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Research Article

Prolonged copper depletion induces expression of antioxidants and triggers apoptosis in SH-SY5Y neuroblastoma cells

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Abstract. SH-SY5Y neuroblastoma cells were cultured for up to three serial passages in the presence of the copper chelator triethylene tetramine (Trien). The copper-depleted neuroblastoma cell line obtained showed decreased activities of the copper enzymes Cu, Zn superoxide dismutase and cytochrome c oxidase with concomitant increases in reactive oxygen species. Mitochondrial antioxidants (Mn superoxide dismutase and Bcl-2) were up-regulated. Overexpression and activation of p53 were early responses, leading to an increase in p21. Eventually, copper-depleted cells detached from the monolayer and underwent apoptosis. Activation of up-stream caspase-9, but not caspase-8, suggested that apoptosis proceeds via a mitochondrial pathway, followed by caspase-3 activation. The addition of copper sulfate to the copper-depleted cells restored copper enzymes, normalized antioxidant levels and improved cell viability. We conclude that prolonged copper starvation in these replicating cells leads to mitochondrial damage and oxidative stress and ultimately, apoptosis.

Key words. Copper depletion; apoptosis; neurodegeneration; oxidative stress; neuroblastoma; mitochondria; antioxidant.

Copper is a trace transition metal, essential for the development and function of the central nervous system. In the genetic disturbance of intestinal copper absorption known as Menkes' disease, and in its animal models, the lack of this metal during brain growth results in lethal neurodegeneration [1–3]. Moreover, copper deficiency induced experimentally in the mouse brain during perinatal life leads to impaired brain development and degeneration of neuronal cells [4]. In addition, a copper imbalance and/or its uncontrolled redox activity are increasingly reported to be associated with many neurodegenerative diseases, such as Alzheimer's disease, prion diseases and familial amyotrophic lateral sclerosis [5]. However, the molecular mediators sensing a copper imbalance and triggering the death of neuronal cells have not yet been identified.

Low copper levels in cells are responsible for impaired activities of copper-dependent enzymes, required in crucial metabolic processes [6]. In particular, the impairment of cytochrome c oxidase (Cytox) and Cu, Zn superoxide dismutase (Cu,Zn-SOD) may lead to the production of reactive oxygen species (ROS) and induce oxidative damage to cells. Cytox is the complex IV of the mitochondrial electron transport chain and reduces oxygen to water during cell respiration; its failure may lead to the production of partially reduced oxygen species [7]. Cu,Zn-SOD is an enzyme fundamental for defense against ROS by efficiently intercepting and destroying superoxide, the one-electron reduction product of oxygen [8]. Brain cells are particularly susceptible to oxidative

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insult because their antioxidant levels are low while they contain, simultaneously, high levels of substrates susceptible to oxidation [5].

Neurodegeneration often occurs via apoptosis [9]. This process follows the pathways of programmed cell death, which is an active form of cell self-destruction in metazoa. Apoptosis is important in the physiological control of cell number in developing organs and in eliminating damaged cells, without necrosis or an inflammatory response [10], but, when inappropriate, it may have pathological consequences. Apoptosis has been observed in the neocortex and the hippocampus of the Mottled/Brindled mouse and in the cerebrum of the macular mutant mouse, two models of Menkes' disease [2, 3]. Copper depletion might exert its pro-apoptotic action via oxidative stress, one of the most common stimuli triggering cell death. A low degree of oxidative stress over a protracted time induces apoptosis, while higher, acute levels induce necrosis [11].

To identify the molecular mechanisms underlying apoptotic neurodegeneration triggered by copper deficiency, in a previous study we set up an experimental model comprising SH-SY5Y neuroblastoma cells grown for a short period of time (3 days) in a culture medium supplemented with a specific copper chelator, triethylene tetramine (Trien) [12]. SH-SY5Y is a human catecholaminergic neuroblastoma cell line which has been proposed as a suitable in vitro model of human dopaminergic neurons [13]. It has been used to investigate the mechanisms of neuronal death triggered by diverse, experimentally applied, death stimuli. Trien is used in the therapy of copper overload in humans, and to control copper levels and reactivity in experimental models [14, 15]. Trien treatment was successful in sequestrating copper outside SH-SY5Y cells, thus depleting the intracellular copper content and selectively affecting copper-dependent enzyme activities [12], but, per se, did not cause cell death. However, some molecular factors involved in the apoptotic process were alerted, namely p53 and caspase-3, which are modulated by redox signals. This led us to hypothesize that these conditions of copper deficiency were not sufficient to generate an oxidative stress strong enough to prompt cells to undergo apoptosis.

To investigate this hypothesis, in the present study we challenged SH-SY5Y cells with Trien for a longer period of time, through serial passages in culture, thus obtaining a line of cells depleted in copper. In these cells, mitochondrial molecular factors, which respond to oxidative stress, were strongly up-regulated and cells underwent apoptosis via a mitochondrial-triggered pathway, through the activation of caspase-9, caspase-3 and p53. We therefore conclude that prolonged starvation of copper in these replicating cells elicits mitochondrial damage and oxidative stress, which in turn commit the cells to apoptosis. These results, obtained in an in vitro system, may help in understanding the mechanisms underlying neurodegeneration in the developing brain due to a copper imbalance.

Materials and methods

Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were purchased from the European Collection of Cell Culture (Salisbury, UK) and grown in Dulbecco's modified Eagle's/F12 Medium (Sigma, St. Louis, Mo.) supplemented with 15% fetal calf serum, at 37°C in an atmosphere of 5% CO_2 in air. Cells were routinely trypsinized and plated at a density of 3×10^6 living cells/75-cm^2 plate.

Treatment with the copper chelator was performed as follows. Cells were incubated the day after plating with triethylene tetramine tetrahydrochloride (Trien; Sigma) at a final concentration of 0.125 mM. The concentration of Trien was chosen on the basis of the results obtained in our previous study [12], showing that this concentration is the lowest producing the maximum effect on copper levels and copper enzyme activities in this cell line. Trien was maintained for 3 days, at which time cells reached confluence (end of the first passage in culture). Then the cells were trypsinized and plated again with the same concentration of Trien every 4 days, the time required for control cells to reach confluence, for additional two passages (second and third passages). Trien-treated cells were always handled with solutions supplemented with the chelator, to avoid any exposure to free copper. By the end of the second passage and the third passage, Trien-treated cells partially detached from the monolayer and floated in the medium. Attached cells were detached by trypsin treatment and pooled with detached cells before performing all biochemical analyses. In some experiments, at the third passage in culture, $CuSO_4$ (0.300 mM) was added to the culture medium the day after replating, together with Trien, and maintained until the end of the third passage.

Cell number and viability estimation

Cells in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) were counted in a hemocytometric chamber under a phase-contrast optical microscope. Cell viability of attached and detached cells was evaluated by their impermeability to Trypan Blue (0.2%).

Flow cytometric analysis of cell viability

Cells were treated with a solution of propidium iodide (50 µg/ml), 0.1% sodium citrate, 0.1% Triton X-100 in hypotonic PBS and analyzed by a FACScalibur instrument (Becton Dickinson, St. José, Calif.). Cells in the sub-G1 region were considered apoptotic.

Western blot analyses

Cells were lysed in Tris-HCl buffer (10 mM, pH 7.4) containing 150 mM NaCl, 1% Triton X-100, and 10% Protease Inhibitor Cocktail (Sigma). After centrifugation (23,000 g, 30 min), supernatants were collected and stored at –80 °C. The total protein content was estimated according to Lowry et al. [16]. Western blot analyses were performed by 12% SDS-polyacrylamide denaturing gel electrophoresis (SDS-PAGE) (except for Cu,Zn-SOD, 15%) and by overnight blotting on PVDF membrane (Millipore, Bedford, Mass.). Proteins were recognized with the following antibodies: Bcl-2 (monoclonal, clone Bcl-2-100; Sigma); manganese-dependent superoxide dismutase (Mn-SOD) (polyclonal; Upstate Biotechnology, Lake Placid, N. Y.); Cytox subunit II and the 39-kDa subunit of complex I (monoclonal; Molecular Probes, Eugene, Ore.); β-actin (monoclonal, clone AC40; Sigma); p21 and Cu,Zn-SOD (polyclonal; Santa Cruz Biotechnology, Santa Cruz, Calif.); p53 (monoclonal, clone BP-53-12; Sigma); caspase-8 and -9 (monoclonal; Upstate Biotechnology). The protein load is reported in the figure legends. Specific protein complexes were identified by the Super Signal Chemiluminescent Substrate (Pierce, Rockford, Ill.).

Enzyme activity assays

Cells in PBS were sonicated for 10 s. Total cell lysate was used for the Cytox assay, while 23,000 g supernatant was used for Cu,Zn-SOD assays.

The Cu,Zn-SOD activity assay was performed by a polarographic method, using a 466 Polarographic Analyzer (AMEL, Milan, Italy), as the cyanide-sensitive activity in tetraborate buffer at pH 9.6 [17]. Data are expressed as µg/mg protein, with reference to purified human erythrocyte Cu,Zn-SOD [8]. Alternatively, proteins were separated on native PAGE and Cu,Zn-SOD activity was visualized by the inhibition of gel staining through the conversion of nitroblue tetrazolium to formazane [18]. This method also allowed measurement of the activity of Mn-SOD, which, having a lower anodic mobility than Cu,Zn-SOD, appeared as a separate upper band.

Cytox activity was determined in fresh total lysates in 30 mM phosphate buffer pH 7.4 at 25 °C by following the oxidation of reduced cytochrome c (from horse heart; Sigma) as previously described [12], using a Lambda 9 Perkin Elmer spectrophotometer.

For caspase-3 activity measurements, cell were lysed in a buffer containing dithiothreitol and protease inhibitors [12], kept on ice for 30 min, sonicated and centrifuged at 10,000 g. Activity was assayed in the supernatants by following the cleavage of a fluorometric probe from the specific substrate Ac-DEVD-AFC (Biomol Research Laboratories, Plymouth Meeting, Pa.) in a Perkin Elmer LS-5 fluorimeter (excitation 400 nm; emission 505 nm). Data are expressed as arbitrary units of fluorescence/mg protein; the protein content was assayed by the Biuret method after precipitation by trichloroacetic acid. Bovine serum albumin (Sigma) was used as a standard.

Evaluation of apoptotic cells by Hoechst staining

Chromosomal condensation and DNA fragmentation were evaluated using the chromatin dye Hoechst 33342 (Molecular Probes). 10^5 cells were suspended in PBS, fixed with paraformaldehyde (4% final concentration) and then layered on a slide. After drying, slides were washed in PBS, and dipped in Hoechst solution (1 µg/ml in PBS) for 20 min. They were then stored in the dark at 4 °C until observed under a fluorescence microscope (Nikon-Eclipse TE 200; Nikon, Tokyo, Japan), connected to a Cool Snap video camera. Exposure time, gain and offset were maintained constant.

Flow cytometric analysis of ROS production

The production of ROS was estimated using dihydrorhodamine 123 (DHR). The non-fluorescent DHR can cross cell membranes and react with intracellular ROS to form the highly fluorescent rhodamine 123 [19, 20]. At the end of each passage in culture, cells were washed in the flask twice with serum-free medium and then incubated with 50 µM DHR (Molecular Probes) from a stock solution in dimethylsulfoxide. Culture plates were placed in the incubator for 10 min, and then washed twice with PBS, trypsinized, and then the effect of trypsin was blocked by the addition of PBS enriched with 0.5 mM Ca^{2+}/Mg^{2+}. Cell pellets were then suspended in PBS and analyzed by flow cytometry (excitation 488 nm, fluorescence detection 530 nm). Only fluorescence from viable cells was analyzed. To exclude dead cells, propidium iodide was added after DHR loading. Tert-butyl hydroperoxide (100 µM) served as a positive control.

Other assays

The copper content in cells was evaluated in total cell lysates by atomic absorption spectrometry using an AAnalyst 300 Perkin Elmer atomic absorption apparatus, equipped with a graphite furnace with platform (HGA 800) and an AS-72 autosampler. Total cell lysates were diluted (1 : 2, v/v) with 65% nitric acid and left at room temperature for at least 1 week prior to analysis, the time required for complete digestion of the sample [21]. The ATP content of cells was measured by a luminometric method using the ATP Bioluminescence Assay Kit CLS II (Roche, Indianapolis, Ind.). Cells were treated with hot Tris-HCl buffer pH 7.75 (1 : 4, v/v), boiled for 10 min and centrifuged; the supernatants were stored at –80 °C until assayed by the ATP-dependent oxidative decarboxylation of luciferin by luciferase, following the instructions of the manufacturer.

For measurements of reduced (GSH) and oxidized (GSSG) glutathione, samples were obtained after scrap-

ing attached cells in their culture medium. Cells were pelleted, resuspended in PBS and sonicated. Subsequent steps were performed according to Reed et al. [22]. Briefly, protein-free supernatants were obtained by acid treatment of the samples, followed by centrifugation; samples were then neutralized, immediately mixed with iodoacetic acid to bind thiol groups, thus avoiding oxidation of GSH, and then treated with 2,4-dinitrofluorobenzene. GSH and GSSG were separated and quantified by HPLC.

Data presentation

The results are presented as mean ± SD. Data were analyzed by Student's t test. A value of $p < 0.05$ was accepted as the level of significance. Western blots and cell cycle plots shown in the figures are representative of at least three analyses performed on samples from at least three separate experiments.

Results

As shown in figure 1 A, addition of the copper chelator Trien (0.125 mM) to the culture medium of SH-SY5Y neuroblastoma cells produced a 60% decrease in intracellular copper content within 3 days; no further decrease was obtained by prolonging the treatment up to three passages in culture. The effect of copper shortage on the activity of two key cuproenzymes was estimated. The activity of the cytosolic enzyme Cu,Zn-SOD (fig. 1 B) showed a decrease of 80% at the end of the first and the second passage with Trien, and a further significant decrease at the end of the third passage. The activity of Cu,Zn-SOD was assayed by two independent methods: polarographic analysis (data shown in fig. 1 B) and specific gel staining following separation on PAGE (see also fig. 10) gave superimposable results. Treatment with Trien also produced a time-dependent decrease in the activity of the mitochondrial enzyme Cytox (fig. 1 C) corresponding to 68% at the end of the first passage in culture; a further decrease was evident after the second passage, while the activity of this cuproenzyme was almost undetectable at the end of the third passage. Data reported in figure 2 A demonstrate that the lower activity of Cytox in Trien-treated SH-SY5Y neuroblastoma was accompanied by a decline in the content of its subunit II, measured by Western blot analysis. In contrast, no change in the 39-kDa subunit of the respiratory chain complex I was observed (fig. 2 A).

To evaluate whether the severe impairment of Cytox affects the energy charge of the cells, we measured the ATP content, by comparing the capability of cell extracts to catalyze the conversion of luciferin to its oxidized form. Figure 2 B shows that the ATP level in Trien-treated cells had begun to decrease at the second passage and was reduced to 40% at the third passage.

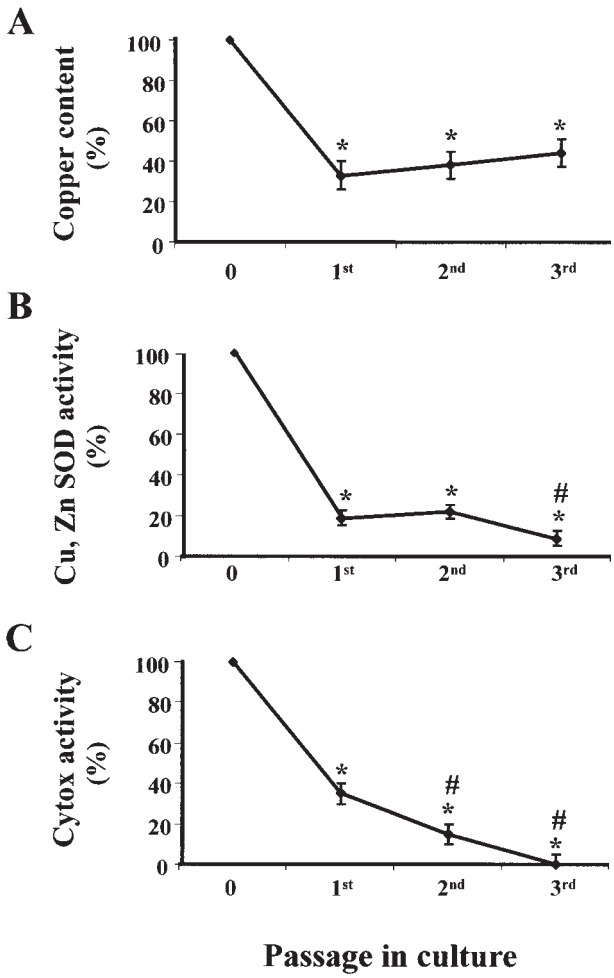

Figure 1. Trien treatment decreases SH-SY5Y copper content, Cu,Zn-SOD and Cytox activities. SH-SY5Y cells were treated with Trien for 3 days (first passage), 7 days (second passage) or 11 days (third passage). Results are expressed as percentage of control value. n = 4, *$0.01 < p < 0.001$ vs control; #$0.01 < p < 0.001$ vs previous passage. (*A*) Copper content of cells (control value: 10.2 ng/mg protein). (*B*) Cu,Zn-SOD activity measured by a polarographic method (control value: 1.4 µg/mg protein). (*C*) Cytox activity measured by a spectrophotometric method (control value: 32.1 mU/mg protein).

Cell counting at the end of each passage with Trien (fig. 3 A) showed that the number of total cells decreased during the treatment. Furthermore, at the end of the second passage, an increasing number of Trien-treated cells started to float in the medium. Cell detachment became more pronounced and significant at the end of the third passage. All the detached cells were dead (Trypan Blue permeable) (fig. 3 B), and they accounted for 30% of all cells. Furthermore, staining by Hoechst 33342 revealed that they had the nuclear morphology of apoptotic cells, showing condensed chromatin (fig. 3 C).

The number of apoptotic cells was estimated at the end of the third passage in culture with Trien by flow cytometric analysis after treatment with propidium iodide. Figure

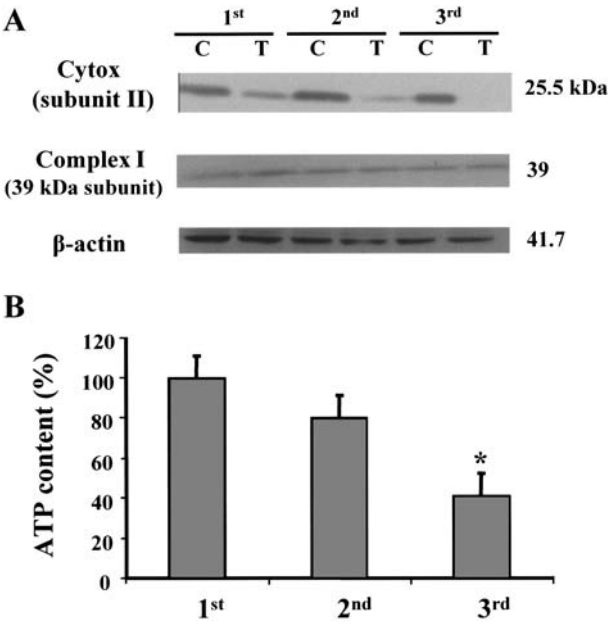

Figure 2. Trien treatment affects Cytox and ATP contents of SH-SY5Y cells. SH-SY5Y cells were treated with Trien for 3 days (first passage), 7 days (second passage) or 11 days (third passage). (*A*) Western blot analysis. Protein (50 µg) was applied to each lane. C, control cells; T, Trien-treated cells. β-Actin was used as a control of the loading. One blot is shown, representative of five separate experiments. (*B*) ATP content of cells. n = 3, $*0.01 < p < 0.001$. Values are expressed as percentage of control level (6.3 nmol/mg protein).

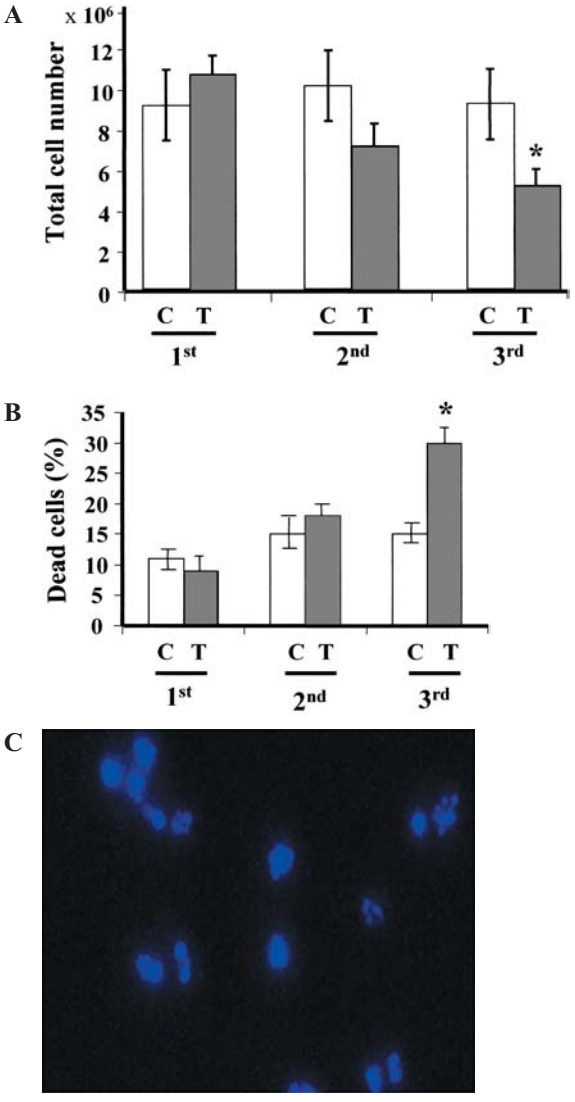

Figure 3. Prolonged Trien treatment produces growth inhibition of SH-SY5Y cells and cell death. Cells were treated with Trien for 3 days (first passage), 7 days (second passage) or 11 days (third passage). (*A*) Total cell count: (*B*) Percentage of Trypan-Blue-permeable cells. (*A, B*) n = 4, $*0.01 < p < 0.001$, versus untreated cells of the same passage: (*C*) Apoptotic nuclei in floating cells at the third passage.

4A shows a representative cell cycle plot of Trien-treated cells, where the sub-G1 region integrates the apoptotic cells. In repeated experiments we found approximately 40% apoptosis (fig. 4B).

Activation and stabilization of the transcription factor p53 plays a role in triggering apoptosis under several conditions [23, 24]. Therefore, we tested the activation of p53 upon Trien treatment of SH-SY5Y neuroblastoma cells. Figure 5 shows a Western blot analysis of p53 in cells treated for three passages with Trien. It reveals a pattern of two bands, the higher-molecular-weight component possibly representing its active, post-translationally modified form (arrow). Copper-depleted cells had an increased p53 content, with the upper band showing the largest increment. This phenomenon was already present at the end of the first passage (not shown), without significant further change up to the third passage. To indirectly evaluate DNA-binding capacity of p53, we measured the expression of the protein product of one of its target genes, p21. Western blot analysis of the p21 content of copper-depleted cells at the third passage is also shown in figure 5: p21 increased upon Trien treatment, following the same trend as p53.

Activation of caspases, a family of cysteine proteases, is considered to be the hallmark of programmed cell death in all cell types. Regardless of the death stimulus, their activation is believed to be the committing step in the cell suicide program [25]. To dissect the molecular mechanisms underlying cell death dependent on copper deficiency, we evaluated the activation of caspase-3, -8 and -9 in Trien-treated SH-SY5Y cells. Caspase-3 activity was tested by a fluorometric assay. As shown in figure 6A, caspase-3 activity increased progressively from the first passage in culture and had been elevated about eightfold by the end of the third passage. Figure 6B shows the analysis of the activation of upstream caspase-8 and -9. The Western blots were performed on the same sample for both caspases. The antibodies recognize both the proforms and the active, cleaved forms of those enzymes, the latter exhibiting a lower molecular weight. In Trien-

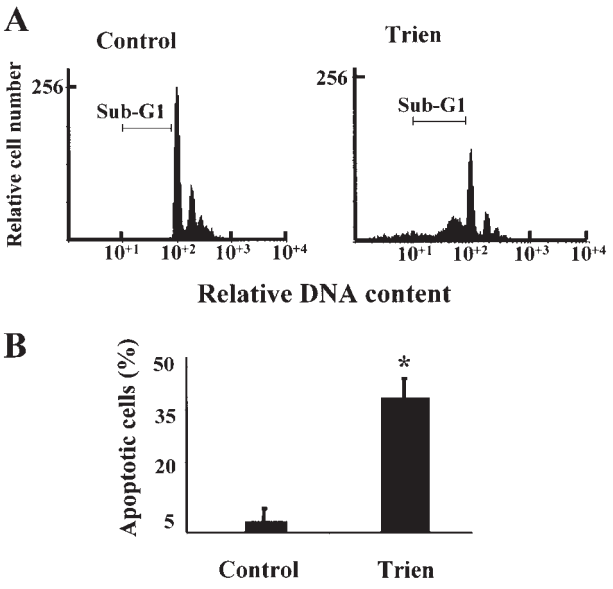

Figure 4. Prolonged copper depletion induces apoptosis in SH-SY5Y cells. Cells were treated with Trien for 11 days (third passage). (*A*) Attached and floating cells were pooled, treated with propidium iodide and analyzed by cytofluorimetry. Typical cell cycle plots are shown; cells undergoing apoptosis localize in the sub-G1 region. (*B*) Percentage of apoptotic cells in the sub-G1 region. n = 4, $p < 0.001$.

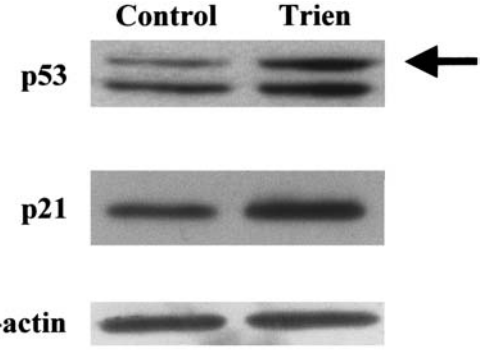

Figure 5. Trien treatment induces increases in p53 and p21 in SH-SY5Y cells. Cells were treated with Trien for 11 days (third passage). Western blot analysis was made with monoclonal antibodies. The arrow indicates the post-translationally activated p53; 50 µg of protein was applied to each lane; *β*-actin was used as a control of the loading. One representative blot is shown, from five separate experiments.

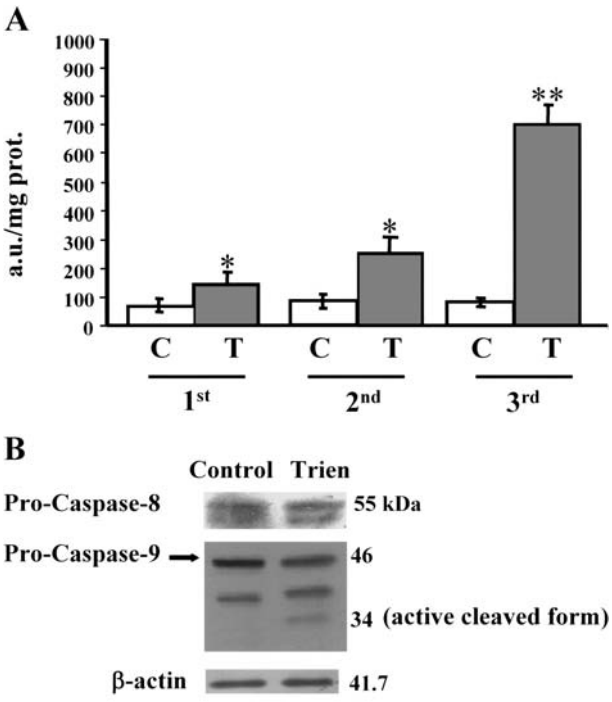

Figure 6. Prolonged copper depletion produces activation of caspase-3 and -9 in SH-SY5Y cells. (*A*) SH-SY5Y cells were treated with Trien for 3 days (first passage), 7 days (second passage) or 11 days (third passage). Caspase-3 activity was assayed by following the cleavage of the fluorogenic substrate Ac-DEVD-AFC; a.u., arbitrary units of fluorescence; n = 4, $*0.05 < p < 0.01$; $**0.01 < p < 0.001$. (*B*) Cells were treated with Trien for 7 days (second passage in culture). The same sample was used for Western blot analysis of caspase-8 and -9; 50 µg of protein was applied to each lane; *β*-actin was used as a control of the loading. One representative blot is shown, from three separate experiments.

treated SH-SY5Y cells, a significant decrease in procaspase-9 is evident, with the appearance of a band with a higher mobility, at 34 kDa, corresponding to the active cleaved form. No change in the pattern of caspase-8 could be detected.

Copper depletion decreases the activity of Cu,Zn-SOD, which is an antioxidant enzyme [8], and of Cytox, the integrity of which is crucial for preventing the leak of partially reduced oxygen species from the mitochondrial electron transport chain [7]. Conceivably therefore, upon Trien treatment, oxidative stress may occur in SH-SY5Y cells. The formation of ROS in attached Trien-treated SH-SY5Y cells was established by measuring the conversion of DHR into its fluorescent oxidized product rhodamine, upon reaction with ROS, by cytofluorimetric analysis [19, 20] (fig. 7). The shift in fluorescence was already evident at the end of the first and second passages (not shown) and remained constant at the third step. Tert-butyl hydroperoxide was added to normal cells as the 100% positive control. As a marker of oxidative stress, we also measured the redox state of glutathione. However, we did not find any change in GSH or GSSG, not even at the third passage in culture (GSH: 23.2 ± 8.3 vs 19.4 ± 6.4; GSSG: 0.48 ± 0.19 vs 0.35 ± 0.14 hmol/mg prot; n = 5).

To compensate for the lack of Cu,Zn-SOD activity, other components of antioxidant defense might increase to cope with ROS. Trien-treated SH-SY5Y cells were therefore analyzed to evaluate the degree of expression of other antioxidant proteins. From the second passage in culture, the activity of the mitochondrial antioxidative enzyme Mn-SOD (measured on native PAGE; fig. 8A) rose, as did its protein content (Western blot analysis, fig. 8B).

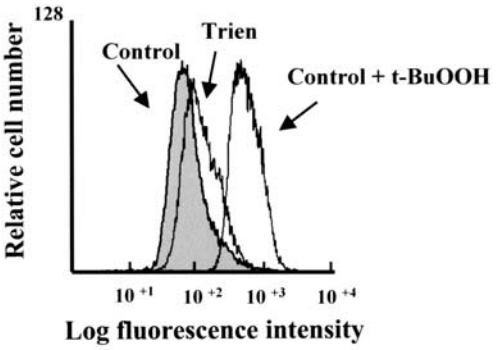

Figure 7. Trien treatment induces production of ROS in SH-SY5Y cells. Cells were treated with Trien for 11 days (third passage). ROS production in cells incubated with dihydrorhodamine 123; 20,000 living cells were analyzed by cytofluorimetry. Test-butyl hydroperoxide (t-BuOOH) (100 µm) was added to untreated cells as a positive control. Results shown are representative of three separate experiments.

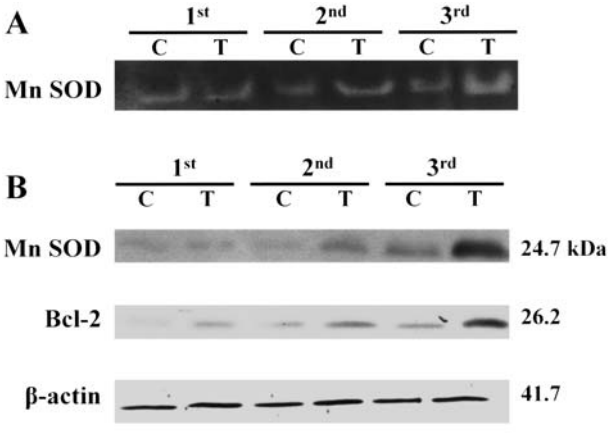

Figure 8. Levels of antioxidants increase in SH-SY5Y cells upon Trien treatment. SH-SY5Y cells were treated with Trien for 3 days (first passage), 7 days (second passage) or 11 days (third passage). (*A*) Mn-SOD activity assayed by specific staining after separation by native PAGE; 100 µg of protein was applied to each lane. One experiment is shown, representative of three. (*B*) Western blot analyses using polyclonal (Mn-SOD) or monoclonal (Bcl-2, *β*-actin) antibodies; 20 µg (Bcl-2) or 50 µg (Mn-SOD and *β*-actin) was applied to each lane; *β*-actin was used as a control of the loading. Blots shown are representative of at least three separate experiments.

The oncogene Bcl-2 can be considered an antioxidant factor, because it is overexpressed under conditions of oxidative stress, and its overexpression protects cells from oxidative insults [26, 27]. Figure 8B shows that the content of Bcl-2 increased strongly in copper-depleted SH-SY5Y cells, starting from the second passage in culture; by the third passage it was highly expressed.

To verify whether addition of exogenous copper could reverse the effects of Trien treatment on cell viability and the molecular parameters, cells at the beginning of the third passage with Trien were supplemented with $CuSO_4$

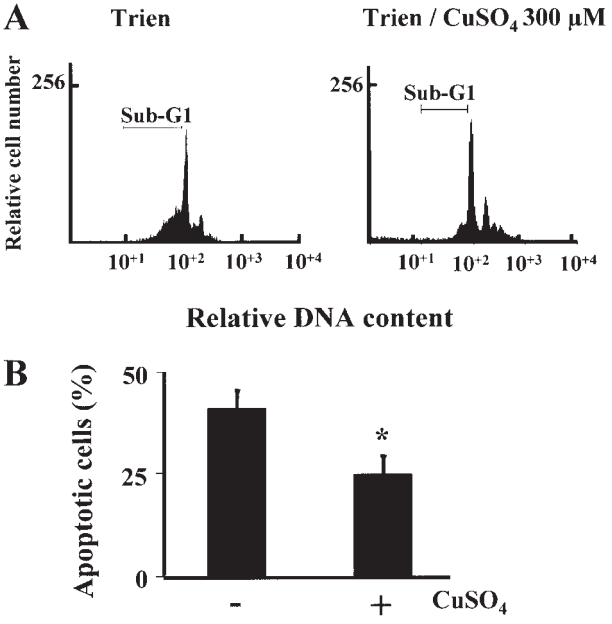

Figure 9. $CuSO_4$ addition to Trien-treated neuroblastoma cells improves cell viability. Cells were treated with Trien for three passages in culture. At the second day of the third passage, 300 µM $CuSO_4$ was added to the medium, together with Trien. At the end of the third passage, attached and floating cells were pooled, treated with propidium iodide and analyzed by cytofluorimetry. (*A*) Typical cell cycle plots are shown; cells undergoing apoptosis localize in the sub-G1 region. (*B*) Percentage of apoptotic cells in the sub-G1 region. n = 4, * $0.01 < p < 0.001$.

in addition to the chelator (Trien/$CuSO_4$). This combination was used to ensure that the phenomena we had observed were specifically due to a lack of copper rather than to other additional effects of Trien itself. $CuSO_4$ (300 µM) was the lowest concentration tested giving positive results. Copper plus Trien were maintained in the culture until the end of the third passage, i.e. for 3 days. Fewer detached cells were detected upon treatment with 300 µM $CuSO_4$ (not shown). Floating and attached cells were pooled, marked with propidium iodide and analyzed by FACScan. Figure 9A reports representative cell cycle plots, where the sub-G1 region, including the apoptotic cells, is markedly smaller in copper-supplemented cultures. In figure 9B, the percentage of cells present in the sub-G1 region is shown: Trien/$CuSO_4$ cells had 25% apoptotic cells versus 40% in Trien-treated cells. Consistently, Trien/$CuSO_4$-treated cells showed recovery of Cu,Zn-SOD activity as well as in the protein content of Cytox subunit II (fig. 10), while the amount of Bcl-2 protein fell back to levels comparable to those of controls. Mn-SOD, instead, remained high, both in terms of activity and protein content (fig. 10).

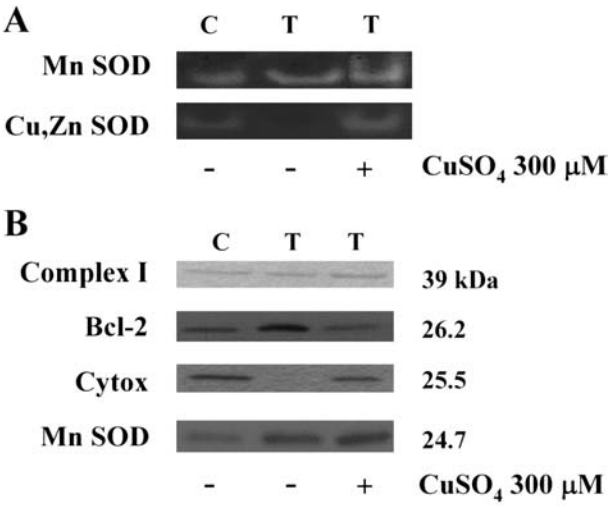

Figure 10. Effect of $CuSO_4$ addition on antioxidants levels of Trien-treated SH-SY5Y cells. Cells were treated with Trien for three passages in culture. On the second day of the third passage, 300 μM $CuSO_4$ was added to the culture medium, together with Trien. (*A*) Mn-SOD and Cu,Zn-SOD activities assayed by specific staining, after separation by native PAGE; 100 μg of protein was applied to each lane. One experiment is shown, representative of three. (*B*) Western blot analyses using polyclonal (Mn-SOD) or monoclonal (Bcl-2, subunit II of Cytox) antibodies; 20 μg (Bcl-2) or 50 μg (Mn-SOD, 39 kDa subunit of complex I, subunit II of Cytox) was applied to each lane. Blots shown are representative of at least three separate experiments. The 39-kDa subunit of complex I is used as a control of the loading.

Discussion

In Mottled/Brindled neonatal mice, copper depletion leads to impaired development of the brain, due to apoptotic death of neural cells through a mitochondrial pathway [2]. The intentions of the present study were to investigate whether this response to copper deficiency may occur more generally in highly replicating neuronal cells and, thus, to better describe the molecular mediators of apoptosis.

The presence of the copper chelator Trien in the culture medium of SH-SY5Y cells produced a decrease in the intracellular level of the metal, which apparently reached an equilibrium by the first passage in culture. The decrease in copper content was about 60%, which corresponds to the decrement observed in the brain of Mottled/Brindled mice [2]. Therefore, Trien-treated neuroblastoma cells may represent a valid in vitro model of copper deficiency in the brain.

The activities of the cuproenzymes Cu,Zn-SOD and Cytox were also decreased, but to a greater extent than copper, and with each exhibiting a different behavior. Intracellular residual copper may be unavailable for binding to the chaperones that mediate the delivery of the metal to these proteins [28]. The differences in behavior of Cu,Zn-SOD and Cytox could be due to the different times required for the turnover of these two proteins, our results indicating that in these cells it may be longer for Cytox than for Cu,Zn-SOD. Otherwise, the differences could be due to the different subcellular distribution of these two enzymes. One needs to bear in mind that an apo-form of Cu,Zn-SOD accumulates during copper deficiency, and this may be ready to catch any available copper [29, 30], thus explaining why Cu,Zn-SOD levels remain higher than those of Cytox. Furthermore, this phenomenon may be facilitated by the overexpression of CCS, the chaperone delivering the metal to Cu,Zn-SOD, which occurs in copper deficiency [31]. Conversely, in copper deficiency, apo-Cytox has never been found, because copper homeostasis is critical for the maturation and assembly of the individual Cytox subunits into the functional holoenzyme [32–35]. In fact, the expression of subunit II of Cytox was severely reduced in Trien-treated SH-SY5Y cells.

Parallel to the depletion of copper and cuproenzymes, Trien-treated SH-SY5Y cells showed overexpression of Mn-SOD protein and activity. Lai et al. [36, 37] demonstrated that mRNA, activity and protein levels of Mn-SOD were increased in copper-deficient rats. Furthermore, increased Mn-SOD immunoreactivity has been observed in the cerebella of patients with Menkes' disease [38]. From the reports of several studies performed in the most diverse experimental systems, Mn-SOD is known to be induced by oxidative stress [27, 39]. Indeed, ROS behave as signaling molecules when produced in sub-toxic amounts, as a sort of second messenger [40, 41], regulating the transcription of antioxidants. Lai et al. [37] also suggested that a higher transcriptional rate of Mn-SOD in rat liver may be ascribable to the increased oxidative stress resulting from copper deficiency.

In copper-depleted SH-SY5Y cells we found evidence for the formation of a constant flux of ROS. In these cells, the lower efficiency of complex IV may arrest the flow of electrons in the mitochondrial transport chain. Electrons accumulate upstream of complex IV, leak from complex I and complex III, and react with oxygen to give superoxide [7]. Since complex I, as well as complex II, appears to be unaltered in copper-depleted SH-SY5Y cells [12], mitochondrial malfunction and ROS production can be ascribed to faulty Cytox.

Copper deficiency in SH-SY5Y cells did not affect the level of GSH. This is in line with our findings in the brain of the Mottled/Brindled mouse [2]. This finding further strengthens the hypothesis that copper-deficient neuroblastoma cells represent a good model for copper deficiency in brain. It also indicates that the glutathione response to copper deficiency differs between the brain and other tissues. In fact, liver and plasma of copper-deficient animals show increased GSH levels, possibly due to modulation of γ-glutamylcysteine synthetase [42, 43]. Furthermore, the oxidative insult produced by copper depletion is not very powerful because GSH was not oxidized.

From the end of the second passage in culture with Trien, a conspicuous number of SH-SY5Y neuroblastoma cells detached from the monolayers and showed the characteristic nuclear morphology of apoptotic cells. This supports our hypothesis that prolonged copper deficiency prompts neural cells to undergo apoptosis, as occurs in the brain of the animal models of Menkes' disease [2, 3]. Furthermore, detached cells were also Trypan Blue permeable, thus, necrosis subsequent to an apoptotic process seems to occur in copper-deficient cells. Low levels of oxidative stress for a prolonged period induce apoptosis, while higher acute levels induce necrosis [11]; we hypothesize that Cytox inhibition in our experimental model produces a low but continuous level of ROS leading to apoptosis. This is supported by the findings that inhibitors of complexes of the mitochondrial respiratory chain induce the production of ROS and apoptosis in various types of cultured cells [44].

The proteases belonging to the caspase family are well characterized among the molecular effectors of apoptosis: they are present in latent forms and are activated in cascades [25]. In SH-SY5Y cells, we observed the early and progressive activation of the downstream caspase-3 during Trien treatment. In fact, it appears to be alerted as early as the first passage, activation becoming prominent at the end of the treatment, leading to apoptosis. Treatment of copper-deficient SH-SY5Y neuroblastomas with the pro-oxidant paraquat leads to an intense activation of this isoform of the caspases [12], which are known to be specifically involved in oxidative-stress-induced programmed cell death [45]. This confirms the idea that chronic treatment with Trien generates a flow of ROS. Furthermore, we also investigated the mechanisms underlying caspase-3 activation. In fact, pro-caspase-3 represents the converging point of diverse pathways leading to programmed cell death, being activated by specific upstream caspase family members. Apoptosis triggered by stimulation of surface receptors proceeds through the activation of pro-caspase-8, whereas mitochondrial factors activate pro-caspase-9 [9, 46]. In our model of apoptosis, we found that caspase-9 was active, while caspase-8 showed no significant changes. This finding reinforces our hypothesis that the main target of chronic copper depletion is the mitochondrion, which in turn triggers apoptosis. It also consolidates the results obtained by analyses of the brain of the animal model of copper deficiency, where we found the release of cytochrome c in the cytosol, an upstream signal of caspase-9 activation [2].

The tumor suppressor protein p53 is a zinc-finger-like transcription factor that controls cell cycle progression and apoptosis [23]. In Trien-treated cells, p53 protein increased strongly, together with its post-translationally activated form. As we have already reported, this is a very early event, because it starts from the first phase of copper deficiency [12], before the appearance of apoptosis. Our data confirm those of Narayanan et al. [47], who found that treating HepG2 cells with a copper chelator for short time increases p53 mRNA abundance and nuclear translocation, without apparent apoptosis. Physiological concentrations of copper are known to change the native protein conformation of p53 and inhibit its DNA-binding capacity [24, 48]. In our system, increased activity of p53 was demonstrated by the parallel increment in the content of one of its downstream products, p21, which binds to a number of cyclin and Cdk complexes allowing the arrest of the cell cycle [24]. We suggest that lowering the intracellular copper concentration may relieve copper-dependent instability and inhibition of p53 action [24, 48]. In this way, the increase in p53 may result in both a decrease in cell growth and in cell death by apoptosis. Furthermore, our results on p53 support the assumption that Trien behaves as a specific copper chelator [12]: Trien-treated SH-SY5Y cells still have zinc to allow p53 activity.

In copper-depleted SH-SY5Y cells we observed a 60% decrease in ATP. Apoptosis is an energy-requiring process; in copper-deficient SH-SY5Y cells, it is not accompanied by a contemporary adequate resynthesis of ATP, possibly due to the lack of activity of Cytox, leading to energy impairment. Of interest would be to clarify why these results differ from those found in organs of copper-deficient animals, showing no change in ATP levels [49, 50]: whether this is due to the different ways to achieve copper deficiency, to the different methods of ATP measurement, to the severity of copper deficiency or to the absence of concomitant apoptosis in those models. Anyway, the results obtained in SH-SY5Y are in line with our previous findings in the copper-deficient brain of the Mottled/Brindled mouse, where ATP decreases by more than 50% [2].

Chronic copper depletion in SH-SY5Y cells induced a large increase in Bcl-2 protein, likely due to ROS induction of the expression of Bcl-2 [27], which is considered to be an antiapoptotic factor [51]. Despite the increase in Bcl-2, SH-SY5Y cells depleted of copper undergo apoptosis. A possible explanation for this incongruity may be the fact that ROS act at the same time both as Bcl-2 inducers and as pro-apoptotic stimuli. The simultaneity of these two events may counteract Bcl-2 prevention of cell death. Indeed, apoptosis induced by ROS has already been reported to be concomitant with a Bcl-2 increase in cultured skin fibroblasts [52] and in several tumor cell lines [44]. The increase in Bcl-2 found in our copper-depleted model system is not in line with our previous findings in the brain of neonatal Mottled/Brindled mice, where low levels of Bcl-2 accompanied apoptosis [2]. The reason for this discrepancy could be the intrinsic differences of the two experimental models (in vitro tumor cells versus in vivo neurons). Tumor cells activate mechanisms of resistance to death stimuli much more than do neurons in a de-

veloping brain, where apoptosis is a physiological process in the remodeling of the growing tissue [53].

Cell death and molecular changes observed under copper deficiency could be partially reversed by copper addition to cell medium. In these experiments, the contemporaneous presence of Trien may prevent the toxic effects of high concentrations of exogenous copper that have been reported to be pro-apoptotic in hepatoma cells [47]. The recovery of Cu,Zn-SOD activity after addition of $CuSO_4$ to Trien-treated cells, together with Cytox rescue, improved cell viability, indicating that impairment of copper-dependent enzymes is a causal factor in the induction of cell death. Indeed, copper supplementation by injection prevents the neurological disturbances present in the Mottled/Brindled mouse [54]. Bcl-2 levels also revert to control ones; the different response of Mn-SOD is probably due to a different turnover of this protein or to an additional specific effect of Trien.

To summarize, in the present report we show that long-lasting depletion of the intracellular copper in SH-SY5Y cells stimulates the production of low levels of ROS, possibly due to alteration of mitochondrial function mediated by Cytox impairment and favored by the decrease of Cu,Zn-SOD activity. To counteract this challenge, cell levels of the mitochondrial antioxidant proteins, namely Mn-SOD and Bcl-2, are increased, but not sufficiently to prevent the cells from ceasing replication and committing suicide through p53 activation. Caspase-9 and -3 carried out the program of cell death, thus confirming the involvement of mitochondial effectors.

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