Nutrition 30 (2014) 96–106

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Copper-induced alterations in rat brain depends on route of overload and basal copper levels

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ARTICLE INFO

Article history:
Received 23 March 2013
Accepted 12 June 2013

Keywords:
Copper
Brain
Oxidative stress
Lipids
Apoptosis

ABSTRACT

Objectives: Copper (Cu) is widely used in industry for the manufacture of a vast range of goods including Cu-intrauterine devices (IUDs), electronic products, agrochemicals, and many others. It is also one of the trace elements essential to human health in the right measure and is used as a parenteral supplement in patients unable to ingest food. Elevated Cu levels have been found in the plasma of women using Cu-IUDs and in farmers working with Cu-based pesticides. However, possible alterations due to Cu overload in the brain have been poorly studied. Therefore, the aim of this study was to investigate the effects of Cu administration on rat brain in Cu-sufficient and Cu-deficient animals fed on semi-synthetic diets with different doses of Cu (7 or 35 ppm).
Methods: We aimed to investigate the effects of Cu administration using two routes of administration: oral and intraperitoneal (IP). Male Wistar rats were feeding (one month) a complete (7 ppm) or a deficient (traces) Cu diets subdivided into three categories oral-, intraperitoneal- (or both) supplemented with copper carbonate (7 to 35 ppm). Cu content in plasma, brain zones (cortex and hippocampus), antioxidant enzyme activities, and protease systems involved in programmed cell death were determined.
Results: The results show that Cu levels and the concentration of Cu in plasma and brain were dose-dependent and administration route-dependent and demonstrated a prooxidative effect in plasma and brain homogenates. Oxidative stress biomarkers and antioxidative enzyme activity both increased under Cu overload, these effects being more noticeable when Cu was administered IP. Concomitantly, brain lipids from cortex and hippocampus were strongly modified, reflecting Cu-induced prooxidative damage. A significant increase in the activities of calpain (milli- and micro-) and caspase-3 activity also was observed as a function of dose and administration route.
Conclusion: The findings of this study could be important in evaluating the role of Cu in brain metabolism and neuronal survival.

Introduction

Copper (Cu) is an essential trace element in living organisms, functioning as a cofactor for many enzymes [1,2]; however, excessive amounts of Cu are potentially hazardous to human health because Cu can participate in the Fenton reaction, producing oxidative stress (OS) and subsequent damage to biomolecules [3,4]. Cu-derived substances are used in a wide range of industries, in the production of agrochemicals, and in Cu-containing intrauterine devices (Cu-IUDs) [5–8]. It has been shown that chronic exposure to Cu ions by farmers handling agrochemicals and women using Cu-IUDs leads to elevated levels of Cu in the plasma [7–11]. Cu is administered parenterally to individuals unable to ingest food by oral or enteral routes and in cholestatic infants [12,13]. Doses of up to 1.3 mg/d (20 mmol/d) can be warranted in patients with excessive gastrointestinal losses and major burns [14].

Various studies have demonstrated that patients with neurodegenerative diseases had elevated Cu concentrations in their plasma [15,16], suggesting a direct or indirect involvement of Cu overload in the progression and/or the etiology of neurologic diseases [17]. Moreover, a recent study reported that the

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence this work. The authors declare that there are no conflicts of interest.

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http://dx.doi.org/10.1016/j.nut.2013.06.009

ingestion of excessive Cu through drinking water and vitamin supplements is at least partly responsible for the development of Alzheimer's disease in developed countries, which is reaching epidemic proportions [18]. Cu deficiency also has been associated with neurodegenerative processes [19] and it is speculated that it may affect Cu uptake and/or metabolism at the blood–-brain barrier or in the brain itself. The focus of neurologic diseases is now on understanding the homeostatic process of metals associated with these disorders and how excess or deficiency can induce neurologic dysfunction [20–27]. The question of the role of Cu excess or deficiency in neurologic illnesses remains a matter of debate [23–25,27] and requires further investigation. Based on the previous experimental and epidemiologic evidence, we hypothesize that Cu availability is crucial in the etiology of specific neurologic illnesses, such as Alzheimer's disease, and that not only the excess or the defect of Cu but also the route of access to the organism (in the case of the overload) are key factors to be considered. The intestine plays a crucial role in maintaining the homeostasis of this metal. So, we also hypothesize that the overload produced by Cu-based IUDs, inhalation or dermal exposure during agrochemical-related activities, or the manipulation of Cu-derived supplies from industrial production devices may be considered more dangerous than the oral acquisition of excess Cu, such as drinking tap water with elevated Cu levels.

Thus, the aim of the present study was to investigate the effects of Cu administration on the brain of rats fed on Cu-sufficient, Cu-deficient, or Cu-excessive oral diets. In some cases, rats also were cosupplemented with parenteral Cu doses. Specifically, we aimed to determine the following:

1 The concentration of Cu in plasma and brain tissue as a function of the administration route and doses assayed;
2. The Cu overload-associated damage to lipids and proteins in brain;
3. The response of the main Cu-homeostatic proteins (metallothioneins [MTs] and ceruloplasmin [CRP]) in brain and plasma;
4. The changes in the antioxidant (enzymatic and non-enzymatic) defense system in the brain; and
5. The involvement of the main proteolytic system associated with programmed cell death during Cu-induced OS.

Materials and methods

Chemicals

All chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina or Palo Alto, CA, USA), Merck (Darmstadt, Germany), or Carlo Erba (Milan, Italy).

Animals and treatments

Certified pathogen-free male Wistar rats were used. The rats were maintained under controlled temperature conditions (25°C) with a relative humidity of 60%, forced ventilation, and a normal photoperiod of 12-h darkness and 12-h light. The health of animals was verified in accordance with the internationally recommended practices of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council [28]. Solid food and drinking water were provided ad libitum. The experimental diets were prepared in our laboratory according to the recommendations for Wistar rats [29]. All procedures for handling the animals followed the National Institutes of Health regulations [28]. The experimental protocol was reviewed and approved by the Bioethics Committee of the Faculty of Medical Sciences, UNLP (COBIMED # 00382/11).

Experimental protocols

Groups of six 21-d-old rats were randomly assigned to the following protocols and treated during 30 d. Cu was administered in the form of $CuCO_3$. A concentration of 7 or 35 ppm of $CuCO_3$ in solid food was considered sufficient or moderately excessive, respectively, in accordance with the recommendations of previous authors [30–32]. The groups were (Table 1): control (C) rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; and D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group. It is important to note that the dose delivered to the rats fed on D groups depends on the volume injected. The present doses delivered are related to the doses that the rats receiving Cu in the diet (control groups) ingested day by day. This obviously changed along the experimental period. So, we had to measure the volume of water and the amount of food ingested by the rats in order to adequate the delivery of Cu and maintain the extension of Cu overload initially planned for our protocol.

Solutions for IP injections were prepared by dissolving pure $CuCO_3$ (Riedel-de Haën, ultra-pure, Buckinghamshire, UK) in PBS pH 7.40/Tween®-20 (1%) and sterilized by ultrafiltration through 0.22 μm Millipore membranes (Pal Alto, CA, USA). The rats received the daily injections on alternate abdominal sides using non-traumatic sterile/disposable needles type BD 0.30 × 13–30G ½ (BD Precision Glide, Becton Dickinson Industries Ltd., New York, NY, USA). The rats were controlled during the experimental period to observe their behavior, quantify water and food consumption, and determine their body weight gains. Samples of feces and blood from the tail vein were taken weekly to determine the Cu concentration and Cu homeostatic proteins. Samples of drinking water and each batch of food (prepared at 5-d intervals) were analyzed for Cu content in triplicate. The Cu concentration in the tap water was 0.06 ± 0.01 mg/L (or ppm). Considering that each animal drank no more than 15 mL water/d, a maximum of 1 ppm/d was probably acquired from the water, a negligible amount in the experimental model. Linear regression curves and the analysis of variance (ANOVA) test for Cu content in food demonstrated that there were no significant variations between the six experimental preparations (Table 1 shows representative values). The actual doses of Cu administered to each experimental treatment were calculated considering the amount of food consumed daily, the injected Cu (in the corresponding groups), and the weight of the animals (Table 1).

Sample collection

At the end of the treatments, animals were sacrificed by decapitation. Brains were taken out, washed, weighed, and quickly homogenized in HEPES 50 mM pH 7.4 with CHAPS 5 mM, dithiothreitol 5 mM, and aprotinin 10 mg/mL, in a proportion of 6 mL buffer to each 100 mg tissue. Blood was collected using heparin as anticoagulant in ice-cold polypropylene tubes. Plasma samples were immediately prepared by centrifugation (4000*g*, 10 min) and then stored at −70°C until analyzed.

Analytical methods

To determine Cu concentration, samples (100 μL) were digested with 1 mL of a mixture of concentrate H_2SO_4:HCl:HNO_3 (1:1:2) at 90°C overnight. The digests were cooled, diluted with ultrapure water (18 mΩ cm, Carlo Erba, Milan, Italy), and ultrafiltered with a 0.22 μm Millipore membrane (Milli-Q Purification System, from Millipore, Palo Alto, CA, USA) [33]. Ultrafiltered dissolutions were directly aspirated into the flame of a Perkin-Elmer 1100B Spectrophotometer equipped with a Perkin-Elmer cathode lamp (Perkin-Elmer Corp., Norwalk, CT, USA) at a spectral width of 1 nm. Calibrations were performed with a standard solution of $Cu(NO_3)_2$ in HNO_3 0.5 N (Tritrisol from Merck Co., Darmstadt, Germany). All measurements were carried out in peak height mode (324.7 nm line). The intra- [(SD/×).100] and inter- [(ΔSD/Δ×).100] assay coefficients of variation were 15.5 and 6%, respectively. Cu in feces was determined using the same methodology, except that the material was previously treated at 100°C overnight to dryness, weighed and then mineralized as described before. CRP was analyzed by the conversion of *p*-phenylenediamine into a colored product measured at 550 nm at 37°C in a buffer glacial acetic/sodium acetate (50 mM, pH 5.5) directly into flat-bottom plates, using a Multi-Detector Microplate Reader SpectraMax M2/m2^e model from Molecular Devices Analytical Technologies (Sunnyvale, CA, USA) [34]. Intra- and interassay coefficients of variation were 8.3% and 4.4%, respectively. CRP concentrations were calculated by comparison with the reaction rate of human pure CRP standard (Sigma Chem. Co., Buenos Aires, Argentina). MTs were determined by adding an excess of Ag^+ (500 μL/20 μg Ag^+/mL of sample). The mixture was incubated with 100 μL of an hemolysate of red

Table 1
Nomenclature of the experimental groups and main characteristics of the diets

Nomenclature of diets	C	CuO	CuI	D	D-I7	D-I35
Routes and amounts of $CuCO_3$ (ppm) supplementation	7, orally	35, orally	7 orally plus 35 IP	None	None	35, IP
Cu via IP	None	None	35 ppm	None	Equivalent to that ingested by C	Ingested by C x 5
Diet Cu content (mg/Kg)	7.7 ± 0.1^a	37.6 ± 0.3^b	7.5 ± 0.2^a	0.1 ± 0.0^b	0.1 ± 0.0^b	0.1 ± 0.0^b
Initial body weights (g)	175.6 ± 0.5	177.1 ± 1.4	174.3 ± 1.5	170.2 ± 1.1	178.8 ± 2.5	181.0 ± 2.5
Final body weights (g)	280.1 ± 6.0	282.2 ± 3.6	283.3 ± 3.2	279.0 ± 2.7	285.7 ± 4.6	288.4 ± 3.0
Total body weight gain (g)	104.4 ± 4.0	105.1 ± 3.5	109.0 ± 2.9	108.8 ± 2.2	106.9 ± 2.0	107.4 ± 3.1
Rate of body weight gain (g/d)	3.0 ± 0.2	3.0 ± 0.3	3.1 ± 0.2	3.1 ± 0.4	3.0 ± 0.3	3.0 ± 0.2
Initial food intakes (g/d/rat)	11.5 ± 0.8	10.8 ± 1.1	10.8 ± 0.5	11.1 ± 0.7	10.8 ± 1.1	10.9 ± 1.1
Final food intakes (g/d/rat)	30.0 ± 2.3	27.9 ± 1.9	26.6 ± 0.8	25.8 ± 1.2	27.9 ± 1.9	25.8 ± 0.6
Cu doses (mg/kg/d) (mean, extreme values)	0.67 (0.51–0.82)	3.00 (2.29–3.71)	162.82 (124.32–201.31)	0.015 (0.01–0.02)	0.67 (0.50–0.84)	3.43 (2.63–4.22)
Initial food efficiency ratio*	9.0 ± 0.4	9.7 ± 0.5	10.0 ± 0.3	9.8 ± 0.5	9.9 ± 1.0	9.8 ± 0.7
Final food efficiency ratio*	3.5 ± 0.2	3.8 ± 0.2	4.1 ± 0.3	4.2 ± 0.5	3.8 ± 0.5	4.6 ± 0.3
Diet TBARS content (nmoles MDA/kg)	0.29 ± 0.02	0.30 ± 0.03	0.28 ± 0.02	0.29 ± 0.04	0.31 ± 0.02	0.30 ± 0.04

ANOVA, analysis of variance; Cu, copper; IP, intraperitoneal; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances
Each value was expressed as the mean ± SD of six independent determinations. Cu and TBARS contents were determined according to the methods described in the Materials and method section. Results (for Cu content in diets) significantly different between them were indicated with distinct superscript letters (ANOVA + Tukey; $P < 0.01$)
* Body weight gain (g)/food intake (g).

blood cells (2% in buffer Tris/HCl 30 mM pH 8.0), heated (2 min at 100°C) and the denature proteins were discarded by centrifugation (5 min at 15 000*g*). MTs were quantified in the supernatant fraction previously acidified with HNO_3 using an atomic absorption spectrometer Avanta Ultra Z (GBC Scientific Equipment, Hampshire, IL, USA). For calculation, the stoichiometry of Ag^+-thionein was assumed to be 17g-at. Ag^+ per mole of MTs [35].

Biomarkers of ROS production

Thiobarbituric acid-reactive substances (TBARS) were measured in brain homogenates as previously described [36]. TBARS (mainly malondialdehyde [MDA]) were reacted with 2-thiobarbituric acid (TBA) to yield TBA-MDA adducts and quantified at 532 nm. The concentration of the chromophore was calculated from a calibration curve prepared with fresh tetramethoxypropane (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina). Nitrate and nitrite [NOx] concentrations were measured using the method of Griess on samples previously reduced with vanadium chlorohydrate [37]. Quantification was performed after calibration with standard solutions of sodium nitrate from Merck Co. (Darmstadt, Germany). Protein carbonyls (PCs) were determined by the method of Reznick and Packer [38]. PC concentrations were calculated from a calibration curve prepared with a stock solution of sodium pyruvate (Sigma Chem. Co.).

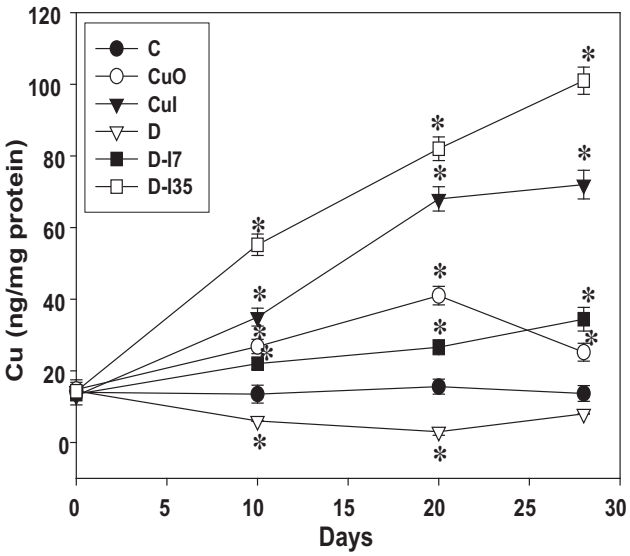

Fig. 1. Evolution of copper (Cu) plasma concentrations during the experimental period. Results are the mean of six independent measurements assayed in duplicate ± SD using plasma obtained from tail blood. Data shown correspond to days 0, 10, 20, and 28 of the dietary treatments. Significant differences between data of each curve and the basal value at zero time are indicated by asterisks ($P < 0.01$). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

Antioxidant defense system enzymes

The activity of catalase (CAT) was determined using the method of Aebi [39]. *K* values from the curves were electronically calculated using the equation $k = (2.30/t).\log (DO_f - DO_i)$. The specific activity of the enzyme was obtained considering the concentration of proteins corresponding to each sample. Superoxide dismutase (SOD) was determined using the method of Misra and Fridovich [40]. To calculate the activity of SOD in the samples we performed a reaction without sample homogenate in order to obtain the rate of the autoxidation of epinephrine. An enzymatic unit was defined as the amount that inhibits 50 % of auto-oxidation. To obtain the specific activity of SOD the ratios between the values of enzymatic units of each sample and the corresponding values of protein concentration (mg/mL) were calculated. The activity of glutathione transferase (GST) was determined by a method used in an earlier study [41]. The specific activity of the enzyme was calculated for each sample in terms of nanomoles of product/min•mg of total cellular protein, with $\varepsilon = 9.6\ mM^{-1} \bullet cm^{-1}$ (340 nm). Glutathione peroxidase (GPx) was determined by the method of Flohé and Gunzler [42]. To calculate the enzymatic units (in terms of nmoles of nicotinamide adenine dinucleotide phosphate-oxidase/min), $\varepsilon = 6.22\ mM^{-1} \bullet cm^{-1}$ (340 nm) was considered. The specific activity was obtained as units of enzyme activity/corresponding value of protein content (mg/mL). Glutathione reductase (GR) was determined by the method of Carlberg and Mannervik (1985) [43]. The specific activity of the enzyme was calculated for each sample in terms of micromoles of product/min.mg protein, with $\varepsilon = 6.22\ mM^{-1} \bullet cm^{-1}$ (340 nm).

Antioxidant molecules

The levels of reduced (GSH) and oxidized (GSSG) glutathione were determined by the glutathione reductase/dithio-nitrobenzoic (DTNB) method [44]. To calculate the ratio GSH/GSSG, samples were reanalyzed after derivatization with divinyl-pyridine (3 mM final concentration). The vitamin E (α-tocopherol) concentration was measured after extraction with the Buttriss and Diplock method [45] using a high-performance liquid chromatography technique [46] and expressing the results in pmol/mg protein. Total antioxidant reducing ability (ferric reducing ability of plasma [FRAP] assay) was measured according to Benzie and Strain [47]. FRAP values were obtained by comparing the change in absorbance at 593 nm (slope of the initial velocity reaction from 0 to 2 min) in the test sample mixtures with standards containing known concentrations of ferrous ions.

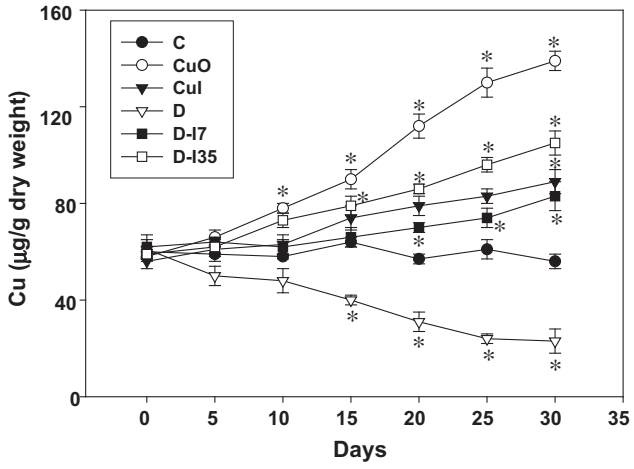

Fig. 2. Copper content in feces during the experimental period. Results are expressed as the mean of six rats assayed in triplicate ± SD [µg Cu/dry weight (110°C/12 h). Data from curves significantly different from the control value at zero time are indicated by asterisks ($P < 0.01$). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

Lipid analysis

Total lipids were extracted [48] and the phospholipid fraction was separated from the extracts by a microcolumn chromatography method [49]. Capillary gas-liquid chromatography (GLC) of the fatty acid methyl esters (FAME) was performed as indicated in a previous paper [50] using a capillary column (Omegawax 250, from Bellefonte, Supelco, PA) mounted on a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA) equipped with a terminal computer integrator and data station. The FAMEs were identified by comparison of their relative retention times with authentic standards and mass distribution was calculated electronically by quantification of the peak areas. Eicosamonoenoic acid (21:0) was used as the internal standard. An aliquot of each Folch extract was evaporated and the residue was dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing digitonin 1%. Aliquots of this solution were taken to measure enzymatically cholesterol (Cho), phospholipids (PL), and triglycerides (TG) using commercial kits from Wienner Lab. (Rosario, Argentina).

Programmed cell death biomarkers

Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA) by caspase-3 (Sigma Chem. Co., Buenos Aires, Argentina). The released *p*-nitroaniline (p-NA) was monitored at 405 nm. Each assay was run in parallel with inhibitor-treated cell lysate (to measure the non-specific hydrolysis of the substrate) and caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit manufacturer). A calibration curve using a standard solution of p-NA also was run for each assay to calculate the activity of the protease expressed as µmol p-NA released/min•mL of sample. The activities of milli- (m) and micro- (µ) calpains also were measured. The technique involves the hydrolysis of ultra-pure casein (Sigma, Chem. Co., CA, USA) by calpain(s) and the subsequent detection of trichloroacetic acid (TCA)-soluble peptidic fragments at 280 nm [51]. To select the activity of each calpain isoform, the level of calcium in the medium was regulated (5 mM or 500 µM of $CaCl_2$ for m-calpain or µ-calpain, respectively). The activities of calpains were calculated considering a unit of calpain to be the amount of enzyme that produces a change of absorbance of 0.01 at 280 nm. Results were expressed as units/min•mg of protein.

Statistical analysis

All values represent the mean of six rats assayed in triplicate expressed as mean ± SD. Data were analyzed by ANOVA plus the Tukey test with the aid of SPSS 11.0.1 software (SPSS Inc., Chicago, IL). Differences in the accumulation of Cu over time were analyzed by two-way ANOVA including time as a measure. Results also were plotted and analyzed using Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO). The statistical significance ($P \leq 0.05$) of differences is indicated by asterisks or letters, as appropriate.

Results

Figure 1 shows the evolution of the Cu concentration in plasma of rats during the treatment. Higher levels were observed in the case of IP treatment than for oral supplementation. Those rats treated IP with five times the normal intake of Cu (D-I35) showed an almost linear increase in Cu over time. On the contrary, in rats injected with an equivalent amount of Cu to that in the control diet (CuI), the level of Cu enters a plateau by day 20 of treatment. Oral supplementation (CuO) showed biphasic behavior: At first there was a clear and almost linear increase, after which the Cu content tended to decline until it reached levels similar to the control. Rats fed on the D diet showed significantly lower levels of Cu than those of the C group during the first 20 d of treatment. However, the levels had normalized by the end of the experimental treatment. The concentration of

Table 2
Oxidative stress biomarkers in various tissues from rat fed the experimental diets

Tissues	Diets					
	C	CuO	CuI	D	D-I7	D-I35
Plasma						
Cu (ng/mg protein)	13.7 ± 2.1^{a}	25.2 ± 2.4^{b}	71.9 ± 4.0^{c}	8.0 ± 0.1^{d}	34.4 ± 3.3^{e}	101.2 ± 3.9^{f}
MTs (ng/mg protein)	3.5 ± 0.3^{a}	15.2 ± 0.9^{b}	7.1 ± 0.7^{c}	3.3 ± 0.3^{a}	4.2 ± 0.1^{d}	9.3 ± 0.5^{e}
CRP (ng/mg protein)	48.0 ± 3.7^{a}	74.1 ± 5.0^{b}	86.0 ± 4.8^{c}	31.1 ± 4.4^{d}	63.4 ± 4.0^{e}	111.3 ± 5.5^{f}
Brain						
Cu (ng/mg protein)	6.0 ± 0.2^{a}	7.5 ± 0.1^{b}	11.2 ± 0.2^{c}	3.9 ± 0.1^{d}	7.9 ± 0.1^{b}	13.5 ± 0.3^{e}
MTs (ng/mg protein)	5.0 ± 0.2^{a}	5.5 ± 0.2^{a}	6.1 ± 0.2^{a}	3.9 ± 0.1^{b}	4.4 ± 0.2^{a}	7.3 ± 0.1^{c}
CRP (ng/mg protein)	2.8 ± 0.1^{a}	4.4 ± 0.1^{b}	5.6 ± 0.1^{c}	2.3 ± 0.2^{a}	4.8 ± 0.1^{b}	6.7 ± 0.1^{c}
[NOx] (nmoles/mg protein)	1.5 ± 0.1^{a}	2.9 ± 0.1^{b}	4.7 ± 0.2^{c}	0.8 ± 0.1^{b}	2.7 ± 0.1^{b}	4.6 ± 0.1^{c}
TBARS (nmoles/mg protein)	2.8 ± 0.1^{a}	4.6 ± 0.3^{b}	6.1 ± 0.2^{c}	1.1 ± 0.1^{d}	3.2 ± 0.1^{a}	6.7 ± 0.2^{c}
PCs (nmoles/mg protein)	3.4 ± 0.1^{a}	6.3 ± 0.2^{b}	8.1 ± 0.2^{c}	2.2 ± 0.2^{d}	4.5 ± 0.03^{e}	8.8 ± 0.2^{c}

ANOVA, analysis of variance; Cu, copper; CRP, ceruloplasmin; IP, intraperitoneal; MT, metallothionein; [NOx], nitrate and nitrite; PC, protein carbonyls; TBARS, thiobarbituric acid-reactive substances

Results (obtained day of sacrifice) were the mean of six independent rats analyzed in triplicate ± SD. They were expressed in the units detailed in each case. Determinations were performed according to the methodologies described in the Materials and method section. Statistical differences between results of the same raw were indicated with distinct superscript letters (ANOVA + Tukey test; $P < 0.01$). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily IP injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group

Table 3
Antioxidants in brain from rats fed the experimental diets

Tissue	Diets					
	C	CuO	CuI	D	D-I7	D-I35
GSH + GSSG	3.8 ± 0.2[a]	4.5 ± 0.2[b]	6.2 ± 0.2[c]	6.0 ± 0.3[c]	6.2 ± 0.3[c]	6.2 ± 0.2[c]
GSH	3.0 ± 0.2[a]	3.3 ± 0.1[a]	4.1 ± 0.1[b]	5.5 ± 0.3[c]	4.0 ± 0.1[b]	3.8 ± 0.1[b]
GSSG	0.8 ± 0.1[a]	1.2 ± 0.1[b]	2.1 ± 0.2[c]	0.5 ± 0.2[a]	2.2 ± 0.1[c]	2.4 ± 0.1[c]
R: GSH/GSSG	3.4 ± 0.1[a]	2.8 ± 0.1[b]	1.9 ± 0.1[c]	11.1 ± 3.2[d]	1.8 ± 0.1[c]	1.6 ± 0.1[c]
α-Toc (pmoles/mg protein)	490 ± 11[a]	342 ± 8[b]	329 ± 14[c]	485 ± 12[a]	350 ± 10[b]	288 ± 10[d]
FRAP (μmoles/mg protein)	325 ± 10[a]	295 ± 7[b]	269 ± 9[c]	315 ± 10[a]	278 ± 14[b]	244 ± 11[d]

α-Toc, α-tocopherol; Cu, copper; FRAP, ferric reducing ability of plasma; GSH, glutathione; GSSG, oxidized glutathione; IP, intraperitoneal
Results (mean of six animals assayed in triplicate ± SD) for GSH +GSSG and GSH were expressed as μmoles/mg protein. Determinations were performed according to the procedure described in section 2.8. Results statistically significantly different ($P < 0.01$) between them are indicated with distinct superscript letters. C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily IP injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group

Cu in feces (Fig. 2) increased along the experimental period in all groups with respect to C with the exception of group D, in which it declined progressively up to the end of the treatment.

Table 2 shows that plasma CRP and MTs were both higher with Cu overload. In contrast, the D diet produced a decrease in Cu and CRP plasma levels without any change in MTs. There was a concomitant and sharp increase in the concentration of CRP and MTs in the plasma and brain of group D rats IP-supplemented with Cu (D-I7 and D-I35) with increased Cu concentration. Similar behavior was observed for the OS biomarkers: higher TBARS, PCs, and [NOx] associated with increased levels of Cu in brain and lower for the D treatment but with increases after the IP administration of Cu.

Table 3 shows the concentration of the two main antioxidant molecules in brain, α-tocopherol and glutathione (GSH, GSSG). α-Tocopherol levels decreased after Cu supplementation but total glutathione (GSH + GSSG) levels rose after all treatments. When GSH and GSSG were analyzed separately, the concentration of both was observed to increase. The increment in the latter being more pronounced than in the former caused a decrease in the GSH/GSSG ratio. On the contrary, in the D group the level of GSH increased more than that of GSSG, causing an increase in the GSH/GSSG ratio. Furthermore, Table 3 shows a decrease in FRAP values that can be indirectly correlated to the increase in Cu. The consumption of the main antioxidant molecule for the lipid compartment of the cell (α-tocopherol) followed a similar behavior to that observed for TBARS, a biomarker of lipid peroxidation (Table 2) and paralleled the changes observed for the FRAP assay in brain homogenates, indicating the total antioxidant potential.

Table 4 shows the activity of different enzymes of the antioxidant defense system. The higher concentration of brain GSSG is in agreement with the observed increment in the activity of GR. Other enzymes of the antioxidant defense system such as GPx, SOD, and CAT were also altered by Cu overload (Table 4), their activities being higher in the brain after Cu overload, especially in those groups receiving IP administration of Cu. Cu deficiency (D) decreased GPx and SOD activities, whereas CAT and GR did not change significantly with respect to the C diet. GST activity remained unaltered under all treatments.

Figure 3 shows the results obtained after analyzing the main lipid classes in the brain after treatments. A lower level of phospholipids (PL; grey bars) was observed after Cu supplementation, more pronounced for the CuI and D-I35 diets. No significant changes were observed for the neutral lipids (NL, dark grey bars) after Cu addition. However, D diets produced a significant increase in NL content. The cholesterol (Cho, dark bars) level increased with IP-administered Cu (CuI and D-I35). We also evaluated the possible differential behaviors of these main groups of lipids in two key brain regions: cortex and hippocampus (Tables 5 and 6, respectively). NL showed no changes after Cu supplementation and increased when Cu was deficient. PL decreased and Cho increased particularly in CuI and D-I35. The Cho to PL ratio therefore increased significantly after treatment with all diets (CuO, CuI, and D-I35). Analyzing the esterification percentage of Cho we observed significant increments in all Cu supplementary diets. The level of total lipids was not modified in the cortex whereas in the hippocampus it increased significantly after CuO, CuI, and D-I35 treatments. Total lipids to PL and total lipids to Cho ratios increased as a consequence of Cu

Table 4
Activities of the main antioxidant defense enzymes assayed in brain homogenates from rats fed the experimental diets

Parameters	Diets					
	C	CuO	CuI	D	D-I7	D-I35
GPx (μmoles NADPH/mg protein min)	1.7 ± 0.1[a]	2.5 ± 0.2[b]	4.3 ± 0.2[c]	0.9 ± 0.03[d]	2.9 ± 0.1[b]	4.7 ± 0.2[c]
GR (nmol/mg protein min.)	66.6 ± 2.0[a]	122.5 ± 2.3[b]	170.0 ± 2.4[c]	70.2 ± 4.2[a]	133.7 ± 3.1[b]	166.5 ± 2.2[c]
GST (μmoles/mg protein min)	1.1 ± 0.1[a]	1.1 ± 0.3[a]	0.8 ± 0.2[a]	0.8 ± 0.1[a]	1.0 ± 0.1[a]	1.1 ± 0.3[a]
SOD (U/mg protein)	2.8 ± 0.2[a]	5.5 ± 0.1[b]	6.8 ± 0.1[c]	1.1 ± 0.1[d]	4.8 ± 0.1[b]	5.6 ± 0.2[c]
CAT ($k[min^{-1}]$/mg protein)	7.2 ± 0.3[a]	9.5 ± 0.1[b]	11.6 ± 0.2[c]	6.3 ± 0.2[a]	9.2 ± 0.2[b]	12.5 ± 0.4[c]

ANOVA, analysis of variance; CAT, catalase; Cu, copper; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione transferase; IP, intraperitoneal; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; SOD, superoxide dismutase
Results were expressed as the mean of six rats assayed in triplicate ± SD. Units were indicated between parentheses for each parameter. Different superscript letters correspond to data significantly different at $P < 0.01$ (ANOVA + Tukey test). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily IP injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group

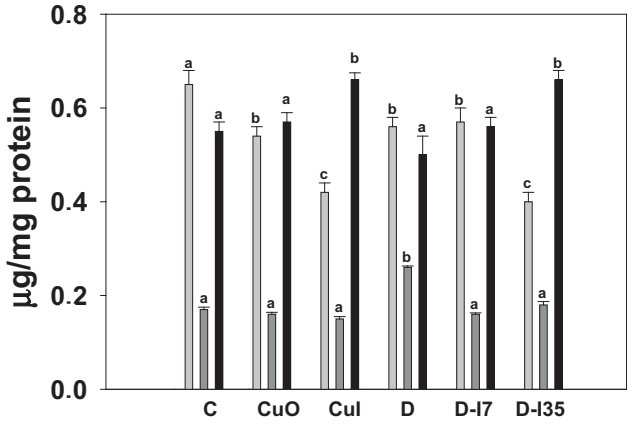

Fig. 3. Major lipid subclasses analyzed in whole brain tissue homogenates from rats fed the different diets. Results are expressed as mean ± SD (six animals assayed in triplicate). Lipids were isolated by Folch extraction and separated by means of TLC, eluted and quantified enzymatically as described in section 2.9. Groups of bars represent the content of total phospholipids (grey), neutral lipids (dark grey), and cholesterol (black), respectively. Statistically different results (ANOVA + Tukey test; $P < 0.01$) between the dietary groups are indicated by different superscript letters. C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

overload in both brain regions. Figure 4 shows the results of the fatty acid composition of the groups according to the brain region analyzed (Fig. 4 A, B, respectively). The behavior of the two areas was similar after Cu treatment: a decrease in 16:1, 18:1, and 18:2n-6—more evident in the case of IP Cu supplementation—and a generalized decline in polyunsaturated fatty acid (PUFA) content.

Figure 5 shows the activity of caspase-3 and Figure 6 of the isoforms of m-calpain and μ-calpain in rat brains obtained after each experimental treatment. Effector caspase-3 was significantly higher in brains from rats fed on CuO or D-I7 diets (Fig. 5), whereas m-calpain and μ-calpain activity was higher in all other cases (Fig. 6).

Discussion

Our results demonstrate that increases in plasma Cu are proportional to the dose administered and strongly influenced by the route of entry (the IP route is less regulated than the oral route). Oral supplementation led to a decrease in Cu levels after 20 d of treatment that could be attributed to the adaptation/induction of the endogenous system(s) involved in Cu homeostasis [52,53]. Although the D diet group showed a significant decrease in the level of plasma Cu with respect to the control, the level tended to normalize toward the end of the experimental period. These facts are in agreement with the observed pattern in feces: Cu content was higher in rats supplemented orally or by IP injection and lower in those animals receiving a D diet. These compensation mechanisms appear to strongly depend on the route of administration and reinforce the idea that the intestine plays a key role during Cu overload, as demonstrated in previous studies [54–57]. The increase in Cu ions in plasma is reflected in the concentration of this metal in brain. Cu enters the brain probably through the choroid plexus [58]. The subsequent

Table 5
Relationships within lipids in homogenates from brain cortex of rats fed the different diets

Parameters	Diets					
	C	CuO	CuI	D	D-I7	D-I35
Phospholipids/protein (μg/mg)	0.66 ± 0.05^a	0.59 ± 0.05^a	0.41 ± 0.03^b	0.57 ± 0.03^a	0.58 ± 0.10^a	0.39 ± 0.05^b
Neutral lipids/protein (μg/mg)	0.18 ± 0.03^a	0.16 ± 0.05^a	0.17 ± 0.07^a	0.29 ± 0.04^b	0.19 ± 0.04^a	0.20 ± 0.04^a
Cholesterol/protein (μmol/mg)	0.57 ± 0.05^a	0.60 ± 0.07^a	0.68 ± 0.03^b	0.54 ± 0.06^a	0.59 ± 0.03^a	0.71 ± 0.05^b
Cholesterol ester*/protein (μmol/mg)	0.01 ± 0.002^a	0.03 ± 0.004^b	0.04 ± 0.001^c	0.01 ± 0.001^a	0.02 ± 0.002^a	0.03 ± 0.002^c
Total lipids/protein (mg/mg)	1.43 ± 0.07^a	1.37 ± 0.05^a	1.31 ± 0.08^a	1.45 ± 0.05^a	1.35 ± 0.04^a	1.34 ± 0.04^a
Cholesterol/phospholipids (μmol/μmol)	0.86 ± 0.03^a	1.02 ± 0.02^b	1.65 ± 0.02^b	0.95 ± 0.04^b	1.02 ± 0.05^b	1.82 ± 0.06^c
Cholesterol/cholesterol ester (% esterification)	57.0 ± 2.8^a (1.7 ± 0.03)^a	20.0 ± 1.1^b (5.0 ± 0.08)^b	17.0 ± 0.4^c (5.9 ± 0.23)^c	54.0 ± 3.1^a (1.8 ± 0.06)^a	29.5 ± 2.4^b (3.4 ± 0.11)^c	23.7 ± 1.2^b (4.2 ± 0.12)^b
Total lipids/phospholipids (mg/μmol	2.16 ± 0.04^a	2.32 ± 0.07^b	3.20 ± 0.07^c	2.54 ± 0.05^b	2.33 ± 0.04^b	3.44 ± 0.05^c
Total lipids/cholesterol (mg/μmol)	2.51± 0.06^a	2.28 ± 0.04^b	1.92 ± 0.02^c	2.69 ± 0.07^c	2.29 ± 0.03^b	1.88 ± 0.05^c
Neutral lipids/phospholipids (μg/μg)	0.27 ± 0.03^a	0.27 ± 0.06^a	0.41 ± 0.08^b	0.51 ± 0.06^c	0.32 ± 0.04^a	0.51 ± 0.05^c

ANOVA, analysis of variance; Cu, copper; IP, intraperitoneal
Results were expressed as the mean ± 1 SD of six animals assayed in triplicate. For details Materials and methods section. Results along a row with different superscript letters are significantly different between them (ANOVA + Tukey test: $P < 0.01$). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily IP injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group
* Calculated as oleoyl-cholesterol.

Table 6
Relationships within lipids in homogenates from brain hippocampus of rats fed the different diets

Parameters	Diets					
	C	CuO	CuI	D	D-I7	D-I35
Phospholipids/protein (μg/mg)	0.51 ± 0.04^{a}	0.46 ± 0.05^{a}	0.40 ± 0.02^{b}	0.50 ± 0.05^{a}	0.53 ± 0.06^{a}	0.42 ± 0.04^{b}
Neutral lipids/protein (μg/mg)	0.14 ± 0.02^{a}	0.17 ± 0.04^{a}	0.17 ± 0.08^{a}	0.21 ± 0.04^{b}	0.15 ± 0.05^{a}	0.18 ± 0.06^{a}
Cholesterol/protein (μmol/mg)	0.48 ± 0.03^{a}	0.51 ± 0.04^{a}	0.58 ± 0.03^{b}	0.45 ± 0.05^{a}	0.47 ± 0.06^{a}	0.59 ± 0.04^{b}
Cholesterol ester*/protein (μmol/mg)	0.01 ± 0.002^{a}	0.02 ± 0.004^{b}	0.03 ± 0.001^{c}	Traces	0.01 ± 0.001^{a}	0.03 ± 0.003^{c}
Total lipids/protein (mg/mg)	1.15 ± 0.04^{a}	1.21 ± 0.04^{b}	1.23 ± 0.05^{b}	1.16 ± 0.08^{a}	1.14 ± 0.05^{a}	1.21 ± 0.03^{b}
Cholesterol/phospholipids (μmol/μmol)	0.94 ± 0.02^{a}	1.11 ± 0.02^{b}	1.45 ± 0.03^{b}	0.90 ± 0.02^{a}	0.89 ± 0.04^{a}	1.40 ± 0.05^{b}
Cholesterol/cholesterol ester (% esterification)	48.0 ± 2.6^{a} $(2.1 \pm 0.05)^{a}$	25.5 ± 1.8^{b} $(3.9 \pm 0.11)^{b}$	19.3 ± 0.6^{c} $(5.2 \pm 0.12)^{c}$	nc	47.0 ± 2.2^{a} $(2.1 \pm 0.07)^{a}$	19.7 ± 0.9^{c} $(5.1 \pm 0.09)^{c}$
Total lipids/phospholipids (mg/μmol)	2.22 ± 0.05^{a}	2.63 ± 0.04^{b}	3.08 ± 0.10^{c}	2.32 ± 0.06^{a}	2.15 ± 0.05^{a}	2.88 ± 0.07^{b}
Total lipids/cholesterol (mg/μmol)	2.39 ± 0.11^{a}	2.37 ± 0.04^{a}	2.12 ± 0.03^{b}	2.32 ± 0.03^{a}	2.42 ± 0.05^{a}	2.05 ± 0.07^{b}
Neutral lipids/phospholipids (μg/μg)	0.28 ± 0.04^{a}	0.37 ± 0.03^{b}	0.43 ± 0.05^{c}	0.42 ± 0.04^{c}	0.28 ± 0.01^{a}	0.43 ± 0.02^{c}

ANOVA, analysis of variance; Cu, copper; IP, intraperitoneal
Results were expressed as the mean ± 1 SD of six animals assayed in duplicate. For details see Materials and methods section. Results along a row with different superscript letters are significantly different between them (ANOVA + Tukey test; $P < 0.01$). "Trace" amounts below detection limits; nc, non-calculable. C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily IP injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group
* Calculated as oleoyl-cholesterol.

increases in CRP and MTs can be attributed to the elevated Cu levels because it is known that the synthesis of MTs [2,53,59–61] and CRP [2,53,62–65] are induced by Cu overload. We also observed the development of a prooxidative condition with increasing oxidative/nitrative stress biomarkers like TBARS, PCs, and [NOx]. These findings are in agreement with previous studies in which rats were treated with different triggers of OS [66–68]. However, other groups have reported lower SOD, CAT, and GPx activity after OS [69]. This discrepancy could be attributed to the different design of the experimental models in terms of factors such as the causative agent that generates the OS, dose and route of administration, and the period of exposure, thus highlighting the paramount importance of these variables in clarifying the mechanism(s) of Cu-induced damage to the central nervous system (CNS).

The Cu-induced stimulation observed for the biosynthesis of glutathione under excessive reactive oxygen species (ROS) production is in agreement with the results of other authors, specifically in brain [70]. The increment in the total amount (GSH + GSSG) of glutathione also has been attributed to adaptation to exacerbated OS and the subsequent response of the main regulatory enzyme of the glutathione biosynthetic pathway, the γ-glutamyl-cysteinyl synthetase [70,71]. The marked alteration in brain glutathione metabolism under Cu overload may have important connotations due to its involvement in the etiopathogenesis of neurodegenerative disorders hallmarked by a generalized OS condition [72].

Furthermore, the level of OS explains the consumption of α-tocopherol, with important consequences in terms of lipid peroxidation/fatty acid damage. The results of the FRAP assay also correlated with a general depletion of water-soluble and lipid-soluble antioxidants. Because the samples had been treated before the assay with an excess of uricase, this depletion actually reflects the endogenous antioxidant pool content in the CNS [47]. As a direct consequence of the failure of the antioxidant system, we observed a significant decrease in PUFA content in line with TBARS levels. The alteration of complex lipids and PUFAs is also important because of their major role in maintaining the integrity and physiology of neuronal and glial biomembranes [73]. Additionally, the modification of the fatty acyl pattern might have crucial consequences for the precise equilibrium that characterizes the formation of eicosanoids derived from the PUFAs of the ω-6 (proinflammatory) and ω-3 (antiinflammatory and neuroprotective) essential series [74]. Moreover, many neurologic diseases are considered inflammatory pathologies involving glial activation [21,74]. Of even greater importance is the loss under Cu overload of docosahexenoic acid, which plays a crucial and unique role as metabolic precursor of the neuroprotectine D1, directly involved in neuroprotection and cognitive performance [75].

In agreement with our findings, other groups have reported increased cholesterol biosynthesis in neurons under OS [76,77]. Our results show that excess Cu in brain tissues increases the proportion of Cho at the expense of cholesteryl esters. This significant increase affects the total lipid to Cho ratio and also raises the question as to the kind of fatty acyl chains that are acylated to this "new" pool of cholesteryl esters. It is known that cholesteryl esters are stored in cytoplasm lipid droplets and are involved in neurologic illnesses [78]. Alteration of the lipid annulus in rafts is expected to modify multiple parameters involved in central nervous physiology such as the metabolism of neurotransmitters, receptors for neuroprotective factors and neurotransmitters molecules, ion channels, proteins, and enzymes involved in transduction signal pathways, and others

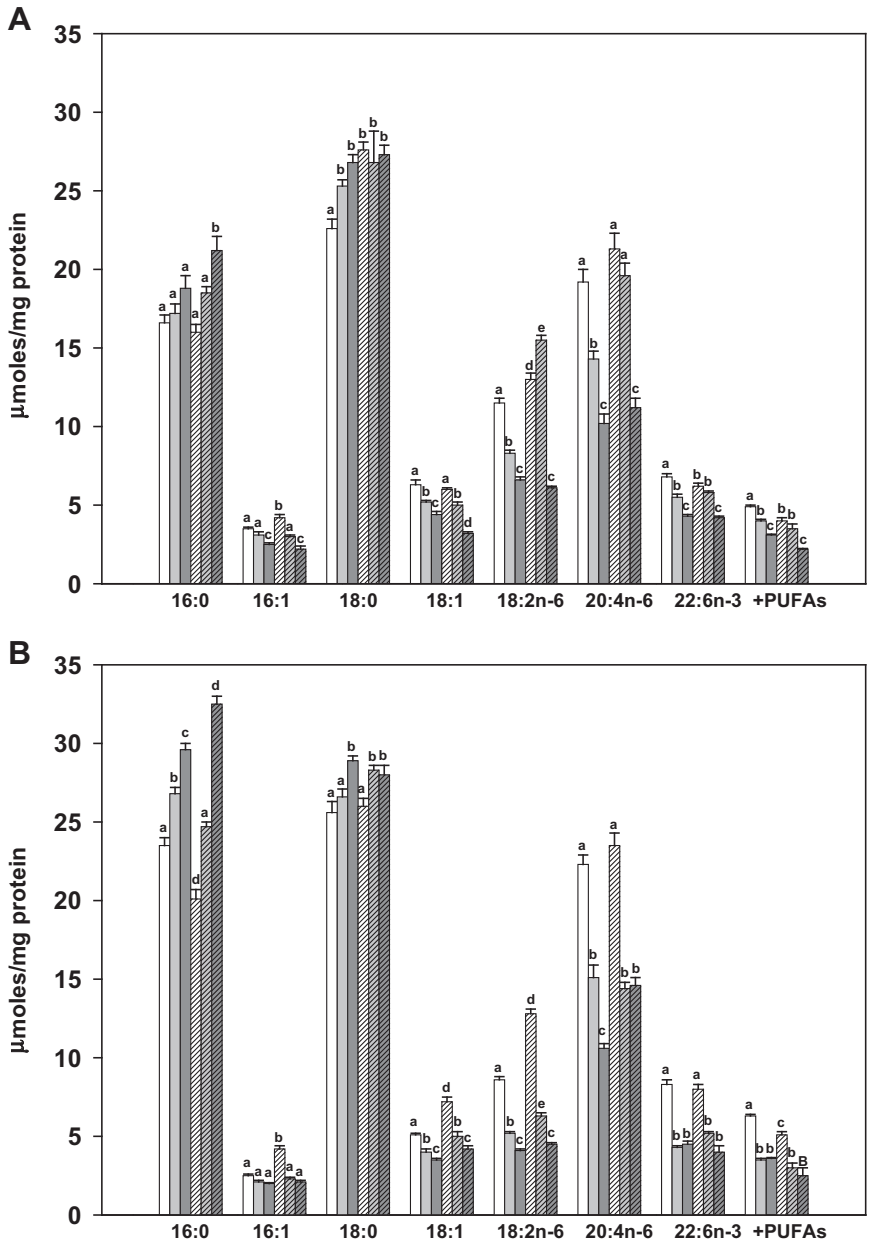

Fig. 4. Major fatty acids in total lipids from brain cortex (A) and hippocampus (B) of rats fed the experimental diets. Results are expressed as µmoles/mg protein (mean ± SD of six independent rats analyzed in duplicate). Analyses were performed as indicated in the Material and methods section using quantitative capillary gas-liquid chromatography (c-GLC) with 21:0 as internal standard. "+PUFAs" indicates the sum of other polyunsaturated fatty acids not included in the histograms. Diets are indicated by different colors and patterns. Without pattern: white bars, C; grey, CuO; dark grey, CuI. With diagonal lines: white bars, D; grey, D-I7; and dark grey, D-I35. Results showing statistical differences within a group are indicated by different superscript letters (ANOVA + Tukey test; $P < 0.01$). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

[74,75]. The responsibility of the aforementioned modifications in the etiology of neurodegenerative process has been widely discussed and it is clear that the maintenance of lipid homeostasis is a crucial factor for normal neuronal function [72–79].

We also investigated the biological activities of the two main protease systems directly linked to the programmed cell death pathway, both of which are directly influenced by ROS overproduction, especially by Cu overload-induced ROS [80]. Previous studies [68,81] reported the importance of Cu dyshomeostasis in proapoptotic events. In this study, we observed a differential response in the activities of the two proteases (caspase-3 and calpains) under the same stimulus, confirming previous experimental data demonstrating a complex interrelationship between the two systems [82,83]. In fact, activation of one of these proteases may lead to inactivation of the other, in other words they function as interdependent and exclusive effectors able to replace one another during the programmed cell death cascade [84]. Additionally, neuronal apoptosis could be aggravated by the Cu overload resulting not only from the exogenously administered Cu but also from the "local" increase in this metal brought about by the loss of neurons [85].

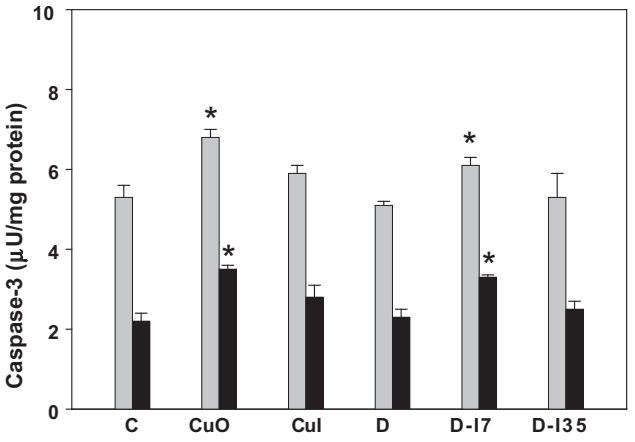

Fig. 5. Caspase-3 activity in whole rat brain homogenates prepared from animals fed on the experimental diets. Results are the mean of six rats assayed in triplicate ± SD (*$P < 0.01$ compared with C). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

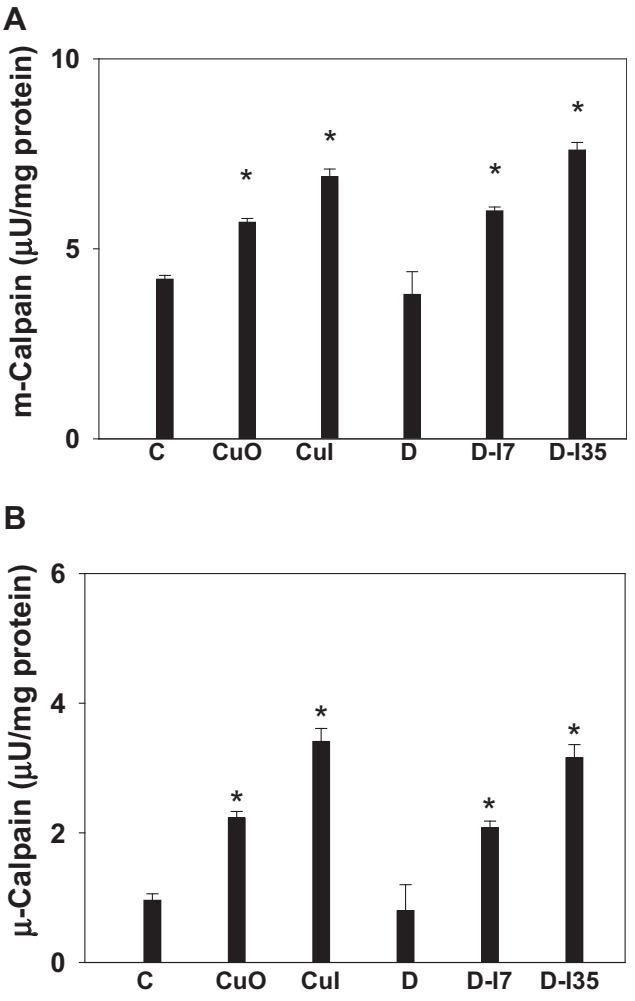

Fig. 6. Milli- (A) and micro- (B) calpaine activities in whole rat brain homogenates prepared from animals fed on the experimental diets. Results are the mean of six rats assayed in triplicate ± SD (*$P < 0.01$ compared to C). C, control rats fed orally on standard pelleted diet containing 7 ppm of Cu; CuO, rats fed orally on a moderate Cu overload (35 ppm); CuI, rats fed orally on 7 ppm plus daily intraperitoneal (IP) injections of 35 ppm of Cu (rats received 7 ppm orally + 35 ppm IP); D, rats fed on a Cu-deficient diet (containing trace amounts of the metal); D-I7, rats fed on a Cu-deficient diet plus daily injections of equivalent Cu solutions to those orally administered to the C group; D-I35, rats fed on D diet plus daily IP injections of five times the amount of Cu solution orally administered to the C group.

The alterations observed as a consequence of Cu overload are more pronounced in IP treatments than in orally treated animals. Oral Cu supplementation must pass through the intestine, which acts as a filter, lowering the levels of Cu entering into the plasma. However, when Cu is administered IP, this regulatory barrier is obviously lost. D-I7 showed only a minor detrimental effect probably due to the fact that IP administration merely compensates for the lack of Cu in the oral diet. However, the detrimental effects of overload become evident when an additional dose of CuI plus D-I35 was administered, an important observation taking into account the findings of other groups concerning the effects of Cu supplementation in hospitalized patients receiving parenteral nutrition [14]. The acquisition of Cu also can be observed in women using Cu-IUDs [7,10] or people exposed to Cu-based pesticides [8]. We also assume that the population at large may accumulate excess Cu through pesticide residues in food and contaminated tap water [7–9,87]. The present study could be a contribution to the awareness of the consequence of Cu overload either by Cu pollution or parenteral nutrition [6–10,14,86–88].

This study, however, has some limitations due to the fact that we do not know exactly if the experimental conditions simulated in our rats could be extrapolated to those associated with real human situations. At first, the deficient conditions settled in our design are rarely observed in human populations because this is an extreme condition of almost total depletion of oral Cu supply. Also, the results strongly suggest that Cu overload by IP route is more dangerous than oral acquisition of the metal. However, a large longitudinal study in people exposed to different degrees of Cu content in tap water, for example, could be an interesting way for the ratification or rectification of our assumption. Another important limitation is related to the chemical structure of the compound we use to simulate the Cu overload ($CuCO_3$). Very probably, humans are exposed to several types of Cu-based compounds of different chemical structures with differences in their physical stabilities, solubility, absorption capacities, life time into the organism, and many other particularities related to their excretion or bioaccumulation rates. Thus, there are a lot of question to be answered before drawing a realistic conclusion about this matter with an undoubtedly nutritional and clinical interest. Unfortunately there is little data available on Cu overload and potential excess exposure in humans as most of the available experimental and epidemiological data derive from animal models. However, the regulatory framework for chronic Cu exposure in large populations indicates that food, drinking water, Cu-containing supplements, and parenteral nutrition are the main sources of human exposure [13,14,21]. The dietary reference intake of this metal in the United States, United Kingdom, other countries in Europe, and in Australia varies from 0.16 to 0.98 Estimated Average Requirements/Recommended Dietary Allowance expressed in mg Cu/kg body weight, varying greatly as a function of age. The Population Reference Intakes for these countries was reported to be between 0.3 and 1.5 mg Cu/kg body weight [21]; however, as described here, under certain circumstances these limits can be significantly exceeded.

Conclusions

Cu overload induced a prooxidative condition in brain, increasing cholesteryl esters, modifying the fatty acyl pattern of complex lipids and causing alterations in proteins. These changes are related to the activation of neuronal apoptosis that may have important implications in Cu-associated brain diseases, thus calling for more in-depth studies in this area.

Acknowledgments

This study was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CCT-CONICET) PIP #0697 and UNLP/M-145. We acknowledge Norma Cristalli, Cristina Pallanza, and Eva Illara de Bozzolo for their excellent technical assistance.

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