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Influence of Copper-(II) on Colloidal Carbon-Induced Kupffer Cell-Dependent Oxygen Uptake in Rat Liver: Relation to Hepatotoxicity

JORGE SANS[a], ANA MARIA AGUILERA[b], PAOLA FAUNDEZ[b], PILAR TRONCOSO[b], VIRGINIA FERNANDEZ[b] and LUIS A. VIDELA[b,*]

Programas de [a]Biología Celular y Molecular, [b]Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago 7, Chile

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Formation of reactive O_2 species in biological systems can be accomplished by copper-(II) (Cu^{2+}) catalysis, with the consequent cytotoxic response. We have evaluated the influence of Cu^{2+} on the respiratory activity of Kupffer cells in the perfused liver after colloidal carbon infusion. Studies were carried out in untreated rats and in animals pretreated with the Kupffer cell inactivator gadolinium chloride ($GdCl_3$) or with the metallothionein (MT) inducing agent zinc sulphate, and results were correlated with changes in liver sinusoidal efflux of lactate dehydrogenase (LDH) as an index of hepatotoxicity. In the concentration range of 0.1–1 μM, Cu^{2+} did not modify carbon phagocytosis by Kupffer cells, whereas the carbon-induced liver O_2 uptake showed a sigmoidal-type kinetics with a half-maximal concentration of 0.23 μM. Carbon-induced O_2 uptake occurred concomitantly with an increased LDH efflux, effects that were significantly correlated and abolished by $GdCl_3$ pretreatment or by MT induction. It is hypothesized that Cu^{2+} increases Kupffer cell-dependent O_2 utilization by promotion of the free radical processes related to the respiratory burst of activated liver macrophages, which may contribute to the concomitant development of hepatocellular injury.

Keywords: Liver, Kupffer cell, respiratory burst activity, hepatotoxicity

INTRODUCTION

Kupffer cells are resident macrophages of the liver playing significant roles in the presentation of antigens, immunomodulation, phagocytosis, and biochemical attack.[1] Uptake of particles by liver macrophages is mediated by receptors present in the plasma membrane, a process that is accompanied by the release of specific molecules including proteases, cytokines, and reactive oxygen and nitrogen species.[1,2] Among the latter species, superoxide radical ($O_2^{\bullet -}$) is predominantly formed in the respiratory burst of activated Kupffer cells,[3] a phenomenon that involves the protein kinase C-dependent activation of NADPH oxidase.[1] Kupffer cells also generate nitric oxide ($^{\bullet}NO$) in an arginine-dependent

* Corresponding author.

reaction catalyzed by the inducible isoform of NO synthase, at rates of about $\frac{1}{8}$ of those of $O_2^{\bullet -}$.[3] In these conditions, formation of peroxynitrite ($ONOO^-$) may occur by the $^{\bullet}NO-O_2^{\bullet -}$ combination reaction known to proceed at an almost difussion-controlled rate,[4] as reported in activated alveolar lung macrophages.[5] Thus, exacerbation of Kupffer cell functioning involving an enhanced respiratory burst activity is thought to represent a key factor in the hepatotoxicity induced by endotoxin,[6] xenobiotics such as acetaminophen,[7,8] ischemia–reperfusion,[9] hyperthyroid state,[10] or acute iron overload.[11]

Formation of reactive oxygen species in biological systems can be accomplished by transition metal catalysis, provided that a suitable concentration of free redox-active transition metals such as iron or copper is available.[12] In fact, cupric ions (Cu^{2+}) at physiological concentrations can promote the formation of hydroxyl radical ($HO^{\bullet}$), or a species of equivalent reactivity, in a reaction requiring a reducing agent (i.e., $O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) known as the copper-catalyzed Haber–Weiss reaction.[13,14] $HO^{\bullet}$, in turn, can initiate a free-radical chain reaction with polyunsaturated fatty acids in phospholipids, leading to the formation of the respective hydroperoxides that can undergo decomposition in the presence of copper ions into additional free-radical moieties.[12] Moreover, the interaction of $ONOO^-$ with chelates of copper can elicit its heterolytic cleavage to yield a species with the reactivity of the nitronium ion (NO_2^+).[15] Thus, copper-catalyzed free radical processes may lead to the generation of highly reactive species capable of oxidizing and/or nitrating a number of key biomolecules, with the consequent alteration of their functions and the conditioning of cytotoxicity.[12,15,16] In view of these considerations, we tested the hypothesis that Cu^{2+} may exacerbate the respiratory activity of Kupffer cells by promoting free radical processes related to the respiratory burst of liver macrophages in the intact organ. For this purpose, rates of colloidal carbon-induced O_2 uptake by isolated perfused livers were measured in the absence and presence of Cu^{2+}, both in control rats and in animals subjected to either Kupffer cell elimination by gadolinium chloride ($GdCl_3$)[17] or induction of the copper-binding protein metallothionein (MT) by $ZnSO_4$[18] pretreatments. Results obtained were correlated with changes in the sinusoidal release of lactate dehydrogenase (LDH) as an index of hepatotoxicity.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague-Dawley rats (Instituto de Salud Pública, Santiago, Chile), fed *ad libitum* and weighing 150–200 g, were used. Experiments were carried out both in untreated rats and in animals subjected to either $GdCl_3$ (24 h after the administration of 10 mg/kg by tail vein injection) or $ZnSO_4$ (17 h after the administration of three doses of 10 mg/kg i.p. in a 24 h time interval), and the respective controls received equivalent volumes of 0.9% wt/vol NaCl. All animals used received humane care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication No. 86-23).

Liver Perfusion and Assessment of Parameters Related to Kupffer Cell Functioning, Tissue Viability and Morphological Studies

Livers were obtained under sodium pentobarbital anesthesia (50 mg/kg, i.p.) and perfused at 37°C with hemoglobin-free Krebs–Henseleit bicarbonate buffer ([in mM]: NaCl, 118; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 10; equilibrated with a 19 : 1 vol/vol O_2/CO_2 mixture to give pH 7.4) via a cannula placed in the portal vein as described previously.[8,10,11] Perfusions were performed for 50 min at constant flow

rates (3.5–4.0 ml/g liver/min) and temperature (36–37°C) without recirculation of the perfusate. O_2 consumption was determined polarographically in the effluent perfusate collected via a cannula placed in the vena cava and allowed to flow past a Clarke-type O_2 electrode.[10] Livers were allowed to equilibrate for 15 min, and perfusate samples were taken every 5 min to measure LDH activity (one unit corresponds to 1 μmol/min at 25°C).[19] Rates of sinusoidal LDH efflux (in mU/g liver/min) were calculated by multiplying the perfusate activity by the perfusion flow, and LDH activity in the liver[19] was determined at the end of perfusion. Colloidal carbon (Rotring, Hamburg, Germany), at the concentration of 0.5 mg/ml, was infused between the 30- and 45-minute time interval, either in the absence or presence of 0.1–1.0 μM Cu^{2+} added at 20 min (see Figure 1). Uptake of carbon was assessed by determining its absorbance at 623 nm (specific extinction coefficient of 0.97 $[mg/ml]^{-1}$) in perfusate samples taken every 5 min, and rates of carbon uptake (in mg/g liver/min) were calculated from influent minus effluent concentration difference, referred to the perfusion flow.[20] Total carbon uptake and carbon-induced O_2 consumption were obtained by the integration of the respective curves between 30- and 45-minute perfusion and expressed as mg of carbon/g liver and μmol of O_2/g liver, respectively (Figure 1).[8,10]

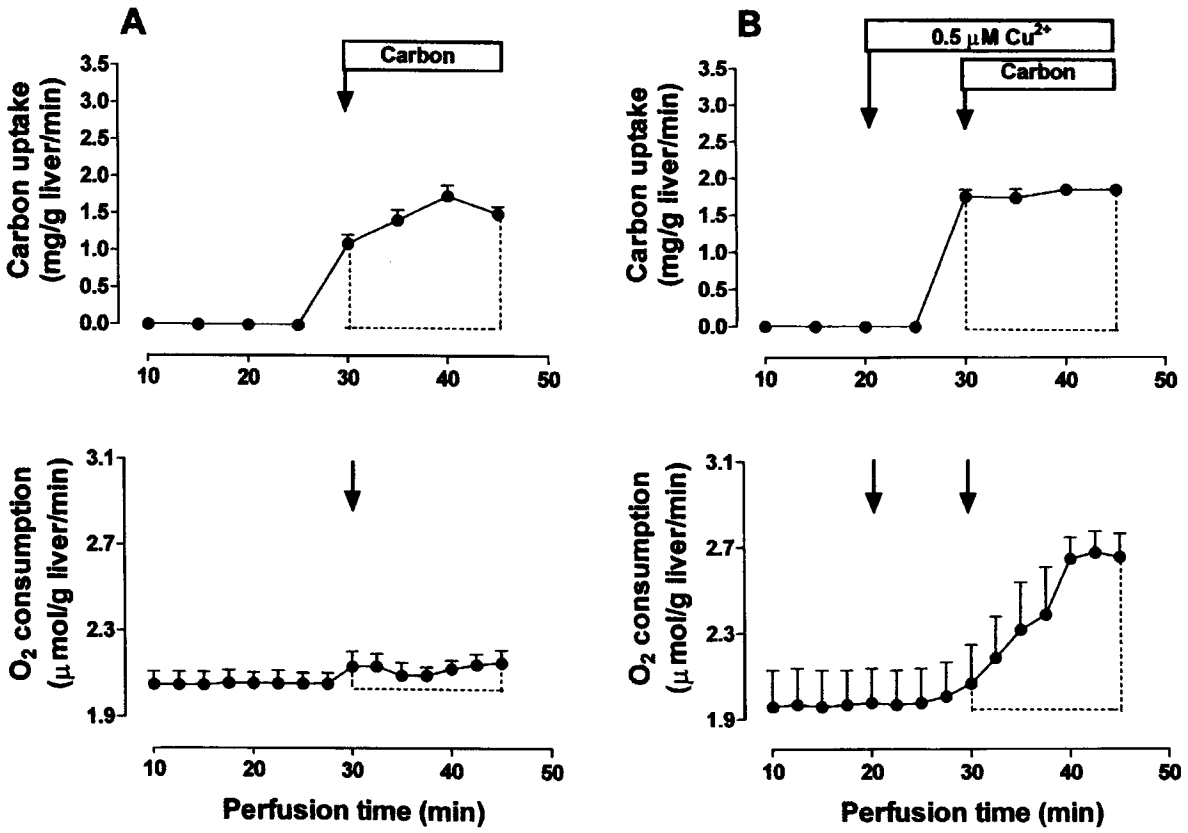

FIGURE 1 Rates of carbon uptake and O_2 consumption by rat livers perfused in the absence (A) and presence (B) of 0.5 μM Cu^{2+}, after infusion of 0.5 mg of carbon/ml. Total carbon uptake and carbon-induced O_2 consumption were obtained by the integration of the respective curves between 30- and 45-minute perfusion and expressed as mg of carbon/g liver and μmol of O_2/g liver, respectively. These integrated values were used to calculate the corresponding carbon-induced O_2 uptake/carbon uptake ratios, expressed as μmol O_2/mg carbon (see Figure 3). Results represent the means ± SEM for four to six animals per each experimental condition.

These integrated values were used to calculate the corresponding carbon-induced O_2 uptake/carbon uptake ratios, expressed as μmol O_2/mg carbon (see Figure 3). Similar calculations were done for the sinusoidal efflux of LDH in the presence of carbon, and results were expressed as units/g liver. Light microscopy studies were carried out in liver samples taken after perfusion with 0.5 mg of carbon/ml in the absence of Cu^{2+} infusion, fixed in Dubosq Brazil for 24 h, embedded in Paraplast, and stained with hematoxylin-eosin.

Biochemical Determinations

Control rats and animals pretreated with $ZnSO_4$ as described in the above section were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the livers were perfused *in situ* with 150 ml of ice-cold 1.15% (wt/vol) KCl containing 10 mM tris pH 7.4 to remove blood. Samples of liver tissue were used to determine the content of total glutathione (GSH) by the catalytic assay of Tietze[21] and that of metallothionein (MT) as described by Eaton and Toal.[22]

Chemicals and reagents used were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Values shown correspond to the means ± SEM for the number of separate experiments indicated. The statistical significance of differences between mean values was assessed either by one-way ANOVA and the Neuman–Keuls' test or by the Student's *t* test for unpaired data as indicated.

RESULTS

Infusion of colloidal carbon into perfused rat livers led to a significant uptake of the particles with a parallel enhancement in the rate of O_2 consumption of the organ over basal values (Figure 1A), the latter parameter being substantially increased by the infusion of 0.5 μM Cu^{2+} (Figure 1B). Histological assessment of the livers from control rats revealed that carbon phagocytosis primarily occurs in Kupffer cells of the periportal areas of the hepatic lobule (Figure 2A;

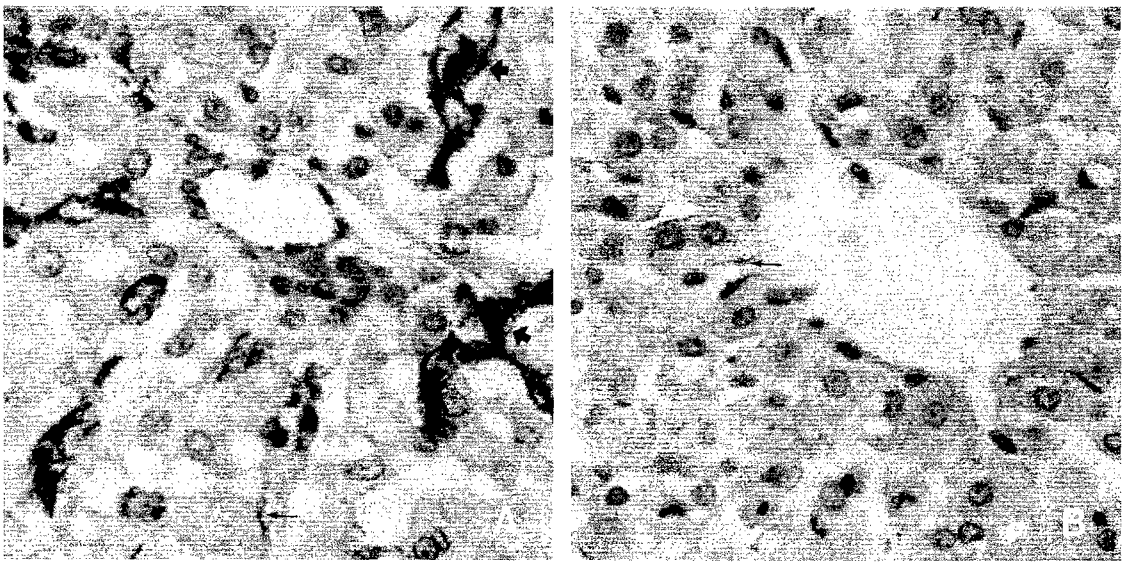

FIGURE 2 Structural characteristics of rat liver parenchyma perfused *in vitro* with 0.5 mg of colloidal carbon/ml as described in Figure 1, in the absence of Cu^{2+} infusion. Studies were performed both in periportal (A) (magnification ×630) and centrilobular (B) (magnification ×630) regions of the hepatic lobule.

big arrow), whereas that by endothelial cells of the periportal (Figure 2A; small arrow) and centrilobular (Figure 2B; small arrow) regions is comparatively smaller; these features were not altered by Cu^{2+} infusion (data not shown). In these conditions, no detectable carbon is observed in parenchymal cells (Figure 2A and B). In the concentration range of 0.1–1.0 μM, Cu^{2+} did not significantly modify the rate of carbon uptake (Figure 3A) or the basal rate of O_2 consumption by perfused livers (control no additions, 2.06 ± 0.06 ($n = 5$) μmol/g liver/min; 0.1 μM Cu^{2+}, 2.17 ± 0.22 ($n = 4$); 0.25 μM Cu^{2+}, 1.99 ± 0.06 ($n = 4$); 0.5 μM Cu^{2+}, 1.97 ± 0.17 ($n = 6$); 1.0 μM Cu^{2+}, 2.01 ± 0.09 ($n = 5$)]. However, carbon-induced O_2 uptake (Figure 3B) and the respective carbon-induced O_2 uptake/carbon uptake ratios (Figure 3C) exhibit a sigmoidal-type kinetics, with half-maximal values being attained at 0.23 μM Cu^{2+}.

Carbon uptake (Figure 4A) and carbon-induced O_2 consumption (Figure 4B) were significantly diminished over control values by Kupffer cell elimination by $GdCl_3$ pretreatment, a rare earth metal that drastically suppressed the Cu^{2+}-dependent enhancement in carbon-induced O_2 uptake (Figure 4B). Pretreatment with $ZnSO_4$ did not alter the hepatic content of GSH, whereas that of MT exhibited an 89-fold enhancement, with a net 63% increase in the content of tissue sulfhydryls derived from GSH and MT (Table I). Induction of liver MT by $ZnSO_4$ pretreatment did not modify carbon uptake by perfused livers, either in the absence or presence of Cu^{2+} (Figure 5A), however, all the increase in the carbon-induced O_2 consumption elicited by the infusion of 0.5 μM Cu^{2+} relative to control values was abolished by $ZnSO_4$ pretreatment (Figure 5B).

Livers perfused with 0.5 μM Cu^{2+} prior to carbon infusion exhibited a 75% increase in the sinusoidal efflux of LDH over control values [control no additions, 1.02 ± 0.11 ($n = 5$), U/g liver; 0.5 μM Cu^{2+}, 1.78 ± 0.22 ($n = 6$); $P < 0.05$]. However, carbon-induced liver LDH efflux in the presence of 0.5 μM Cu^{2+} showed a 5.2-fold enhancement in the absence of changes in the

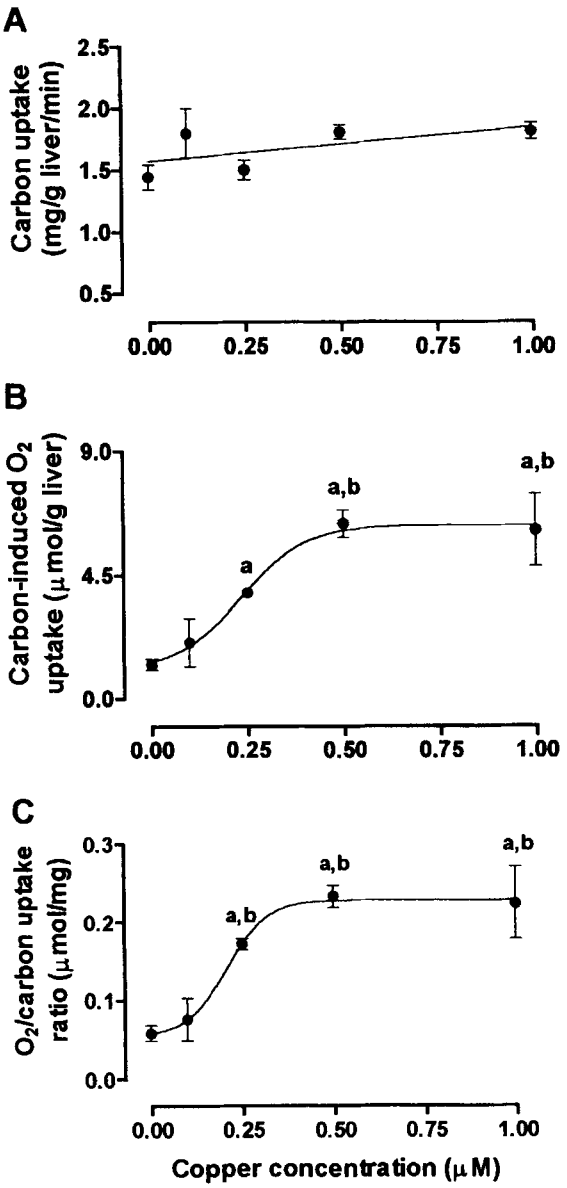

FIGURE 3 Concentration-dependent effects of Cu^{2+} on the (A) rate of carbon uptake, (B) carbon-induced O_2 uptake, and (C) the respective O_2/carbon uptake ratios by isolated perfused rat liver. Livers were perfused in the absence (Cu^{2+} concentration = 0) or presence of 0.1, 0.25, 0.5, and 1.0 μM Cu^{2+}, and infused with 0.5 mg of carbon/ml as described in Figure 1. Carbon-induced O_2 uptake/carbon uptake ratios were calculated by dividing the respective integrated values obtained as described in Figure 1. Results represent the means ± SEM for four to six animals per each experimental condition. The statistical analysis was carried out by one-way analysis of variance and the Neuman–Keuls' test: $^{a}P < 0.05$ compared with controls (zero Cu^{2+} concentration); $^{b}P < 0.05$ compared with 0.1 μM Cu^{2+}.

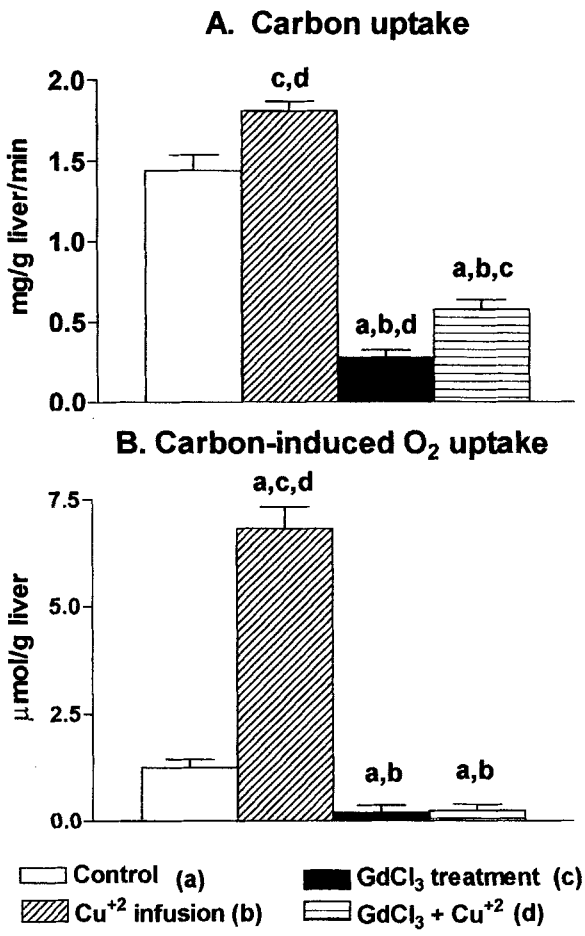

FIGURE 4 Effect of gadolinium chloride ($GdCl_3$) pretreatment on the (A) rate of carbon uptake and (B) carbon-induced O_2 consumption by rat livers perfused in the absence and presence of 0.5 μM Cu^{2+}. Studies were performed in separate groups of rats, 24 h after pretreatment with either $GdCl_3$ (10 mg/kg by tail vein injection) or equivalent volumes of 0.9% wt/vol NaCl (controls), as described in Figure 1. Results represent the means ± SEM for four to six animals per each experimental condition. The significance of the differences between mean values ($P < 0.05$) was carried out by one-way analysis of variance and the Neuman–Keuls' test, and is shown by the letters identityfing each experimental group.

hepatic activity of the enzyme, an effect that was suppressed by pretreatment of the animals with either $GdCl_3$ or $ZnSO_4$ (Figure 6). Values of the carbon-induced liver sinusoidal efflux of LDH obtained under the various experimental conditions used exhibited a significant direct correlation with the respective carbon-induced O_2 uptake values (Figure 6, inset).

TABLE I Effect of zinc sulphate ($ZnSO_4$) pretreatment on the content of liver glutathione (GSH) and metallothionein (MT) in the rat[a]

Parameters	Control rats	$ZnSO_4$-pretreated rats	P
GSH (μmol/g liver)	5.59 ± 1.38	7.02 ± 0.35	N.S.
MT (nmol/g liver)	1.2 ± 0.4	108.2 ± 39.2	0.025
Total -SH[b] (nmol/g liver)	5618 ± 1393	9184 ± 533	0.05

[a]Animals were subjected to three doses of 10 mg of $ZnSO_4$/kg i.p. equally spaced in 24 h time interval or equivalent volumes of 0.9% wt/vol NaCl (controls), and measurements were performed 17 h after treatment. Results represent the means ± SEM for three animals per group, and the significance between mean values was assessed by the Student's *t* test for unpaired data (N.S., not significant).
[b]The content of total sulfhydryls (-SH) was calculated by the sum of the nmol of GHS/g liver and those of (MT/g liver) ×20 considering that one molecule of MT contains 20 cysteinyl residues.[18,22]

DISCUSSION

Infusion of Cu^{2+} concentrations in the range of 0.1–1 μM into the isolated perfused rat liver prior to carbon addition did not significantly modify the basal rate of O_2 consumption of the organ, as would be expected for a redox-active metal ion able to catalyze the aerobic generation and reactions of reactive oxygen and nitrogen species.[12,15,16] This is probably due to the low Cu^{2+} concentrations used that seem to be adequately handled by the hepatic pathways of copper metabolism, namely, sinusoidal and canalicular secretion mechanisms and glutathione-dependent storage in MT.[16,23] Colloidal carbon infused into the perfused liver is readily taken up by sinusoidal cells, predominantly Kupffer cells of the periportal regions of the hepatic lobule,[24] while it is absent in hepatocytes. The process occurred with the concomitant increase in the rate of oxygen uptake,[10,20] parameters that are substantially diminished by $GdCl_3$-induced liver macrophage elimination.[17] Particle-induced hepatic $GdCl_3$-sensitive O_2 uptake is largely accounted for by the respiratory burst of Kupffer cells,[1–3] with minor mitochondrial respiratory components in liver macrophages for energy supply needed for carbon phagocytosis[20] or at

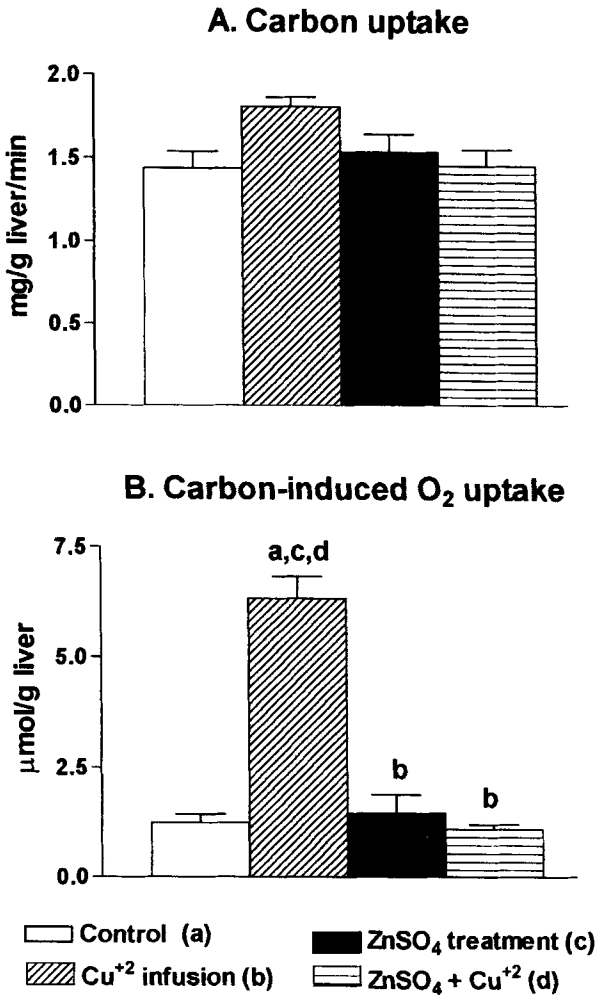

FIGURE 5 Effect of zinc sulphate ($ZnSO_4$) pretreatment on the (A) rate of carbon uptake and (B) carbon-induced O_2 consumption by rat livers perfused in the absence and presence of 0.5 µM Cu^{2+}. Studies were performed in separate groups of rats, 17 h after pretreatment with either $ZnSO_4$ (three doses of 10 mg/kg i.p. equally spaced in a 24 h time interval) or equivalent volumes of 0.9% wt/vol NaCl (controls), as described in Figure 1. Results represent the means ± SEM for four to six animals per each experimental condition. The significance of the differences between mean values ($P < 0.05$) was carried out by one-way analysis of variance and the Neuman-Keuls' test, and is shown by the letters identifying each experimental group.

the parenchymal cell level possibly mediated by prostaglandins released by activated Kupffer cells.[25]

Colloidal carbon stimulation of liver macrophages in the presence of Cu^{2+} elicited a concentration-dependent sigmoidal enhancement in O_2 uptake, with a half-maximal concentration of 0.23 µM. A similar kinetic pattern is observed for the carbon-induced O_2 consumption/carbon uptake ratios at different Cu^{2+} concentrations, which, in addition to the lack of changes of Cu^{2+} on particle phagocytosis, suggest that Cu^{2+} is promoting O_2-dependent processes associated with the carbon-induced respiratory burst of Kupffer cells. Primarily, the phenomenon may involve the interaction of Cu^{2+} with $O_2^{\bullet -}$ generated by liver macrophage NADPH oxidase activity stimulated by carbon infusion, leading to higher rates of the copper-catalyzed Haber–Weiss reaction and $HO^{\bullet}$ formation,[13,14] which could trigger secondary O_2-dependent processes such as lipid peroxidation.[26–28] Considering that Cu^{2+} is known to effectively activate endothelial NO synthase,[29,30] the possibility that the respiratory response observed upon carbon stimulation in the presence of Cu^{2+} could involve an enhanced reactive nitrogen species formation cannot be discarded, however, further studies in isolated liver macrophages are needed to clarify this proposal. The above contention is strongly supported by the abolishment of the Cu^{2+}-induced respiratory activity of activated Kupffer cells by either liver macrophage elimination by $GdCl_3$ or MT induction by $ZnSO_4$ pretreatment. Enhancement of hepatic MT levels is known to take place both in parenchymal cells as well as in endothelial and Kupffer cells,[31,32] and represents a maximal copper binding capacity of 1284 nat g Cu^{2+}/g liver ([107 nmol MT/g liver] × [12 nat g Cu^{2+}/nmol MT]),[18] that largely accounts for all the Cu^{2+} infused into the liver (50 nat g Cu^{2+}/g liver, calculated considering a concentration of 0.5 µM Cu^{2+} infused at 4 ml/g liver/min for 25 min). In addition to the role of MT as a copper storage mechanism, MT can also act as an effective free radical scavenger due to the high rate constants for reaction between its cysteinyl residues and oxyradicals.[18] In fact, MT induction by $ZnSO_4$ leads to a net 63% increase in the content of hepatic sulfhydryl groups comprising GSH and MT, over

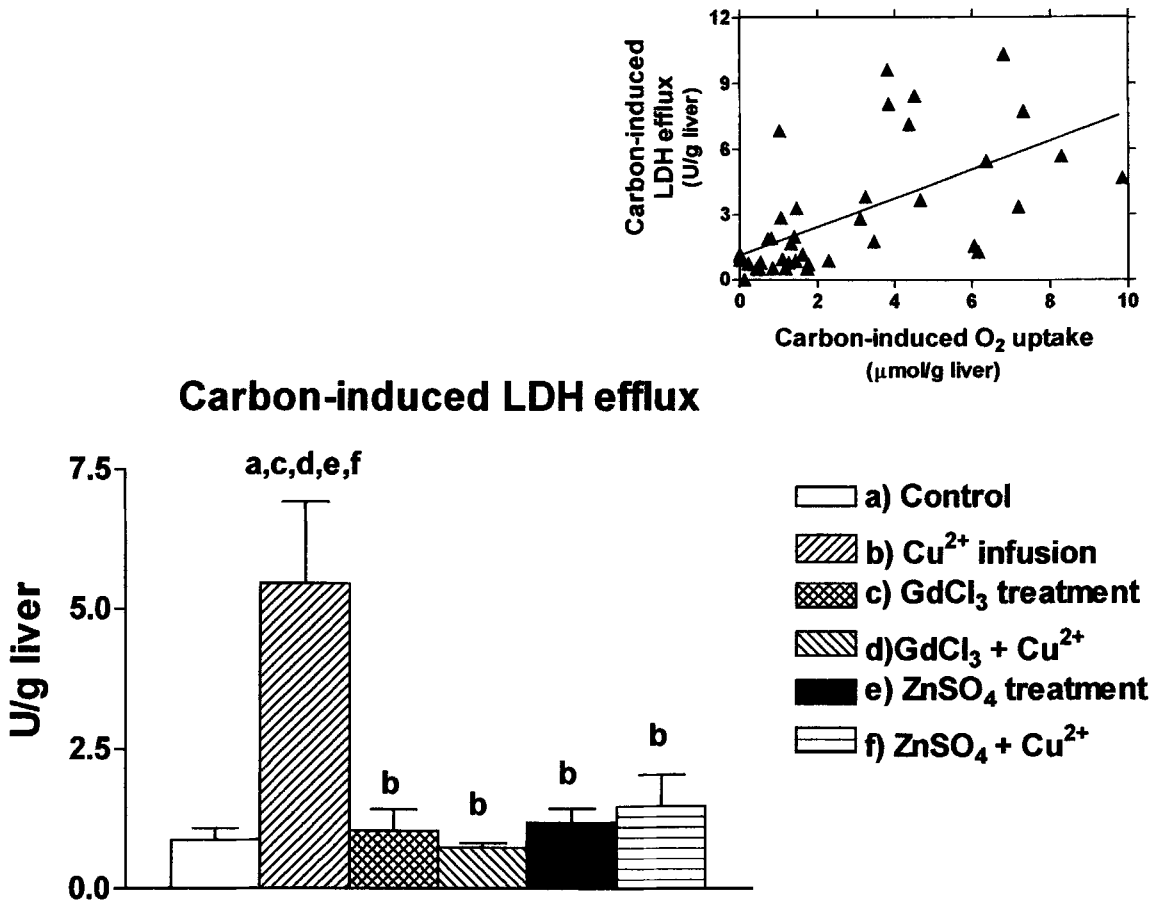

FIGURE 6 Influence of Cu^{2+} infusion (0.5 μM) on the carbon-induced sinusoidal efflux of lactate dehydrogenase (LDH) in perfused livers from control rats and animals pretreated with either gadolinium chloride ($GdCl_3$) or zinc sulphate ($ZnSO_4$). Animals were pretreated with $GdCl_3$ or $ZnSO_4$ as described in Figures 4 and 5, respectively, and liver perfusions were carried out as described in Figure 1. Rates of sinusoidal LDH efflux (in U/g liver/min) during carbon infusion were calculated by multiplying the perfusate activity by the perfusion flow, integrated between 30- and 45-minute perfusion, and expressed as U/g liver. Values of liver LDH activity were determined at the end of perfusion and were comparable among the different groups studied [controls (a), 333 ± 38 ($n = 5$) U/g liver; Cu^{2+} infusion (b), 354 ± 27 ($n = 6$); $GdCl_3$ pretreatment (c), 291 ± 31 ($n = 4$); $GdCl_3$ pretreatment + Cu^{2+} infusion (d), 399 ± 27 ($n = 4$); $ZnSO_4$ pretreatment, 348 ± 23 ($n = 4$); $ZnSO_4$ pretreatment + Cu^{2+} infusion, 388 ± 37 ($n = 4$)]. Results represent the means ± SEM for four to six animals per each experimental condition. The significance of the differences between mean values ($P < 0.05$) was carried out by one-way analysis of variance and the Neuman–Keuls' test, and is shown by the letters identifying each experimental group. Inset: correlation between carbon-induced sinusoidal efflux of LDH and the respective carbon-induced O_2 uptake in untreated rat livers perfused in the absence and presence of different Cu^{2+} concentrations as well as in those from animals pretreated with either $GdCl_3$ or $ZnSO_4$ and perfused with 0.5 μM Cu^{2+} (regression line: $Y = 0.04 + 0.66$ X; $n = 40$; $r = 0.61$; $P < 0.001$).

control values, an effect that may contribute to the normalization of the Cu^{2+}-induced enhancement in the carbon-dependent oxidant burst of Kupffer cells.

Data presented in this work establish an association between the extent of liver injury and the magnitude to the Kupffer cell respiratory burst activity, as the values of carbon-induced liver O_2 uptake and the respective sinusoidal LDH effluxes exhibit a significant direct correlation under the experimental conditions studied. Of special interest is the finding that Cu^{2+}-induced exacerbation of the respiratory activity of carbon-stimulated Kupffer cells is paralleled by

a 5.2-fold increase in the sinusoidal efflux of LDH compared to control values. This Cu^{2+}-induced hepatotoxicity seems to be dependent on the respiratory burst activity of Kupffer cells, due to its $GdCl_3$-sensitivity, and on the availability of the metal ion at the liver macrophage level, due to its abolishment by MT induction, although actions of the metal ion at the parenchymal cell level cannot be discarded. MT induction by zinc pretreatment is known to protect the liver against Cu^{2+}-induced hepatotoxicity by storing Cu^{2+} in a nontoxic form.[33,34] This would reduce the Cu^{2+}-dependent promotion of free radical processes, with the consequent diminution in hepatic lipid peroxidation,[35] as found for α-tocopherol.[36] In agreement with the findings presented in this work, acute iron overload has been recently reported to elicit a derangement in the Kupffer cell functional status represented by early increases in macrophage-dependent respiratory activity involving similar free radical-mediated mechanisms to those proposed for copper.[11] These early changes elicited by iron overload may contribute to the concomitant hepatotoxicity that develops and to the impairment of both total liver respiration and the Kupffer cell response to particle stimulation seen at later times after treatment.[11]

In conclusion, infusion of low concentrations of Cu^{2+} into the isolated perfused rat liver increases the Kupffer cell-dependent O_2 uptake and the sinusoidal release of LDH over control values, effects that are sensitive to Kupffer cell elimination and MT induction. Cu^{2+}-induced Kupffer cell O_2 consumption upon carbon stimulation may represent an important hepatotoxic mechanism of the transition metal, probably due to promotion of free radical processes related to the respiratory burst of activated liver macrophages. In line with this view, tissue damage in patients in the active stage of Behcet's disease, a multisystem vasculitis, has been associated with a prooxidant status found in polymorphonuclear leukocytes represented by decreased activities of superoxide dismutase, catalase, and glutathione peroxidase, and enhanced activity of the $O_2^{\bullet -}$-generator NADPH oxidase.[37] The prooxidant activity of these neutrophils may be exacerbated by the elevated levels of plasma copper found,[37] leading to a depression of the antioxidant defenses of plasma with the consequent enhancement in lipid peroxidation indexes.[38] In addition to this oxidative stress mechanism, exacerbation of Kupffer cell function by Cu^{2+} may also involve the production and release of proinflammatory cytokines, chemical mediators considered essential in the development of tissue injury, as demonstrated for acetaminophen-induced hepatotoxicity.[39]

Acknowledgements

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