 50 μM Cu^{2+}/ascorbate when microsomes were preincubated for 30 min with these Cu^{2+}/ascorbate mixtures prior to determination of the UDGT activity (Fig. 7B).

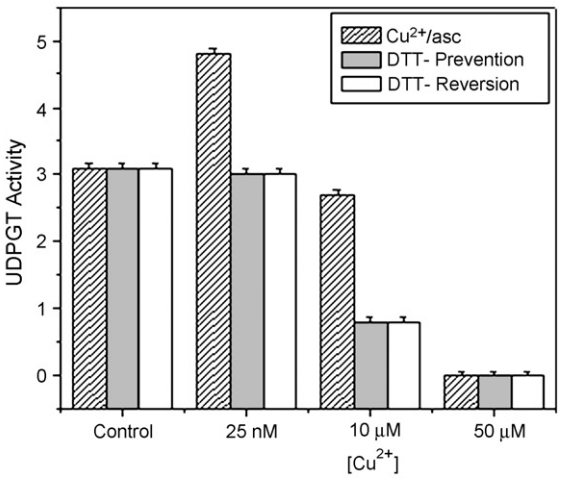

Fig. 8. Effect of DTT on UGT activation induced by Cu^{2+}/ascorbate. [ascorbate]: 1 mM; [DTT]: 2 mM. DTT-protection: microsomes incubated during 15 min with DTT and then 30 min Cu^{2+}/ascorbate before to determine UGT activity. DTT-reversion: microsomes incubated during 30 min with Cu^{2+}/ascorbate and then 15 min with DTT before to determine UGT activity according to Section 2. Control: microsomes incubated without Cu^{2+}/ascorbate. Values represent UGT activity expressed as nmoles of conjugate/min/mg of microsomal protein (mean ± S.E.M. of at least four independent experiments).

3.5. Effect of DTT on microsomal UGT activity in the presence of Cu^{2+}/ascorbate

To evaluate the oxidative changes induced by Cu^{2+}/ascorbate on UGT activity, we studied the effect of a known thiol reducing agent, DTT; Fig. 8 illustrates these results. Treatment of microsomes with 2 mM DTT for 15 min before (prevention) and after (reversion) to add Cu^{2+}/ascorbate, abolished the UGT activation induced by the preincubation of microsomes for 30 min with 25 nM Cu^{2+}/ascorbate. In the same conditions (prevention and reversion), DTT increased UGT activity inhibition caused by 10 μM Cu^{2+}/ascorbate since ∼13% to ∼74% and did not modify the UGT activity inhibition elicited by 50 μM Cu^{2+}/ascorbate.

4. Discussion

Recently, there has been an increase in reports regarding copper-associated diseases in both man and animals. Wilson's disease is an autosomal recessive disorder that results from pathological accumulation of copper predominantly in the liver and brain. Copper also has a role in fatal, non-Wilson liver diseases affecting young children with a genetic abnormality of copper metabolism. Excess accumulation of copper also occurs because of chronic liver diseases such as primary biliary cirrhosis, and chronic hepatitis in both humans and animals [19–23]. Thus, disruption of the normal copper homeostasis or accumulation of copper in excess of metabolic requirements can lead to copper toxicity. Thus, copper toxicosis can be classified as primary when it results from an inherited metabolic defect, and as secondary when it is the consequence of an abnormally high intake, increased absorption, or reduced excretion of copper due to underlying pathologic processes.

In the present study, we studied the molecular mechanisms by which copper ions alter one of the most relevant enzymatic activities in liver biotransformation of xenobiotics. UGT is a relevant enzyme in the biotransformation of drugs; glucuronides are the major metabolites excreted by urine. Previous studies demonstrate that the Fe^{3+}/ascorbate system elicits microsomal UGT activation, due to the generation of reactive oxygen species via Fenton and/or Haber-Weiss reactions [11]. Similarly, Cu^{2+}/ascorbate may induce redox effects [12,24]. Unlike iron ions, however, copper ions may also indiscriminately bind to thiol groups of proteins other than the ones related to its transport and/or storage. Furthermore, such effect seems to depend on copper ions concentration, as it has been observed only with μM but no with nM concentrations [12].

UGT is represented by a super family of thiol proteins and modification of their cysteine alter it enzymatic activity; i.e. substitution of the three cysteine residues within the C-terminal cytosolic tail had minimal effect on basal UGT1A1 activity, but prevented UGT1A1 activation by UDP-GlcNAc physiological activator, which stimulates the import of the donor substrate UDPGA [1,3]. Moreover, the microsomal UGT-activation provoked by μM Fe^{3+} concentration in the presence of ascorbate was totally prevented and reversed by reducing agents, such as, DTT, GSH and cysteine, indicating that only redox changes on the UGT seem be involved in its activation [11]. Furthermore, thiol-alkylating agents such as NEM and mersalyl may alter UGT activity [11,25]. UGT cysteine residues can bind copper ions, a process that alters its enzymatic activity; this phenomenon seems to depend, however, on copper ion concentration. Thus, Cu^{2+}/ascorbate could cause simultaneously UGT oxidative activation and UGT inhibition by its redox effects and binding of copper ions to the enzyme, respectively. It is necessary to note that the binding phenomenon may cluster the copper ions, hindering their pro-oxidant capacity. The extension of both binding-induced inhibition and oxidative activation, should determine the final effect on microsomal UGT activity.

Consistent with our hypothesis, in the absence of ascorbate, μM Cu^{2+} inhibited the microsomal UGT activity while it remained unchanged following nM Cu^{2+} treatment (Fig. 1). In the presence of ascorbate, only nM Cu^{2+} caused UGT activation, low μM Cu^{2+} led to a transient activation by inhibition of UGT activity, and high μM Cu^{2+} abolished this activity (Fig. 2). We have previously demonstrated, with other enzymatic activities, that nM Cu^{2+} concentrations in the presence of ascorbate induce only oxidative changes while μM Cu^{2+}, in either the presence or absence of ascorbate, elicited mainly Cu^{2+} binding to microsomal protein [12]. The corollary of these observations is that intermediate copper ions concentration will have mixed effects. The data presented here is completely consistent with a model in which copper behaves as a pro-oxidant when a reducing agent is present (i.e. ascorbate) and at very low concentrations (i.e. nM) while at higher concentrations (i.e. μM), the copper-binding effect is prevalent, even in the presence of reducing agents.

Contrary to our results, probably because the assay conditions were different, Ikushiro et al. [26] report activation of rat liver microsomes UGT provoked by DTT (5 mM). These authors postulate the reduction of a disulphide bond within the enzyme as a possible mechanism explaining their observations. Ghosh et al. [1] who reported that treatment with 5 mM DTT did not significantly affect UGT activity towards bilirubin in native, digitonin-permeabilized or UDPGlcNAc-treated human liver microsomes, indicating that intra- or inter-molecular disulphide bonding is not required for UGT1A1 activity towards bilirubin, have challenged these results.

Another question we addressed in this study is whether UGT activity relies on the presence of critical cysteinyl residues that need to be in the free form. Copper-binding proteins are able to bind different metal ions (Zn^{2+}, Cd^{2+}, Ag^{+} and Hg^{2+}) as well in virtue of the occurrence of thiol clusters in their amino acid sequences [27–33]. UGT isoenzymes appear to display reactivity towards thiol alkylating agents, such as NEM and mersalyl acid. Apparently, these agents act in a concentration-dependent biphasic manner and differently towards different UGT isoforms [1,11,25]. Micromolar Cu^{2+} concentrations alone and in the presence of ascorbate decreased microsomal thiol content (Fig. 5) and inhibited UGT activity (Fig. 1). We have reported that μM Fe^{3+} concentrations alone did not modify both UGT activity and microsomal thiol content; similar to nM Cu^{2+}/ascorbate, μM Fe^{3+}/ascorbate elicited UGT activation [11].

It is known that different detergents induce a significantly UGT activation which induce conformational changes in the microsomal membrane [2,4,5]. These compounds alter the fluidly of microsomal membrane changing the conformation of their components. These changes may provoke the exposure of new protein cysteine residues, making them available for oxidative or other modifications; on the other hand, membrane fluidity changes may favour the access of UDPGA into de lumen of endoplasmic reticulum, thus increasing the UGT activity. Noteworthy, in our conditions (microsomes treated with Triton X-100), UGT activity remains completely associated to the microsomal membrane, but it was three fold higher than that of native microsomes (Fig. 3). The conformational changes of the microsomal membrane components-including UGT- may explain the activation induced by detergents. The UGT activity inhibitory effects and the decrease of the microsomal thiol content provoked by Cu^{2+} and Cu^{2+}/ascorbate were significantly higher in microsomes treated with Triton X-100 than native microsomes (Figs. 5B and 6B). In light of these data, our results confirm the relevance of free thiol groups in UGT activity.

In copper-overload diseases, free copper ions may reach so high concentrations that may alter the biological functions of several hepatic proteins -including UGT [34,35]. Due to its similarities with iron, an excess of copper is thought to result in cell oxidative damage, including lipid peroxidation. The level of thiobarbituric acid reactive substance (TBARS), a measure of lipid peroxidation, appears increased in copper-loaded rats [34,35]. A study by Aburto et al. [36], however, did not find significant alterations in the levels of malondialdehyde, a lipid peroxidation by product, prompting the study authors to postulate that lipid peroxidation does not play a major role in copper toxicity although it may occur as a terminal event because of cell injury. Copper-binding to proteins, such as UGT, may underlie the non-oxidative mechanism underlying copper-overload damage. This is a very novel proposal, and we are actively testing this model for copper-related consequences in biological systems.

Oxidative UGT activation may represent a relevant physiological mechanism for xenobiotic biotransformation processes since: i) metabolites generated of the xenobiotics biotransformation catalyzed by the cytochrome P450 system are substrates of UGT, and ii) several lipophilic xenobiotics metabolized by cytochrome P450 system, cause oxidative stress (solvent, pesticides and drugs). In this regard, cytochrome P450 reductase, a member of the cytochrome P450 oxidative system, catalyzes the nitro-reduction of

Nitrofurantoin (antibacterial drug) and Nifurtimox (antichagasic drug). The nitro-reduction of these drugs generate ROS, which in turn lead to microsomal lipid peroxidation and UGT activation similar to that observed in our studies, in the presence of Fe^{3+}/ascorbate and 25 nM Cu^{2+}/ascorbate [11,37]. Probably, the oxidative UGT activation phenomenon may account for a physiological oxidative crosstalk between phase I and II drug metabolizing enzymes in order to enhance detoxication of lipophilic substances. If copper ions inactivate UGT, not only drugs glucuronidation may be affected, also those lipophilic drugs metabolized by cytochrome P450 system. This is an interesting field of research and we are currently testing this hypothesis.

A major subject of our research focuses on the potential differences in iron-related and copper-related damage. Our data show once more that Cu^{2+} has the ability to cause damage to biological systems through two very different mechanisms: as a prooxidant (only in the presence of reducing agents, such as ascorbate) and by indiscriminate binding to thiol groups of proteins. In the case of UGT, Cu^{2+}/ascorbate cause both activation and inhibition of the UGT activity. It is necessary to note that DTT totally prevented and reversed the activation of UGT caused by oxygen free radicals generated by Cu^{2+}/ascorbate (Fig. 8), thus indicating that this phenomenon is mediated by oxidative changes on microsomal protein, possibly UGT. Noteworthy, in our assay conditions, microsomal lipoperoxidation also occur and data exist that in this condition microsomal UGT activation also occur (11). Lipoperoxidation is not a redox reversible phenomenon and UGT activation was totally reversed by DTT (Fig. 8), making it an unlikely player in this phenomenon.

These data suggest that ROS generated by Cu^{2+}/ascorbate are likely to be the responsible agents of UGT activation, either directly or indirectly. On the other hand, our data show that concomitant UGT inhibition is explained most likely by copper binding to thiol groups of microsomal protein, which may include UGT itself. We are actively working to address whether these are universal mechanisms for copper-related damage to biological systems.

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