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Early histological and functional effects of chronic copper exposure in rat liver

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Abstract

Cu is an essential trace element capable of producing toxic effects in animals and man when ingested acutely or chronically in excess. Although chronic Cu exposure is increasingly recognized as a public health issue, its early effects remain largely unknown. We approached the significance of a moderate chronic Cu load in young rats to correlate early hepatic histopathological changes with functional alterations of liver cells. For this purpose, supplementation with 1200 ppm of Cu in rat food for 16 weeks was chosen. In these conditions, Cu load elicited a significant decrease in growth curves. There were mild light microscopy alterations in Cu-treated rats, although increasing intracellular Cu storage was correlated with longer Cu exposure both by histological and biochemical measurements. Ultrastructural alterations included lysosomal inclusions as well as mitochondrial and nuclear changes. Liver perfusion studies revealed higher rates of basal O_2 consumption and colloidal carbon-induced O_2 uptake in Cu-treated rats, with enhanced carbon-induced O_2/carbon uptake ratios and NF-κB DNA binding activity. These changes were time-dependent and returned to control values after 12 or16 weeks. It is concluded that subchronic Cu loading in young rats induces early hepatic morphological changes, with enhancement in Küpffer cell-dependent respiratory burst activity and NF-κB DNA binding, cellular responses that may prevent or alleviate the hepatotoxicity of the metal.

Introduction

Copper (Cu) is an essential trace element capable of producing toxic effects in animals and man when ingested acutely or chronically in excess (Bremner 1998). In humans, the autosomal recessive Wilson's disease is characterized by the pathologic accumulation of Cu, mainly in brain and liver tissues (Brewer & Yuzbasiyan-Gurkan 1992). Similarly, the Long-Evans Cinnamon (LEC) rat suffers from fulminant hepatitis and severe jaundice secondary to accumulation of Cu in the liver (Okayasu *et al.* 1992). Both Wilson's disease and the LEC rat are caused by mutations in the ATPase 7b gene (Bull *et al.* 1993; Wu *et al.* 1994), mutant ATPase 7b being unable to pump Cu towards the bile canaliculi for biliary excretion resulting in progressive hepatic Cu accumulation with development of liver fibrosis and cirrhosis (Brewer & Yuzbasiyan-Gurkan 1992).

Various studies have demonstrated that Cu loading in healthy rats by controlled ingestion induces tissue alterations similar to those occurring in mutant models of Cu disease, including

reduction in body weight (Hebert *et al*. 1993) and enhancement in cell Cu content in the mid-zone and periportal regions of the liver lobule (Fuentealba *et al*. 1989). The accumulation of Cu in liver cells occurs primarily within lysosomes of hepatocytes, which increase in size, number, and diversity (Fuentealba & Haywood 1988), reaching the nucleus and nucleolus when the lysosomal capacity for Cu deposition is overcome (Fuentealba *et al*. 1993; Haywood *et al*. 1996). This in turn is directly injurious to the organelle and leads to cell death (Fuentealba *et al*. 1989; Hebert *et al*. 1993). Interestingly, rats are able to recover from an initial period of cellular damage and develop tolerance as evidenced by the diminished levels of intracellular Cu after ten to fifteen weeks of exposure (Fuentealba *et al*. 1993). In parallel to the events described, rats exposed to elevated levels of Cu in the diet show increasing levels of metallothionein (MT) in hepatocytes, a protein involved in Cu storage and free radical scavenging (Mullins Fuentealba *et al*. 1998).

Intracellular Cu homeostasis is tightly regulated as minimal amounts of free Cu may generate reactive oxygen species (ROS) such as hydroxyl radicals, through Fenton and Haber-Weiss reactions (Bremner 1998). These ROS in turn can damage biomolecules within cells including lipids, proteins, and nucleic acids, eventually leading to cell death (Bremner 1998). In the liver, Küpffer cells release various cytotoxic mediators such as pro-inflammatory cytokines, proteases, and ROS when become primed, which may sensitize liver sinusoidal cells to injury via paracrine or autocrine actions (Tsukamoto & Lin 1997). In this respect, previous studies by our group revealed that both *in vivo* acute iron overload (Tapia *et al*. 1998) and *in vitro* Cu exposure (Sans *et al*. 1999) increase Küpffer cell-dependent O_2 utilization by promotion of the free-radical processes related to their respiratory burst activity, which may contribute to the concomitant development of hepatocellular injury (Videla *et al*. 2003).

Although chronic Cu exposure is increasingly recognized as a public health issue (Araya *et al*. 2003), the characterization of early toxic actions of Cu is largely unknown due to lack of reliable markers of functionally relevant biological effects. In the current study, we approached the significance of a moderate chronic Cu load in young rats to correlate early hepatic histopathological changes with functional alterations of liver cells. For this purpose, a dose of 1200 ppm of Cu in rat food for up to 16 weeks was chosen, considering that Cu doses above this level elicit marked hepatic inflammation and necrosis (Aburto *et al*. 2001). Thus, morphological characteristics of the liver were assessed by light and electron microscopy and were correlated with either the hepatic Cu content attained, the basal rate of O_2 consumption of the liver and parameters related to Küpffer cell functioning, and liver NF-κB DNA binding.

Materials and methods

Animals and treatments

Sprague-Dawley rats (Bioterio Central, Facultad de Medicina, Universidad de Chile, Santiago, Chile) weaned at an average weight of 90 g were used in this study. Cu-treated rats were given a diet containing 1200 parts per million (ppm) of Cu added as $CuSO_4$ for 16 weeks. Control rats of the same strain were fed normal rodent diet (Champion S.A.) with a Cu content < 10 ppm. Both groups received their food and water *ad libitum*, and weight was recorded weekly. All animals used in this study received humane care according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication No. 86–23). Studies were performed in groups of control rats and Cu-treated animals after 6, 9, 12, and 16 weeks of treatment.

Liver perfusion and assessment of parameters related to Küpffer cell functioning

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and their livers perfused at 37 °C with haemoglobin-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 (Tapia *et al*. 1997). 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 that allowed to pass a Clarke-type O_2 electrode (Tapia *et al.* 1997). Uptake of carbon (Rotring R-591217) by perfused livers was measured after infusion of 0.5 mg carbon/ml in the 30–45-min time interval by determining the absorbance of carbon at 623 nm (specific extinction coefficient of 0.97 mg/ml^{-1}) in perfusate samples taken every 5 min (Cowper *et al.* 1990). Rates of carbon uptake (in mg/g liver/min) were calculated from influent minus effluent concentration difference, referred to as the perfusion flow. The carbon-induced O_2 uptake was assessed by the integration of the area under the O_2 consumption curves between 30- and 45-min perfusion and was expressed as micromoles per gram of liver (Tapia *et al.* 1997).

Liver biopsy studies

Biopsies were obtained from perfused and unperfused rats rapidly following cervical dislocation. Tissue samples were cut into three pieces for light and electron microscopy and for measurement of tissue Cu content. One piece was fixed in 4% formaldehyde for 8–12 h at room temperature, then washed twice in buffer and progressively dehydrated with graded ethanol and xylene. Tissue was embedded in paraffin, serially sectioned at 5 μm and stained for with hematoxylin-eosin (HE), for Cu with rhodamine, and with Perls' method for iron, following routine procedures. Two observers (MTV and MA) performed all histological evaluations in a blind fashion and their evaluations showed more than 95% agreement. Results were expressed in an arbitrary semiquantitative scale between 0 (no staining) and 3 (intensely positive staining); intermediate staining patterns were classified as 1 or 2. A second piece of tissue was snap frozen and maintained at −70 °C until analyzed for Cu content.

Electron microscopy studies

For transmission electron microscopy, a third piece of tissue was fixed for 8–10 h in 4% paraformaldehyde plus 0.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4), at room temperature. After washing three times in phosphate buffer, the tissue was dehydrated with graded ethanol and embedded in LR-Gold resin as described previously (Sierralta 2001). After peroxide-induced polymerization in the cold, the resin blocks were cut with a Reichert ultramicrotome, the 70 nm thin sections were collected on formvar-coated 300 mesh grids and post-stained with uranyl acetate and lead citrate. The specimens were inspected with a Philips CM100 electron microscope operating at 80 kV.

Measurement of Cu content in liver biopsies

Liver samples were weighed and dried for 12 h at 200 °C. The tissue was processed according to AOAC (AOAC 1997). Briefly, the tissue was digested with a mix of ultrapure nitric acid (2 ml) and sulfuric acid (2 ml) and boiled in a micro Kjeldahl digestion unit (Labconco Corp.) for 15 min. The digest was cooled and perchloric acid (2 ml) was added and boiled again until the sample was colorless and transparent. The digest was diluted with 10 ml of de-mineralized and double-distilled water. The Cu content was determined by an atomic absorption spectrometer equipped with graphite furnace (SIMAA 6100, Perkin–Elmer). MR-CCHEN-002 (*Venus antiqua*) and DOlt-2 (*Dogfish liver*) preparations were used as reference materials to validate the mineral analyses.

Electromobility shift assay (EMSA) and supershift assay

Nuclear protein extracts were prepared from liver samples (Deryckere & Gannon 1994) homogenized in 5 ml buffer A [containing 10 mM HEPES (pH 7.9), 0.6% Nonidet P-40 (NP-40), 150 mM NaCl, and 0.5 mM phenylmethylsulfonylfluoride (PMSF)] and centrifuged for 30 s at 2000 rpm and 4 °C. The supernatant was incubated for 5 min on ice and centrifuged for 5 min at 5000 rpm and 4 °C. The pellet was resuspended in buffer B [containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiotreitol (DTT), 0.5 mM PMSF, 2 mM benzamidine, and 5 μg/ml of the protease inhibitors pepstatin, leupeptin, and aprotinin], incubated on ice for 20 min, and centrifuged for 30 s at 13,000 rpm and 4 °C. The supernatant was stored at −80 °C. The samples were subjected to EMSA for assessment of NF-κB activity (Wong *et al.* 1997) using the NF-κB probe 5′-GATCTCAGAGGGGACTTTCCGAG-3′ (Genset Corp.), labelled with α–^{32}P-dCTP using the Klenow DNA Polymerase Fragment I (Gibco

BRL). For this purpose, 8 μg nuclear protein extracts were preincubated on ice for 15 min with 10 μl of a solution containing 2 μl footprinting buffer [250 mM HEPES (pH 7.6), 50 mM $MgCl_2$, and 340 mM KCl], 3 μl nuclear dialysis buffer [25 mM HEPES (pH 7.9), 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 1 mM DTT, and 0.1 mM NaF], 0.5 μl polydI:dC (2 mg/ml), 2 μl 0.1% NP-40, 1 μl 150 mM NaCl, 1 μl α–^{32}P-labelled NF-κB probe, and 0.5 μl ultra pure water.

The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabeled DNA probe. To assess the subunit composition of DNA binding protein, specific antibodies were used for supershift assay (goat and rabbit immunoglobulin-G raised against NF-κB p50 and p65, respectively; Santa Cruz Biotechnology Inc.) (Van Leeuwen *et al*. 2001). Protein–nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel (6% acrylamide) and run in a 0.5× TBE buffer [45 mM Tris–HCl, 45 mM boric acid, and 1 mM EDTA (pH 8.0)] for 4.5 h at constant voltage (100 V). Gels were transferred to Whatman 3 M paper, dried upon vacuum at 80 °C for 1 h, and subjected to autoradiography. The relative intensity of each band was estimated using densitometry (Scion Image; Scion Corp.).

Statistics

Values represent mean ± SEM. Statistical analysis was performed by multivariate repeated measures analysis between and within groups (weight); two-factor analysis of variance (2-way ANOVA) with a Bonferroni post-test or *t*-test (liver Cu concentration and perfusion data); and Kruskal–Wallis one-way ANOVA (histology numerical measures) using SYSTAT version II. $P < 0.05$ was considered statistically significant.

Results

The Cu-supplemented diet was well tolerated, intake did not change throughout the study period in experimental animals in comparison to controls; and there was a significant interaction ($P < 0.001$) between age and Cu-treatment on weight (Figure 1). Livers from rats loaded with Cu for 6 to 16 weeks stained strongly with rhodamine whereas those of control rats did not (Figure 2a,b). There was no significant difference in iron content between Cu-loaded rats and controls at any time point (data not shown). There were no uniform pathological changes seen by light microscopy in Cu-loaded or control rats, although a few Cu-treated animals had mild areas of inflammatory cell accumulation with a single necrotic foci identified in one (data not shown).

By electron microscopy, irregularly shaped nuclei containing dense chromatin were first seen at 6 weeks of Cu supplementation; the picnotic nuclei became more frequent in animals exposed to Cu for longer periods of time (Figure 2d). Mitochondria became more abundant and polymorphic in size and extent of crest dilation with time in Cu-loaded rats. Hepatocytes in Cu treated animals contained larger numbers of secondary lysosomes with progressing heterogeneity and prominence of inclusions. In many Cu-loaded cells the RER became disorganized and SER was diminished along time. Control rats at all time points displayed a normal ultrastructure of hepatocytes, with well defined and abundant mitochondria, regularly shaped nuclei with a predominance of euchromatin, fewer secondary lysosomes, well defined SER and RER areas (Figure 2c).

The histological intensity of Cu-specific staining in Cu-loaded rats was quantified using the semi-quantitative scale and showed to be higher when compared to controls (Figure 3a), reaching statistical significance at 12 and 16 weeks ($P < 0.01$). After 6 weeks of Cu-supplementation, the liver Cu concentration was 598 ± 82 μg Cu/g dry tissue weight, increased to 612 ± 39 and 2136 ± 510 μg Cu/g dry tissue weight at 9 and 12 weeks, respectively, and decreased to 1545 ± 325 μg Cu/g dry tissue weight at 16 weeks. At all time points the liver Cu concentration of Cu-loaded rats was significantly higher ($P < 0.001$) than controls (Figure 3b). Moreover, there was a significant ($P < 0.05$) effect of time on liver Cu concentration with rats loaded highest at 12 and 16 weeks compared to those at 6 and 9 weeks.

The basal rate of liver O_2 consumption assessed in perfusion studies before carbon infusion was substantially higher in Cu-loaded rats than in controls, reaching significance ($P < 0.05$) at 9 weeks (Figure 4a). Throughout the supplementation period, Cu administration did not significantly modify the rate of carbon uptake [(in mg carbon/g liver)

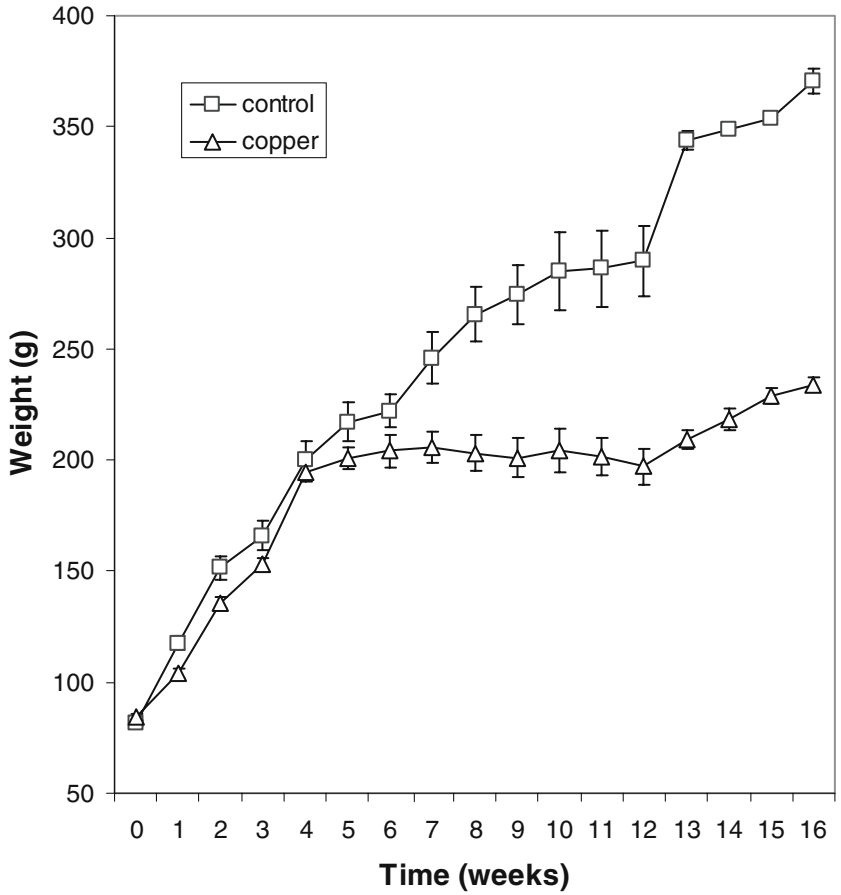

Figure 1. Effect of Cu exposure on weekly weights in weanling rats fed with rat chow *ad libitum* containing 1200 ppm (Cu group) or <10 ppm (control group) of Cu for 16 weeks. Values shown correspond to means $\pm$ SEM ($n=8$–20 animals). The difference between groups was calculated by multivariate repeated measures analysis and found to be treatment- and time-dependent ($P<0.001$).

controls: 6 wks, 1.37 ± 0.12 ($n=5$); 9 wks, 1.28 ± 0.09 ($n=4$); 12 wks, 1.31 ± 0.11 ($n=4$); 16 wks, 1.40 ± 0.03 ($n=3$); Cu-loaded: 6 wks, 1.41 ± 0.06 ($n=4$); 9 wks, 1.40 ± 0.11 ($n=4$); 12 wks, 1.22 ± 0.17 ($n=5$); 16 wks, 1.47 ± 0.07 ($n=5$)]. Carbon-induced O_2 consumption in Cu-treated animals tripled that of controls at 6 and 9 weeks ($P<0.05$) but then decreased to comparable levels at 12 and 16 weeks (Figure4b). The ratio of carbon-induced O_2 uptake/carbon uptake (Figure 4c) and that of carbon-induced O_2 uptake/basal O_2 consumption (Figure4d) were also significantly higher ($P<0.05$) at 6 and 9 weeks, but not at 12 and 16 weeks, between Cu-loaded animals and controls.

NF-κB DNA binding activity, as measured by EMSA in densitometric and arbitrary units and supershift assays, was higher in animals supplemented with Cu than their respective controls, reaching statistical significance at 9 and 12 weeks (Figure 5).

Discussion

In this study, we approached the significance of a moderate controlled Cu load in a murine model to correlate early hepatic histopathologic changes with functional alterations of liver cells. Data presented indicate a divergence in weight gain of the rats after 5 weeks of moderate Cu administration which increased with time. Food was provided *ad libitum* and no changes were detected in consumption during the study, but intake was not measured individually and therefore it is not possible to determine to what extent these results may

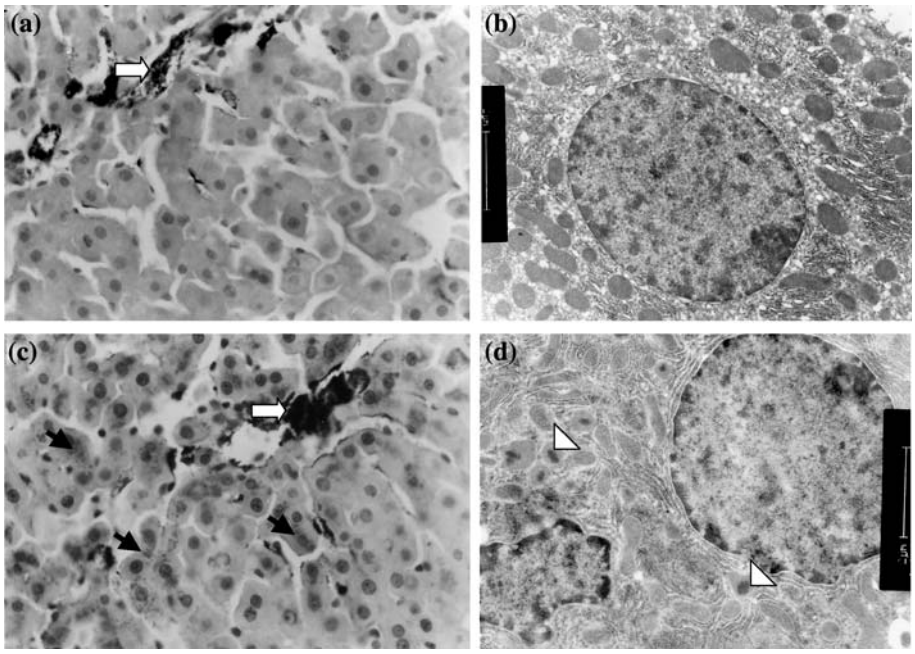

Figure 2. Light and electron microscopic studies in weanling rats fed with rat chow *ad libitum* containing 1200 ppm (Cu group) or <10 ppm (control group) of Cu. Figures show liver parenchyma after 16 weeks of Cu-loading. Rhodamine staining of control (a) and Cu-loaded (b) rat liver showing intracellular Cu accumulation (black arrows) and colloidal carbon (white arrows) (magnification ×40). Electron microscopy photographs of control (c) and Cu-loaded (d) hepatocytes demonstrating changes in nucleus (notice chromatin condensation in the nucleus at the left side) and lysosomal inclusions (white arrow heads) in Cu supplemented rats (dark bar = 2 μm).

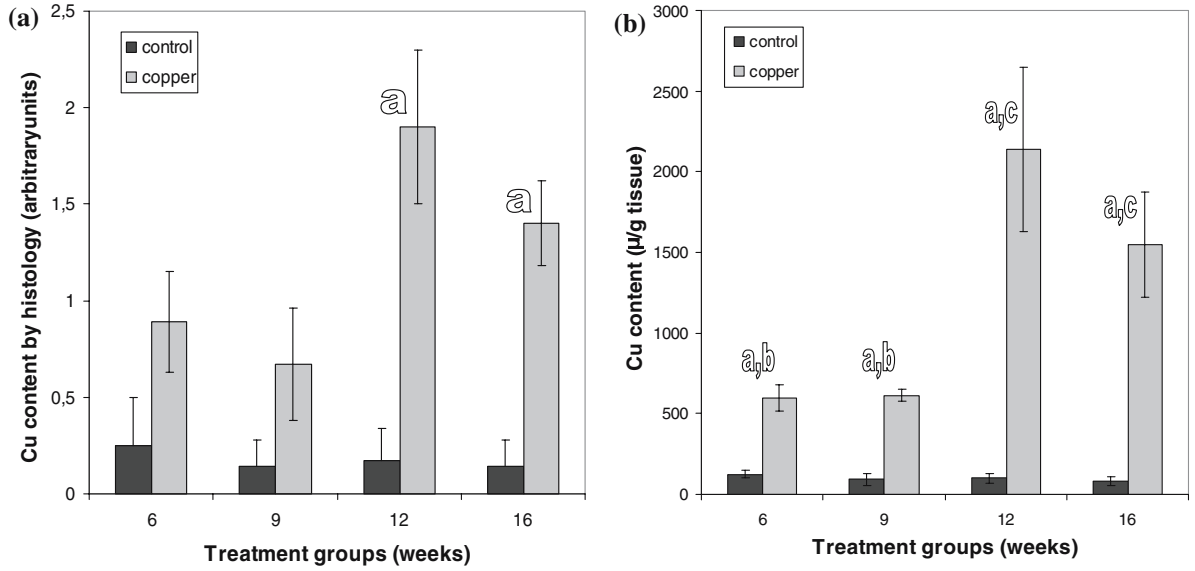

Figure 3. Hepatic Cu content (by atomic absorption spectrometry, SIMAA 6100, Perkin–Elmer) in weanling rats fed with rat chow *ad libitum* containing 1200 ppm (Cu group) or <10 ppm (control group) of Cu for 16 weeks. In panel (a), Cu content was evaluated histologically by rhodamine staining and quantified under blind conditions using an arbitrary scale ranging from 0 to 3. Values shown are means ± SEM for 4 to 10 animals in each group with statistical significance ($^{a}P < 0.05$) between Cu and control animals indicated. Panel (b) shows the means ± SEM of Cu contained within hepatic tissue as measured by spectrophotometry ($n = 3$–10 animals per group). The statistical analysis was carried out using two factor analysis of variance: $^{a}P < 0.05$ comparing Cu-loaded vs controls at each time; $^{b,c}P < 0.05$ comparing Cu-loaded rats at 6–9 weeks with those at 12–16 weeks.

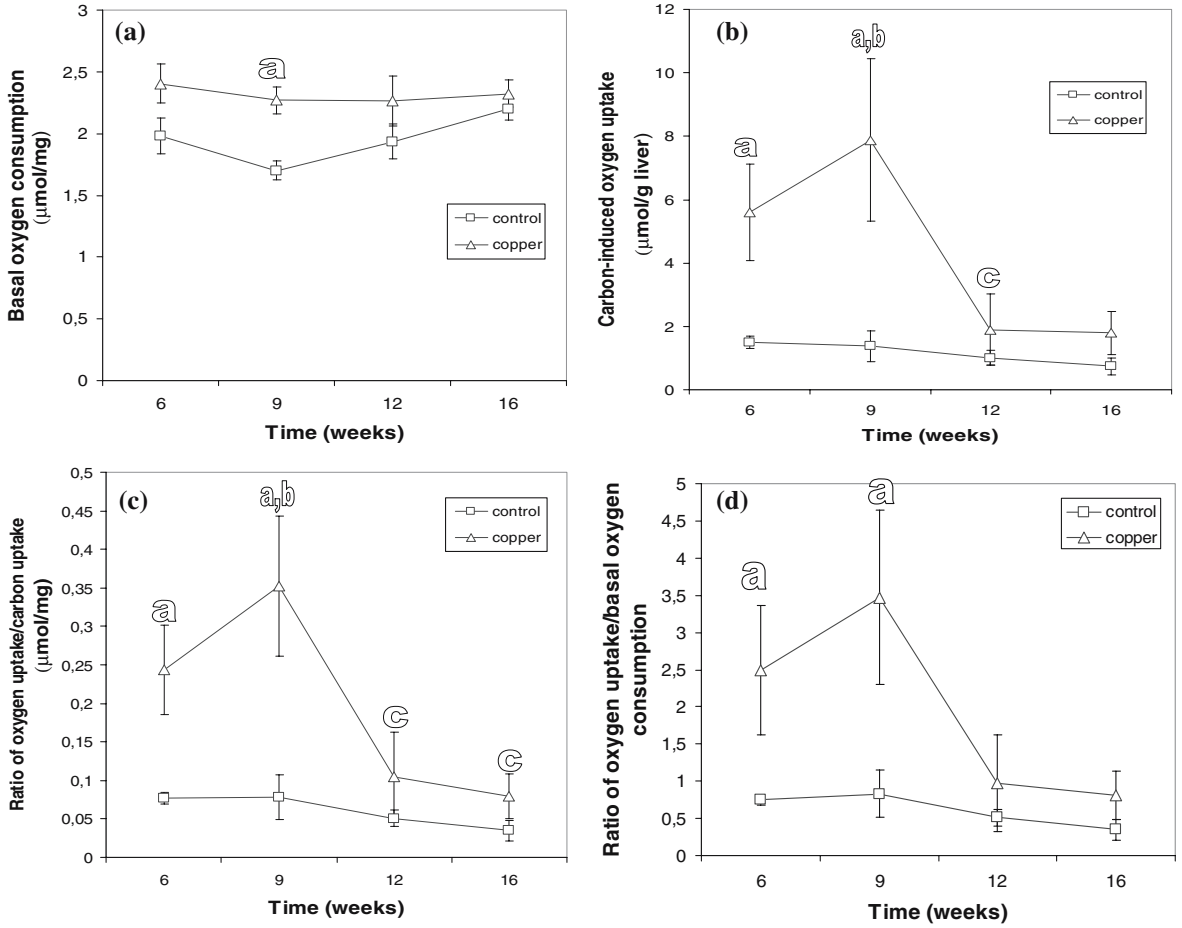

Figure 4. Time course effect of Cu-supplementation on liver perfusion and Küpffer cell functional parameters in weanling rats fed with rat chow *ad libitum* containing 1200 ppm (Cu group) or < 10 ppm (control group) of Cu for 16 weeks. Basal O_2 consumption (a) of perfused livers was obtained previous to carbon particle administration for baseline parameters comparison between Cu-treated and control rats. Total carbon-induced O_2 uptake (b) is calculated as the extra equivalents of O_2 induced by colloidal carbon perfusion. In (c) and (d), ratios of carbon-induced O_2 uptake/carbon particle uptake and that of carbon-induced O_2 uptake/basal O_2 consumption are plotted for Cu-loaded and control rats supplemented for 6–16 weeks. All data points represent means ± SEM of 3 to 6 animals in each group. Statistical analysis was carried out using two factor analysis of variance: $^{a}P < 0.05$ comparing Cu-loaded vs controls at each time; $^{b,c}P < 0.05$ comparing Cu-loaded rats at 9 weeks with those at 12–16 weeks.

be explained by decreased intake or augmentation of the overall energy consumption. Independent of what caused this finding, that the effect became apparent after 5 weeks suggests that either younger animals are better adapted to cope with higher doses of Cu or, that the cumulative effect of Cu requires overpassing a threshold to become apparent.

In agreement with previous studies, Cu-treated animals did not present major histological changes following exposure to 1200 ppm Cu (Hebert *et al.* 1993; Aburto *et al.* 2001). In this respect, Aburto *et al.* showed a dose-dependent hepatic increase in inflammatory and necrotic changes (Aburto *et al.* 2001) whereas a no-observed-adverse-effect level (NOAEL) had been previously described between 1000 and 2000 ppm Cu (Hebert *et al.* 1993). Doses up to 1000 ppm Cu have typically been used in rats for the study of Cu load and metabolism (Evering *et al.* 1990, 1991), whereas Cu above 1250–1500 ppm causes multifocal hepatitis, widespread single-cell necrosis, and apoptosis (Fuentealba *et al.* 2000; Aburto *et al.* 2001). Ultrastructural assessment showed results consistent with early hepatocellular changes due to Cu loading, including an increase in lysosomal

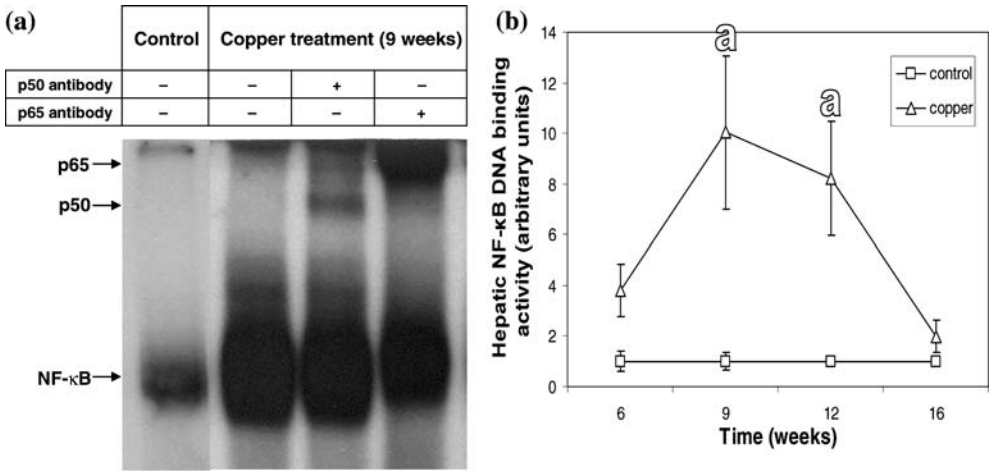

Figure 5. Cu supplementation enhancement of rat liver NF-κB DNA-binding activity in weanling rats fed with rat chow *ad libitum* containing 1200 ppm (Cu group) or < 10 ppm (control group) of Cu for 16 weeks. (a) Representative autoradiographs of NF-κB DNA binding evaluated by EMSA using nuclear extracts from livers from control rats and Cu overloaded animals after 9 weeks of treatment, and supershift analysis using antibodies specific for NF-κB p50 or p65 (b) Densitometric quantification of relative activity of NF-κB evaluated by EMSA in control and Cu-supplemented rats at different times of treatment. Mean value for time controls were arbitrarily set to unity, and values at each time point for Cu-loaded rats were normalized to it. Each data point represents mean ± SEM for 3 to 6 animals. Significance studies: $^{a}P < 0.05$ of Cu-loaded vs control rats at each time point.

number and diversity, appearance of heterochromatin and picnotic nuclei, and varied mitochondrial alterations in accordance to those described in rats loaded with 1500 ppm of Cu (Fuentealba & Haywood 1988). Similarly, it has been described that excess liver Cu in Bedlington terriers is initially sequestered in lysosomes and with increasing saturation of this compartment nuclear accumulation of Cu develops (Haywood *et al*. 1996). It has been proposed that Cu contained within lysosomes is innocuous whereas nuclear Cu is directly injurious to this organelle and responsible for the subsequent cell death (Fuentealba *et al*. 1989; Kumaratilake & Howell 1989). These findings, together with the continued hepatic accumulation of Cu found, indicate that the dose of 1200 ppm of Cu is at the boundary of subacute liver damage and offers the possibility of assessing the functional changes associated with liver Cu load.

A good correlation between histological methods of Cu detection and analysis by absorption spectrophotometry is important in the characterization of Cu storage diseases (Fuentealba *et al*. 1987). In the rat model used in this study a Cu supplemented diet showed a progressive liver accumulation of Cu. This cellular Cu-loading was significant from the sixth week of supplementation and increased maximally at 12 weeks. Most interestingly, there was a good correlation between hepatic Cu content as detected by histological (rhodamine) methods and spectrophotometry, showing that both approaches are useful in the Cu concentration range used in this study. The continuously increasing Cu content in the liver observed along the 16 weeks of this study differs from some of the data previously published (Haywood & Loughran 1985; Fuentealba *et al*. 1993). Weanling rats were used in this study considering that at this age Cu absorption is non-saturable and concentration dependent (Varada *et al*. 1993) and this would favor accumulation of hepatic Cu. Also, the different Cu doses as well as the choice of rat strains and male/female ratio of animals used in each study may partially explain the differences (Nederbragt 1985).

Chronic Cu supplementation augments the liver O_2 consumption as assessed by perfusion studies. This change was maximal at 9 weeks of supplementation in the absence of colloidal carbon with a subsequent return to control values. One expects a rise in O_2 consumption following chronic Cu administration due to its ability to catalyze the aerobic generation and reactions of ROS and reactive nitrogen species (RNS) (Aust *et al*. 1985; Linder& Hazegh-Azam 1996; Tapia *et al*. 1998).

However, a previous study using an acute dose of Cu before liver perfusion failed to demonstrate a rise in basal O_2 consumption, a discrepancy that may be due to the low Cu dose used in this latter study, allowing for its appropriate handling by the hepatic pathways of Cu metabolism (Sans *et al.* 1999). Our histological and biochemical results demonstrate a continuous rise in hepatocellular Cu concentration where the additional metal ion may promote formation of hydroxyl radicals in a Cu-catalyzed Fenton and/or Haber–Weiss reactions thus increasing O_2 use (Samuni *et al.* 1981). The return of basal O_2 consumption to control values after 12–16 weeks suggests an effective hepatic response to the continuous Cu challenge, in the form of sinusoidal and canalicular secretion mechanisms and/or storage in MT (Freedman *et al.* 1989; Linder & Hazegh-Azam 1996).

Infusion of colloidal carbon into perfused rat livers leads to its rapid uptake by sinusoidal cells (Cowper *et al.* 1990; Tapia *et al.* 1997), particularly Küpffer cells of the periportal regions of the hepatic lobule, while it is absent from hepatocytes (Bouwens *et al.* 1986). This uptake generates an increase in hepatic O_2 consumption that is largely accounted for by the respiratory burst of Küpffer cells (Decker 1990; Wang *et al.* 1993), with a minor component from parenchymal cells that could be mediated by prostaglandins released by activated Küpffer cells (Qu *et al.* 1996). Using gadolinium chloride as a Küpffer cell inactivator, we previously demonstrated that the increased hepatic respiratory consumption that followed an acute Cu dose was primarily due to Küpffer cell activation (Sans *et al.* 1999). Chronic Cu supplementation similarly augments the respiratory burst of Küpffer cells as measured by the increased carbon-induced O_2 uptake in treated rats while particle phagocytosis remained constant through time. This change is likely secondary to the interaction of Cu and $O_2^{\cdot -}$, which is generated by liver macrophage NADPH oxidase activity following carbon infusion (Samuni *et al.* 1981; Rowley & Halliwell 1983), in addition to the ability of Cu to effectively activate endothelial NO synthase (Plane *et al.* 1997). Interestingly, this increase was most important at 9 weeks of Cu loading with a subsequent return to control values at 12–16 weeks. Similarly, the ratios of liver carbon-induced O_2 consumption to basal O_2 uptake and that of carbon-induced O_2 consumption to carbon phagocytosis also show an important initial increase in time, thus pointing to a net enhancement in the respiratory burst activity of Küpffer cells. It is tempting to speculate that since the respiratory burst response depend greatly on NADPH oxidase activity (Decker 1990; Wang *et al.* 1993), progressive inactivation of this enzyme by the ROS produced in the process could explain at least part of this result (Jandl *et al.* 1978). Cu-loading studies have previously alluded to the capability of rats to develop resistance and tolerate continuous Cu supplements by changing the intracellular distribution of Cu and by an efficient Cu unloading mechanism (Fuentealba *et al.* 1993). It is well recognized that intracellular Cu metabolism is linked to MT; Sans *et al.* showed that induction of MT by $ZnSO_4$ pre-treatment greatly diminished the carbon-induced O_2 uptake generated from acute Cu exposure in perfused rat livers (Sans *et al.* 1999). MT can act as both a Cu storage protein and as a free radical scavenger diminishing the further generation of ROS from Cu (Mullins & Fuentealba 1998; Viarengo *et al.* 2000). As such, measurement and subcellular localization of hepatic MT should provide new insights into the functional changes that are observed in perfused rat livers.

Cellular oxidative stress greatly influences gene expression (Sen & Packer 1996). Cu, among other trace elements, activates a large number of genes through regulation of transcription factors including the NF-κB signalling pathway (Kudrin 2000). Here we showed that chronic Cu supplementation in rats activated this pathway as measured by NF-κB DNA binding activity. This change was significant at 9 and 12 weeks of treatment and returned to basal values at 16 weeks. Activation of the NF-κB signalling pathway by Cu supplementation is consistent with a previous study where tetrathiomolybdate, a compound used in the treatment of Cu overload, suppresses NF-κB level and transcriptional activity of proangiogenic factors (Pan *et al.* 2002). Interestingly, Murr1, a protein involved in Bedlington Terrier Cu toxicosis (Klomp *et al.* 2003) inhibits HIV-1 growth in unstimulated CD4+ T cells, mediated through its ability to decrease basal and cytokine-stimulated NF-κB activity (Ganesh *et al.* 2003), again linking NF-κB to Cu metabolism. In addition, Cu containing biological implants have been tested in rats and showed to

locally increase levels of activated NF-κB, causing major differences in inflammatory cell accumulation (Suska *et al*. 2005). These results allow us to propose NF-κB as a key marker in the Cu-induced cellular response to oxidative damage. Again, we observe a late decrease of NF-κB activity which coincides with the ability of rats to develop resistance to Cu supplementation (Fuentealba *et al*. 1993). How NF-κB activity relates to cellular Cu loading and alterations of respiratory responses remains to be elucidated.

In conclusion, subchronic Cu loading with 1200 ppm Cu (as $CuSO_4$) in young rats alters their growth curves and induces early hepatic morphological changes as evidenced by light and electron microscopy and enhancement in Küpffer cell-dependent respiratory burst that is paralleled by an activation of NF-κB DNA binding activity. These effects peaked at 9 weeks and diminished after 12–16 weeks Cu of exposure. Mechanisms responsible for developing this apparent tolerance despite progressive liver Cu deposit are currently under study in our laboratory.

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