Chronic exposure of HepG2 cells to excess copper results in depletion of glutathione and induction of metallothionein

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Abstract

Metallothionein (MT) and reduced glutathione (GSH) play an important role in the intracellular handling of copper by preventing the generation and favouring the removal of copper-derived free radicals. The present study addressed the changes in MT and GSH that follow chronic (2 or 5 weeks) exposure of human hepatoblastoma cells (HepG2) to excess copper. Copper treatment (100 μM, 2 weeks) led to a 28-fold elevation in intracellular copper. Concomitantly, cells exhibited a seven-fold increase in total MT and an increment in its saturation with copper from 45 to 86%. Around 38% of copper in the cytosolic fraction could be accounted for by MT. GSH equivalents were substantially lowered (to 37% of basal levels) in treated cells, with only part of it being accounted for by an increase in GSSG. Copper-treatment induced no changes in catalase or GSH-peroxidase activities but it was associated with a small reduction in SOD (20%) and GSH-reductase (26%) activities. Copper-loaded cells did not differ from controls in their basal oxidative tone; however, when exposed to *tert*-butylhydroperoxide they exhibited a markedly greater susceptibility to undergo both oxidative stress and cell lysis. It is proposed that chronic exposure of HepG2 cells to excess copper is accompanied by "adaptive changes" in GSH and MT metabolism that would render cells substantially more susceptibility to undergo oxidative stress-related cytotoxicity.

Keywords: Copper; Chronic exposure; Metallothionein; Glutathione; HepG2 cells; Oxidative stress; Cell lysis

1. Introduction

Copper is an essential trace element in human nutrition. Under physiological conditions, its status is controlled by well-established homeostatic mechanisms (Linder et al., 1998; Peña et al., 1999). However, under certain environmental or genetically defined conditions, such mechanisms can be overridden, leading to a potentially toxic copper accumulation (Luza and Speisky, 1996). As copper toxicity involves its ability to catalyse the generation of free radicals and/or to directly interact with essential biomolecules, copper sequestering is of vital importance for cell protection. Reduced glutathione (GSH) and metallothionein (MT) represent two of the most important molecules in the intracellular handling of copper. The fact that cysteine residues constitute one-third of both molecules is a key structural feature in their abilities to bind copper. GSH is likely to be one of the first molecules with which copper ions interact upon entering cells (Freedman et al., 1989). During such interaction, the tripeptide reduces Cu^{2+} to Cu^{+}, and subsequently sequesters Cu^{+} ions as a Cu–GSH adduct (Jiménez and Speisky, 2000). This latter compound has been suggested to participate in copper metabolism as a copper-carrier to several cupro-proteins among which MT molecules are included (Da Costa-Ferreira et al., 1993; Steinebach and Wolterbeek, 1994). GSH, on the other hand, via its interaction with copper ions, is also considered to play a role in defining

Abbreviations: BSA, bovine serum albumin; DCHF-DA, 2′,7′-dichlorofluorescin diacetate; DTNB, 5,5′-dithio*bis*[2-nitrobenzoic] acid; FBS, fetal bovine serum; GSH, reduced glutathione; GSHeq, glutathione equivalents; GSHperox, glutathione peroxidase; GSHred, glutathione reductase; GSSG, oxidised glutathione; LDH, lactate dehydrogenase; LEC, Long–Evans cinnamon rats; MT, metallothionein; MOPS, 3-[*N*-morpholino] propanesulfonic acid; NADH, β-nicotinamide adenine dinucleotide, reduced form; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; PBS, phosphate buffered saline; PCA, perchloric acid; SDS, sodium dodecylsulfate; SOD, superoxide dismutase; *t*-BOOH, *tert*-butyl-hydroperoxide; TMM, toxic milk mutant mice.

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the susceptibility of cells to excess copper (Freedman et al., 1986, 1989). To the extent that GSH sequesters redox-active copper ions, it would prevent these from catalysing free radical generation, thus serving as a mechanism that protect cells against the deleterious consequences of excessive copper accumulation. In addition, GSH contributes to cell protection by acting directly as a scavenger of free radicals generated during copper metabolism and serving as cofactor in the GSH-peroxidase-dependent removal of peroxides generated in copper-overloaded cells (Dillard and Tappel, 1984; Meister, 1988; Sokol et al., 1990).

Metallothionein is a low molecular weight protein of which one-third of its amino acids are cysteine residues. The latter confer MT the ability to interact with zinc and copper ions forming Zn/Cu–MT complexes (Bremner et al., 1986; Hamer, 1986). Under physiological conditions, MT binds copper and zinc to a roughly similar extent. However, given the considerably higher affinity of the thionein for copper (Kägi, 1991), under copper overloading conditions MT becomes increasingly saturated with the metal, representing the complex an important form of storing copper intracellularly (Bremner, 1987, 1998; Harris, 1991). Pathological conditions featuring a major copper accumulation under the form of Cu–MT are, in humans, Wilson's disease (Hunziker and Sternlieb, 1991; Mulder et al., 1992) and in animals, the hepatitis developed by toxic milk mutant mice (TMM) and by Long–Evans cinnamon rats (LEC) (Schilsky and Sternlieb, 1993). During copper overload, MT synthesis is induced via gene transcription (Bremner, 1987). However, beyond its copper-storing property, recent evidence suggests that the copper-saturated thionein may behave as a pro-oxidant during oxidative stress (Sakurai et al., 1994; Stephenson et al., 1994; Liu et al., 2001). Under these conditions, Cu–MT becomes unstable, releasing into the medium copper ions capable of promoting free-radical generation. Given the potentially opposing actions that GSH and Cu–MT could exert, antioxidant and pro-oxidant, respectively, we decided to investigate in human hepatoblastoma cells the changes that may take place in both thio-molecules under copper-overloading conditions. Also, we examined the influence of copper overloading on the susceptibility of cells to undergo lytic damage induced by oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

Histidine, GSH, GSSG, baker's yeast glutathione reductase, NADPH, NADH, bovine haemoglobin, 5,5′-dithio*bis*[2-nitrobenzoic] acid (DTNB), rabbit liver Cd/Zn–MT, BSA, (±)-epinephrine, DCHF-DA, sodium pyruvate, sodium dodecyl sulfate, Folin–Ciocalteu's reagent, glycine, hydrogen peroxide, *t*-BOOH, silver nitrate ($AgNO_3$), Trizma base, Tris–HCl and MOPS were all obtained from Sigma Chemical Co (St Louis, MO, USA). Copper sulfate ($CuSO_4 \cdot 7H_2O$), PCA, sucrose, sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Merck Química Chilena (Santiago, Chile). [^{109}Cd]$CdCl_2$ (sp. act. 2.95 mCi/mg) was purchased from NEN^{TM} Life Sciences Products. RPMI-1640 complete medium, fetal bovine serum (FBS), trypsin/EDTA and penicillin/streptomycin were obtained from GIBCO BRL (Chile). All other chemicals were p.a. grade.

2.2. Cell culture

HepG2 cells were grown in a water-saturated atmosphere of CO_2/air (5%/95%) mixture, at 37 °C, in RPMI-1640 complete medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were chronically exposed to 100 μM copper in a medium supplemented with Cu:His (molar ratio of 1:10) during 2–5 weeks. Cells reaching 90% of confluence were subcultured (every 2–3 days) after treatment with 0.25% trypsin/27 mM EDTA (5 min, 37 °C). Trypsin was inactivated by addition of FBS-containing medium.

2.3. Obtention of subcellular fractions

Harvested cells were pelleted (500 *g*, 5 min, 4 °C), washed twice with PBS, and centrifuged again to ensure the removal of the added copper, EDTA and FBS excess. Cells were counted in a Neubaüer hemocytometer and their viability assessed by the trypan blue exclusion test or by the lactate dehydrogenase (LDH) leakage assay. Subsequently, cell suspensions were aliquoted (5×10^6 cells/vial) and centrifuged (500 *g*, 5 min, 4 °C). The supernatants were discarded and the pellets stored at −70 °C (samples were assayed within 4 weeks). Cell pellets were defrosted and mechanically lysed in 10 mM sodium phosphate buffer, pH 7.4; lysis completion was assessed by optical microscopic examination (×400). Total cell protein, copper and zinc were measured in whole cell lysates. Total MT, Zn–MT, copper and zinc contents, SOD, catalase, GSHperox and GSHred activities were assayed as described below in supernatants (S-18) obtained after centrifugation of whole cell lysates (18,000 *g*, 20 min, 4 °C). S-18 aliquots (1.0 ml) were treated with 40% PCA (10 μl) and centrifuged again (18,000 *g*, 10 min, 4 °C). Copper, zinc, total GSH equivalents (reduced plus oxidised) and GSSG were determined in supernatants of the PCA-treated S-18 fractions (acidified S-18 fraction). This procedure was followed for all determinations except for total MT. In this case, cells were washed with 50 mM

MOPS (pH 7.4) supplemented with 200 mM sucrose and lysed in 500 mM glycine-NaOH (pH 8.5).

2.4. Determination of metals

Copper, zinc and silver were measured at 325, 214 and 329 nm, respectively, by atomic absorption spectrophotometry (AAS) using metal-specific hollow cathode lamps and an air/acetylene flame (Perkin–Elmer model 2280).

2.5. Determination of metallothioneins

Zn/Cu–MT and total MT were estimated in terms of their metal-binding capacity according to Eaton and Toal (1982) and Scheuhammer and Cherian (1991), respectively. For total MT determination, the reaction mixture contained 50–500 μl of S-18 fraction plus Ag (10 μg) as $AgNO_3$ in 1.3 ml of 500 mM glycine buffer (pH 8.5). The mixture was incubated in darkness (10 min, 20 °C), added 100 μl of a human hemolysate obtained from healthy volunteers (Scheuhammer and Cherian, 1991), and heated (2 min, 100 °C). After a 30-s spin (18,000 *g*), the hemolysate/heat treatment was repeated once. Finally, the mixture was centrifuged (18,000 *g*, 15 min, 20 °C), and the supernatant was analysed for Ag content. Total MT was estimated assuming 17 at-g of Ag (AW 107.9) per mole of MT (MW of 6000 Da). For Zn–MT determination, the reaction mixture contained $[^{109}Cd]CdCl_2$ (0.2 μCi in 0.4 μg of Cd) and 20–200 μl of S-18 fraction in 0.4 ml of 20 mM Tris buffer, pH 7.4. After incubation (20 min, 20 °C), the mixture was twice treated with 100 μl of 2% bovine haemoglobin and heated (100 °C, 2 min). Finally the mixture was centrifuged (18,000 *g*, 15 min, 20 °C) and the radioactivity remaining in the supernatant was measured using an ISODATA γ-counter (model 500). Zn–MT was estimated assuming 6 at-g of Cd (AW 112.4) per mole of MT (Jiménez et al., 1997). Cu–MT content was calculated as the difference between Total MT and Zn–MT contents. Data are expressed as pmoles of MT per 10^6 viable cells.

2.6. Quantification of reduced and oxidised glutathione

Glutathione was assayed as the sum of its reduced and oxidised forms (hereafter referred to as GSHeq), according to Anderson and Meister (1980). In brief, acidified S-18 fractions were neutralised with 250 mM phosphate/5 mM EDTA buffer, pH 7.4, and added to a mixture containing NADPH (0.2 mM), DTNB (0.6 mM) and GSHred (1 U/ml). The GSH-dependent rate of formation of 5-thio-2-nitrobenzoic acid was monitored spectrophotometrically at 412 nm (Shimadzu UV-1601). For GSSG determination, acidified S-18 fractions were neutralised as above and assayed according to the NADPH-GSHred method as previously (Bannach et al., 1996). The reaction was initiated by the addition of GSHred (1 U/ml) to a mixture containing the samples and NADPH (2 mM), and the OD at 340 nm was measured. Data are presented as nmole of GSHeq or GSSG/10^6 viable cells.

2.7. Determination of enzymatic activities

SOD was assayed according to Misra and Fridovich (1972). The reaction was initiated by the addition of S-18 to a mixture containing (±)-epinephrine (0.225 mg/ml) in 50 mM glycine buffer, pH 10.5. Formation of adrenochrome was monitored at 480 nm. One unit of SOD inhibits the rate of epinephrine oxidation by 50% (20 °C, pH 7.4). Catalase was assayed using H_2O_2 as substrate. The reaction was initiated by the addition of S-18 to a solution containing 1 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.4); the decrease in OD at 240 nm ($\varepsilon = 46$ μM/cm) was monitored in the presence and absence of sodium azide (1 mg/ml). One unit of catalase reduces 0.1 mmol H_2O_2/min (20 °C, pH 7.4). GSHperox was assayed with *t*-BOOH as substrate. The reaction was initiated by the addition of S-18 to a mixture containing 1 mM GSH, 2 mM *t*-BOOH, 0.2 mM NADPH and GSHred (1 U/ml) in 50 mM sodium phosphate/2 mM EDTA buffer (pH 7.4); the decrease in OD at 340 nm was monitored. One unit of GSHperox reduces 1 nmol *t*-BOOH/min (20 °C, pH 7.4). GSHred was assayed using GSSG as substrate. The reaction was initiated by the addition of S-18 to a mixture containing GSSG (0.2 mM) and NADPH (0.2 mM) in 50 mM sodium phosphate/2 mM EDTA (pH 7.4); the OD at 340 nm was monitored. One unit of GSHred reduces 1 μmole GSSG/min (20 °C, pH 7.4).

2.8. Measurement of intracellular oxidative tone

The intracellular oxidative tone was assayed by quantifying the relative fluorescence of DCHF-derived 2′,7′-dichloroflurescein (Royall and Ischiropoulos, 1993), as previously carried out (Hu et al., 1995). Confluent cells, grown in six-well plates, were incubated (37 °C, 15 min) with 10 μM DCHF prepared in PBS supplemented with 1 mM $CaCl_2$ (PBS–Ca buffer). Cells were washed twice and incubated with PBS–Ca (60 min, 37 °C) in the absence or presence of *t*-BOOH (0–0.75 mM). Aliquots from each well were recovered and stored at 4 °C until protein and LDH activity determinations (within 24 h). Immediately after, cells were washed twice with PBS, and lysis was induced by the addition of 1 ml of a 0.1% SDS solution to each well. After centrifugation (3000 *g*, 5 min, 4 °C), the relative fluorescence of the supernatants was determined in a spectrofluorometer (Shimadzu 5000) using 504 nm and 525 nm as excitation and emission wavelength, respectively.

Lactate dehydrogenase was assayed in extracellular medium by measuring the decrease in OD at 340 nm that takes place upon pyruvate (2 mM) reduction in the presence of β-NADH (0.136 mM). One unit of LDH represents the μmol of NADH oxidised per min at 20 °C, and was expressed per mg of cell protein.

2.9. Protein determination

Total protein content was measured according to Lowry et al. (1951) using BSA as standard.

2.10. Statistical analysis

The results are presented as means ± S.D. from at least three independent experiments each, conducted in triplicate. Groups of test data were compared using Student's *t*-test for paired observations. Values were considered to differ significantly at the level of $P < 0.05$.

3. Results

3.1. Intracellular copper content and distribution

Figure 1 depicts the intracellular levels and the subcellular distribution of copper in HepG2 cells grown during a 2- or 5-week period in the continuous presence of 100 μM extracellular copper-histidine. Under such exposure conditions, no significant changes in cell morphology or in the cell growth rate were apparent. However, at higher copper concentrations (⩾200 μM) or at longer exposure times (⩾6 weeks), an increase in the time required to reach confluence, from 2–3 days in control cells to 4–5 days, macroscopic alterations in their morphology, and a significant loss in cell viability (near 33%) were seen. Therefore, studies presented here were limited to culturing HepG2 cells during 2 and up to 5 weeks, in the presence of a 100 μM copper ($HepG2_{100}$). As shown in Fig. 1, after 2 and 5 weeks, copper exposure led to a 28- and 32-fold increase, respectively, in total intracellular copper (expressed as nmol of Cu per 10^6 viable cells). Regardless of the exposure length, copper was found to accumulate preferentially (about 70%) in the S-18 fraction. Following PCA-treatment of the S-18 fractions from control cells ($HepG2_c$), and 2 and 5-week $HepG2_{100}$ cells, a 35, 68 and 72% of the copper originally present in S-18 were still associated with their respective acid supernatants (Fig. 1). These changes in intracellular copper took place in complete absence of changes in the total intracellular concentration or subcellular distribution of zinc (data not shown).

3.2. Intracellular metallothioneins

Figure 2 depicts the levels of Zn–MT (assessed as binding of ^{109}Cd to MT), Zn/Cu–MT, namely, total MT (assessed by the saturation of MT with silver which

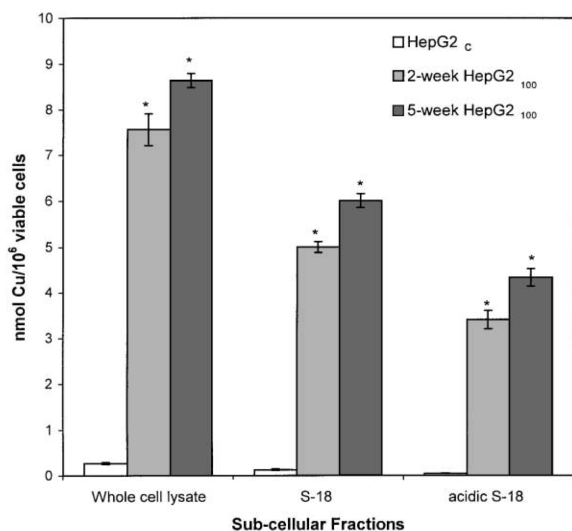

Fig. 1. Intracellular levels and subcellular distribution of copper in HepG2 cells chronically exposed to copper. HepG2 cells were cultured during a 2- or 5-week period in the absence ($HepG2_c$) or presence ($HepG2_{100}$) of copper at a concentration of 100 μM, under the form of a 1:10 Cu–His complex. Copper content was measured by AAS in whole cell lysate, S-18 and acidic S-18 fractions as described in Materials and methods. *Significantly different relative to their controls ($P < 0.05$).

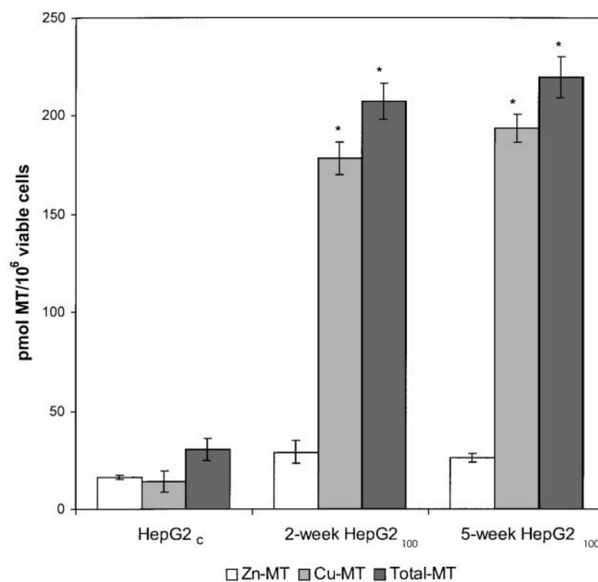

Fig. 2. Zn–metallothionein, Cu–metallothionein and total metallothionein contents in HepG2 cells chronically exposed to copper. Total MT content and MT saturation with zinc (Zn–MT) or copper (Cu–MT), were determined, as described in Materials and methods, in S-18 fractions obtained from control cells ($HepG2_c$) and from cells exposed to 100 μM copper ($HepG2_{100}$) during 2 or 5 weeks. *Significantly different relative to their controls ($P < 0.05$).

displaces Zn and Cu), and Cu–MT (inferred from subtracting Zn–MT from total MT) present in control and copper-treated cells. Copper treatment increased total MT by 6.8- and 8.2-fold, after 2 and 5 weeks, respectively. Such elevations were accompanied by greater saturation of the thionein molecules with copper ions. In fact, while MT molecules in control cells exhibited around 45% saturation with copper (and therefore a 55% saturation with Zn), thionein molecules in $HepG2_{100}$ cells showed 86 and 88% saturation with copper after 2 and 5 weeks, respectively (Fig. 2).

3.3. Reduced and oxidised glutathione

Control cells exhibited a total glutathione content of 18.5 nmol of GSHeq per 10^6 viable cells (equivalent to 37.1 nmol/mg cell protein). Such a level, which is comparable to that reported in HepG2 cells by Duthie et al. (1988), remained essentially unchanged throughout the 2- to 5-week study period (SD equal to 2.3 nmol/mg protein). However, in copper-treated cells, GSHeq were substantially decreased, reaching 37% (6.8 nmol/10^6 viable cells) and 34% (6.3 nmol/10^6 viable cells) of the basal levels, after 2 and 5 weeks of treatment, respectively (Fig. 3). This decrease was accompanied by a significant, but comparatively minor, increment in GSSG levels. In fact, the levels of the disulphide rose from 0.6 nmol/10^6 viable $HepG2_c$ cells, to close to 1.4 and 1.8 nmol/10^6 viable $HepG2_{100}$ cells, after 2 and 5 weeks treatment, respectively (Fig. 3). No significant increments in GSSG were observed in control cells during the 5 week-culture period.

3.4. Enzyme activities related to antioxidant defence

As shown in Fig. 4, copper exposure was not associated with significant changes in GSH-perox and catalase activities (apparent V_{max}) as measured in S-18 fractions from cells treated for 2 weeks. Minor but significant decreases (of 20 and 26%) were seen in the activity of SOD and GSH-red, respectively. Decreases of a similar extent (19 and 24%) were found for these last two enzymes in HepG2 cells treated with copper during 5 weeks (data not shown).

3.5. Cellular susceptibility to extracellular tert-butyl-hydroperoxide

To investigate the effects of chronic copper exposure (during 2 weeks) on the susceptibility of HepG2 cells to undergo oxidative stress and lysis, $HepG2_{100}$ and control cells were incubated in the presence of increasing extracellular concentrations of *t*-BOOH. Minor but not significant differences were observed in the oxidative tone (Fig. 5A) and cell viability (Fig. 5B) of the two cell populations when these were acutely exposed to either 0 (basal condition) or 0.1 mM *t*-BOOH concentrations. Increasing the extracellular peroxide concentration to 0.25 mM led to no changes in the viability of both cell populations, but this was associated with a significant increase (30%) in the oxidative tone of copper-loaded cells. At *t*-BOOH concentrations of 0.35 mM, copper-loaded cells (but not control cells) exhibited a progressive increase in their oxidative tone (Fig. 5A) and a decrease in their viability (Fig. 5B). At concentrations

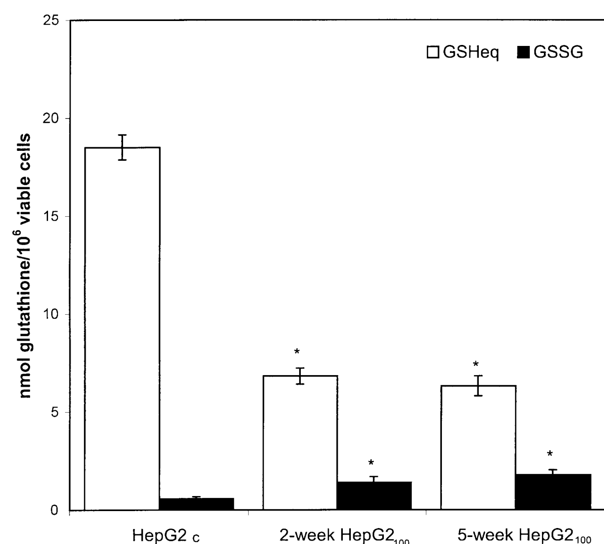

Fig. 3. Reduced and oxidised glutathione levels in HepG2 cells chronically exposed to copper. Total GSHeq and GSSG were assayed in PCA-derived S-18 fractions obtained from control cells ($HepG2_c$) and from cells exposed to 100 μM copper ($HepG2_{100}$) during 2 or 5 weeks. *Significantly different relative to their controls ($P<0.05$).

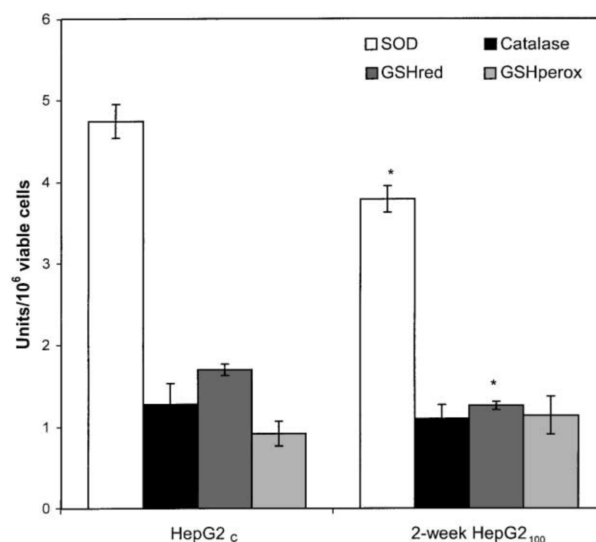

Fig. 4. Antioxidant enzyme activities in HepG2 cells chronically exposed to copper. SOD, catalase, GSHred and GSHperox activities (apparent V_{max}) were determined in S-18 fractions obtained from control cells ($HepG2_c$) and from cells exposed to 100 μM copper ($HepG2_{100}$) during 2 weeks. Units of activity for each enzyme are defined in Materials and Methods. *Significantly different relative to their controls ($P<0.05$).

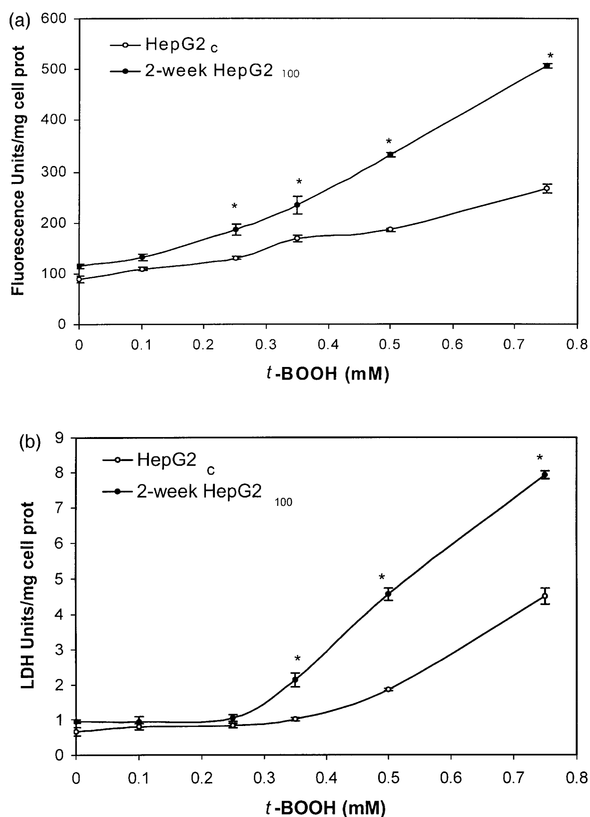

Fig. 5. Susceptibility of copper-exposed HepG2 cells to undergo *t*-BOOH-induced oxidative stress and cell lysis. Control cells ($HepG2_c$) and cells grown during 2 weeks in the presence of 100 µM copper ($HepG2_{100}$) were acutely (60 min) treated with increasing extracellular concentrations of *t*-BOOH (0–0.75 mM). The intracellular oxidative tone (Fig. 5A) was assayed as DCHF-derived fluorescence present in supernatants obtained from whole cell lysates as described in Materials and methods. Values of fluorescence, expressed per mg of viable cell protein, are relative to a 100 units value ascribed to the fluorescence of control cells unexposed to the peroxide. Cell lysis (Fig. 5B) was measured as LDH activity leaked into the extracellular medium, and it is expressed as units of activity per mg of viable cell protein. *Significantly different relative to their controls ($P<0.05$).

equal to or higher than 0.5 mM, the peroxide induced a significant and sustained increase in oxidative tone and a decrease in viability in both cell populations. Noteworthy, when exposed to 0.5 and 0.75 mM *t*-BOOH concentrations, copper-loaded cells exhibited not less than a two-fold greater susceptibility to undergo peroxide-induced cytotoxicity in comparison with $HepG2_c$ cells.

4. Discussion

Hepatoblastoma cells growing in a medium containing toxic concentrations of copper (generally from 200 to 800 µM) initiate homeostatic and protective mechanisms to prevent copper-induced cytotoxicity. In the present study we aimed to address some of the possible "adaptive changes" that follow chronic (2 or 5 weeks) exposure of HepG2 cells to a medium containing high but non-toxic concentrations of copper (100 µM). Under such conditions HepG2 cells remain largely viable despite 28- and 32-fold increased accumulation of copper. Current understanding of the mechanisms by which cells handle an excess of copper is limited; however, existing evidence supports the participation of GSH and MT as two of the major molecules involved in the intracellular sequestering and storing of copper. The increment in intracellular copper seen in $HepG2_{100}$ cells was accompanied by major but opposite changes in the concentrations of these thio-molecules. It was estimated that about 40 and 47% of the cytosolic copper accumulated by 2- and 5-week exposed-cells could be accounted for by its binding to MT, respectively (assuming 12 at-g of Cu per mole of MT). In line with the recognised higher affinity and ability of copper to induce the de novo synthesis of MT (Bremner, 1987), an increased (almost double) saturation of the thionein with copper and an elevated (seven- to eight-fold) number of these molecules were found. Under overloading conditions, MT represents a mechanism to store copper (Hamer, 1986; Bremner, 1987; Kägi, 1991). However, its induction may not be observed until a threshold concentration of intracellular copper is reached (Bremner et al., 1986). Thus, it is likely that at early stages of exposure saturation of pre-existing MT with copper precedes the synthesis of new MT molecules. Our studies suggest that the ability of HepG2 cells to respond to an excess of copper via saturation and induction of MT would approach its maximum after 2 weeks, increasing slightly but not significantly thereafter, with no differences in saturation with copper. A decrease in cell viability was seen in the presence of 100 µM copper for more than 6 weeks, suggesting that under such conditions uptake of copper continues, possibly overriding the secretory and/or the MT-dependent copper storing mechanisms. Given the nature of HepG2 cells as potential metabolic suppliers of copper to non-hepatic cells, it is tempting to speculate that these cells respond to extracellular copper by incorporating it even beyond their capacity to handle the metal. This contention may also apply to the LEC rats, in which a progressive and huge hepatic accumulation of copper under the form of Cu–MT precedes the appearance of hepatitis (Sakurai et al., 1992; Suzuki et al., 1995). The exact mechanisms underlying the onset of liver damage in LEC rats have not been elucidated; however, the occurrence of free copper ions resulting from a limited capacity to store copper may be possible. Removal of copper from Cu–MT by treatment of LEC animals with tetrathiomolybdate (Suzuki et al., 1993, 1994; Ogra et al., 1996) retarded the onset of the injury (Lai and Sugawara, 1997).

The importance of sequestering copper under the form of Cu–MT as a mechanism to resist the continuous extracellular presence of toxic concentrations of

copper was suggested by Freedman et al. (1986, 1989) and by Freedman and Peisach (1989a,b). These authors provided evidence that HAC cells (a human hepatoma cell line) made resistant to high copper concentrations (200–800 μM) exhibited degrees of resistance that were proportional to their elevated intracellular copper and MT levels. Furthermore, subsequent culturing of the resistant cells in the absence of copper led to decreases of their intracellular copper while maintaining their resistance to Cu toxicity. The latter was ascribed to elevated MT levels which presumably also conferred cross-resistance to the toxicity induced by other MT-binding metals. In addition, Schilsky et al. (1989) observed in HepG2 cells that MT induction by exposure to zinc mitigated the toxicity induced by the subsequent exposure to copper. Recently, however, using mutant human hepatoblastoma cell lines, the same authors reported that resistance to copper could also be achieved in the absence of MT induction (Schilsky et al., 1998). Thus, further studies are needed to define whether MT induction is a general and "essential" change towards acquiring resistance to copper toxicity.

Considering the role that GSH seems to play in sequestering copper and in carrying the metal into MT (Freedman et al., 1989; Steinebach and Wolterbeek, 1994), our studies also addressed the possible changes in the GSH pool associated with copper accumulation. Our results show that after 2 or 5 weeks of excess copper exposure, viable HepG2 cells exhibited GSH levels substantially lower than those of control cells. The lower GSH levels seen in $HepG2_{100}$ cells are in line with the low GSH levels occurring in the livers of individuals with Wilson's disease (Summer and Eisenburg, 1985). Although the latter could partially reflect a combination of cause and consequence of damage in Wilson's disease hepatocytes, our results suggest that diminished GSH levels can take place in viable liver cells in the absence of a challenge other than continuous exposure to non-toxic amounts of copper. Within the framework of this study, one mechanism implicated in this decrease could relate to the ability of GSH to interact with copper forming a Cu–GSH adduct. Recently, we presented evidence that copper ions swiftly interact with GSH in vitro (Jiménez and Speisky, 2000). Interestingly, unlike GSH, the adduct showed no reactivity towards the thiol-reactive agent DTNB, yet it fully conserved the free radical-scavenging properties of the tripeptide. However, as also reported by others, attempts to isolate the adduct from cellular systems were limited by its aerobic lability and unstability in the presence of either reducing agents or copper chelating buffers (Freedman et al., 1989; Ciriolo et al., 1990). If the adduct is assumed to have been formed within $HepG2_{100}$ cells, this is likely to have compromised the GSH pool to an extent comparable to that of copper ions needed to be intracellularly sequestered. Thus, on considering the increment in non-MT cytosolic copper that took place in $HepG2_{100}$ cells, up to 30% of the loss in GSHeq could be accounted for by adduct formation. A comparable percentage of GSHeq is also likely to have been oxidatively consumed through the GSH-dependent reduction of Cu^{2+} (Freedman et al., 1989). In fact, $HepG2_{100}$ cells exhibited significantly higher GSSG levels after 2 and 5 weeks. In our results, only 13 and 19% of the lost GSHeq could be accounted by the corresponding increments in intracellular GSSG. However, since part of the GSSG generated by the HepG2 cells is likely to be continuously pumped out (Lu et al., 1993), attempts to establish the exact contribution of such mechanism to the loss of GSHeq becomes difficult to interpret.

Our results on the susceptibility of copper-loaded cells to oxidative stress reveal that, in the absence of added peroxide, $HepG2_{100}$ cells show marginal, but non-significant differences in initial viability and oxidative tone relative to control cells. This was found despite their substantially lower basal GSH levels (as measured by the DTNB–GSH reductase coupled assay). Thus, a part of the GSH molecule may be in the form of a Cu–GSH adduct, contributing to the antioxidant defence (Jiménez and Speisky, 2000). The similarity between $HepG2_c$ and $HepG2_{100}$ cells became apparent in the presence of *t*-BOOH, since copper-loaded cells were more susceptible to undergo both oxidative stress and lytic damage. Increments in the oxidative tone of $HepG2_{100}$ cells were associated with cell lysis at high but not low *t*-BOOH concentrations. Because at low concentrations (0.25 mM) $HepG2_{100}$ cells displayed a significantly higher oxidative tone in the absence of cell loss, an elevated oxidative stress is likely to be the cause, rather than the consequence, of the lytic event.

By acting as cofactor in the GSHperox-dependent removal of peroxides and as scavenger of peroxide-derived free radicals, GSH is of fundamental importance in protecting cells against peroxide-induced cytotoxicity (Meister, 1988). In fact, the cytotoxicity induced by *t*-BOOH in cultured mammalian cells has been shown to be proportional to the depletion of GSH induced by buthionine sulphoximine (Ochi, 1988). Therefore, low GSH levels in copper-loaded cells could be considered a major intracellular determinant of their susceptibility to *t*-BOOH-induced cytotoxicity.

MT is generally regarded as a stable and safe form of copper storage. However, under some particular conditions, it may behave as a pro-oxidant. In fact, in the presence of hydrogen peroxide, MT isolated from of LEC rat liver generates far more hydroxyl radicals than MT from the liver of control animals (Sakurai et al., 1994). Addition of Cu–MT (obtained from TMM livers) to microsomal membranes itself did not cause lipid peroxidation but enhanced substantially the peroxidation initiated by *t*-BOOH (Stephenson et al., 1994). Recently, Liu et al. (2001) reported a nitric oxide-dependent

pro-oxidant and pro-apoptotic effect of Cu–MT in copper-loaded HL-60 cells. Taken together, the above-referred studies indicated that high Cu–MT levels in copper-loaded cells itself may not be sufficient to promote oxidative damage. However, in the presence of a relatively low oxidative stress, high Cu–MT levels are likely to exacerbate damage. Thus, in addition to the low GSH levels, the high Cu–MT levels could also be regarded as an important factor contributing to the higher susceptibility of copper-loaded cells to oxidative stress. Therefore, data presented here suggest that HepG2 cells respond to the continuous presence of copper by undergoing adaptive changes in GSH and MT. These changes per se do not compromise cell viability, but in the presence of oxidative conditions render copper-overloaded cells substantially more susceptible to undergo oxidant-triggered cytotoxicity.

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