Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line

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Abstract
The mechanism of copper (Cu) neurotoxicity was studied in the RCSN-3 neuronal dopaminergic cell line, derived from substantia nigra of an adult rat. The formation of a Cu–dopamine complex was accompanied by oxidation of dopamine to aminochrome. We found that the Cu–dopamine complex mediates the uptake of $^{64}$CuSO$_4$ into the Raúl Caviedes substantia nigra-clone 3 (RCSN3) cells, and it is inhibited by the addition of excess dopamine (2 mM) (63%, $p < 0.001$) and nomifensine (2 µM) (77%, $p < 0.001$). Copper sulfate (1 mM) alone was not toxic to RCSN-3 cells, but was when combined with dopamine or with dicoumarol (95% toxicity; $p < 0.001$) which inhibits DPNH and TPNH (DT)-diaphorase. Electron spin resonance (ESR) spectrum of the 5,5-dimethylpyrroline-N-oxide (DMPO) spin trap adducts showed the presence of a C-centered radical when incubating cells with dopamine, CuSO$_4$ and dicoumarol. A decrease in the expression of CuZn-superoxide dismutase and glutathione peroxidase mRNA was observed when RCSN-3 cells were treated with CuSO$_4$, dopamine, or CuSO$_4$ and dopamine. However, the mRNA expression of glutathione peroxidase remained at control levels when the cells were treated with CuSO$_4$, dopamine and dicoumarol. The regulation of catalase was different since all the treatments with CuSO$_4$ increased the expression of catalase mRNA. Our results suggest that copper neurotoxicity is dependent on: (i) the formation of Cu–dopamine complexes with concomitant dopamine oxidation to aminochrome; (ii) dopamine-dependent Cu uptake; and (iii) one-electron reduction of aminochrome.

Keywords: aminochrome, copper, dopamine, DT-diaphorase, free radicals, quinones.


Copper is an essential trace element which plays an important role in cell physiology as a cofactor of several enzymes, including cytochrome oxidase, CuZn-superoxide dismutase, lysine oxidase, dopamine-β-hydroxylase and ceruloplasmin. Two genetic disorders, Menkes’ and Wilson’s diseases, are related to the mutation of Cu transporting proteins (Tumer et al. 1997). The ability of copper to cycle between a stable oxidized (Cu$^{2+}$) and unstable reduced (Cu$^{+}$) state is essential for the enzymatic activity of CuZn-superoxide dismutase and cytochrome oxidase. However, the ability of copper to redox cycle may also be involved in free radical reactions. The reduced form of copper (Cu$^{+}$) catalyzes the formation of hydroxyl radicals (·OH) in the presence of hydrogen peroxide through the Fenton reaction (Halliwell and Guttridge 1989). Hydroxyl radicals constitute some of the most harmful free radicals, and they induce cellular damage by reacting with lipids, amino acids, thiol groups, RNA and DNA (Halliwell and Guttridge 1989; Chen et al. 1992; Chen et al. 1997; Fu and Dean 1997; Liu et al. 1997; Das et al. 1998). In addition, Cu$^{+}$ and Cu$^{2+}$ can...
accept an electron from and donate electrons to superoxide radicals (Halliwell and Gutteridge 1989).

The formation of Cu–amino acid and Cu–dopamine complexes has been reported (Richter et al. 1977; Kiss and Gergely 1979; Alberghina et al. 1982; Kiss et al. 1984; Barnea et al. 1989; Aoyagi and Baker 1993; Chikira et al. 1997). Copper catalyzes the oxidation of catechol to o-quinone by (i) reaction of free Cu^{2+} with the Cu^{2+}–catechol complex, which favors the transfer of electrons from catechol to reduce Cu^{2+} to Cu^{+} and (ii) formation of Cu(I) O_2\^+, which effectively oxidizes catechol solely or when complexed with copper, as a result of the reaction of Cu^{+} and dioxygen (Balla et al. 1992). This ability of copper to oxidize catechol to o-quinone may have a pathophysiological relevance since the oxidation of dopamine to aminochrome and its one-electron reduction to leucoaminochrome o-semiquinone has been proposed to be responsible for the degenerative process occurring in dopaminergic neurons in Parkinson’s disease (PD) (Segura-Aguilar and Lind 1989; Baez et al. 1995; Segura-Aguilar et al. 1998; Segura-Aguilar et al. 2000). NAD(P)H : quinone oxidoreductase [EC 1.6.99.2, DPNH and TPNH (DT)-diaphorase] has been postulated to be an important neuroprotective enzyme, which prevents the one-electron reduction of aminochrome to leucoaminochrome o-semiquinone by reducing aminochrome to leucoaminochrome (Segura-Aguilar and Lind 1989; Baez et al. 1995; Segura-Aguilar et al. 1998; Segura-Aguilar et al. 2000). DT-diaphorase is a unique flavoenzyme, which reduces quinones to hydroquinones by transferring two electrons. Dicoumarol is a specific and potent inhibitor of DT-diaphorase and, indeed, DT-diaphorase activity is defined as the quinone reductase activity inhibited by dicoumarol (Ernst et al. 1987). The role of copper in neurodegenerative disease is suggested by the increased incidence of parkinsonism in subjects exposed to copper in mining operations in Chile (Roberto Gallardo, Department of Stereotaxic Neurosurgical, Psychiatric Institute, Santiago, Chile, personal communication).

The present investigation tested the hypothesis that copper toxicity is dependent on dopamine oxidation to aminochrome and its one-electron reduction to leucoaminochrome o-semiquinone radical. In this work, we have used a dopaminergic cell line derived from adult rat substantia nigra, cultured in the presence and absence of dicoumarol (Arriagada et al. 1998). Copper uptake and expression of antioxidant enzyme mRNA was also measured in order to assess the mechanism of copper neurotoxicity.

**Experimental procedures**

**Chemicals**

Dopamine, CuSO_4, nomifensine, dicoumarol, DME/HAM-F12 nutrient mixture (1 : 1), menadione, cytochrome C and 5,5-dimethylpyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Thermoscript RT–PCR system and Taq DNA polymerase were obtained from Life Technologies (Grand Island, NY, USA). RNeasy midi system was from QIAGEN (Hilden, Germany). The primers were obtained from T-A-G-Copenhagen A/S (Copenhagen, Denmark). ^{64}\text{CuSO}_4 was obtained from the La Reina Nuclear Plant, National Commission of Nuclear Energy of Chile (Santiago, Chile).

**Cell cultures**

The RCSN-3 cell line was derived from the substantia nigra of a 4-month-old normal Fisher 344 rat. The cell material used to establish primary cultures was then transformed to a permanent cell line by exposure to media conditioned by UCHT1 cells, a process that induces transformation in cell cultures, and the resulting cell line was subsequently cloned (Caviedes et al. 1992, 1993, 1994; Liberona et al. 1998, 1997). The RCSN-3 cell line grows as a monolayer, with a doubling time of 52 h, a plating efficiency of 21% and a saturation density of 41 000 cells/cm². The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO₂. The cells were grown in Dulbecco’s modified Eagle’s medium (DME)/nutrient mixture HAM F₁₂ (1 : 1), 10% bovine serum, 2.5% fetal bovine serum, and 40 mg/L gentamicin sulfate (Dagnino-Subiabre et al. 2000).

**Toxicity**

For CuSO₄ toxicity experiments, the cells were incubated with cell culture medium but in the absence of bovine serum and phenol red for 120 min. The concentrations used in the toxicity experiments were 1 mM CuSO₄, 100 μM dopamine, and 100 μM dicoumarol. The cells were visualized at 100 x magnification in a Nikon Diaphot inverted microscope equipped with phase contrast optics and fluorescence. The CuSO₄ toxicity was measured by counting live and dead neurons after staining the cells with 120 μM fluorescein diacetate (FA) for 4 min at 37°C and 1 min at 37°C after addition of 25 μM ethidium bromide (EB) (Diaz-Trelles et al. 1999). FA is a marker for live cells and EB intercalates the DNA of dead cells. The cells were counted in a phase contrast microscope equipped with fluorescence, using the following filters: FA, 510–560 nm (excitation) and LP-590 nm (emission); EB, 450–490 nm (excitation) and 515–565 nm (emission).

**RT-PCR**

The expression of superoxide dismutase (EC 1.15.1.1; SOD), hydrogen peroxide : hydrogen peroxide oxidoreductase (EC 1.11.1.6; catalase), glutathione peroxidase (EC 1.11.1.9) and DT-diaphorase in the RCSN-3 cell line was studied by RT-PCR. The cells were incubated with 200 μM CuSO₄, 200 μM CuSO₄, and 200 μM dopamine; or 200 μM CuSO₄, 200 μM dopamine, and 100 μM dicoumarol for 10 min before the extraction of total RNA. Total RNA was isolated using a RNeasy Midi kit (QIAGEN). Five micrograms of the total RNA were used for the synthesis of a single strand of DNA with the reverse transcriptase (RT) reaction. The RT-reaction was performed by using a Thermoscript RT-PCR system (Life Technologies) with Oligo (dT)₁₂₀ as primers. The total RNA (1.5 μg) was incubated at 65°C for 5 min in diethylpyrocarbonate (DEPC)-treated water before the addition of 4 μL 5X RT-buffer, 3 mM DDT, 40 U RNaseOUT, 1 μL DEPC water, 0.6 mM dNTP mix, and 15 U Thermoscript reverse transcriptase. The mixture was incubated at 25°C for 10 min, 50°C for 50 min and 85°C for 5 min before adding 2 U of
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RNase H, after which the mixture was incubated at 37°C for 20 min. The amplification of ssDNA of antioxidant enzymes was performed by PCR reaction using the following primers 5′-CTCAGGAAGACATTGCAACCTTGAC-3′ (upstream) and 5′-CTCAGACATGCAAAGGTGACGAGGTG-3′ (downstream) designed from the cDNA sequence of rat CuZn-superoxide dismutase (CuZn-SOD) (Hass et al. 1989); 5′-CGCTTTTCTTTCGCCAGAC-3′ (upstream) and 5′-AGAATTCGACCTTGAGTGTCGAC-3′ (downstream) designed of rat catalase (Furuta et al. 1986); 5′-ACAGTCCACCCGTGTATGCTCTG-3′ (upstream) and 5′-TCTTTGCCATCTCTGTGGATGTCC-3′ (downstream) designed of rat glutathione peroxidase (Christensen and Burgener 1992); 5′-AGTCCAAAAAGCAATGTCGCACTGTG-3′ (upstream) and 5′-CAATTCTGCCTGTGCAG-3′ (downstream) designed of rat GSH-Px (Hass et al. 1991); 5′-CAGAAGCGACATCGAGGGGA-3′ (upstream) and 5′-CAAGCCTCTCTGCAAACGAC-3′ (downstream) designed of rat DT-diaphorase (Bayney et al. 1989). 5′-TTTGGATGCTTGTCAGAAACCAGAG-3′ (upstream) and 5′-GCAAGCATCAAAGGTGAAGGTGACGAGGTG-3′ (downstream-2) designed of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9; GAPDH) (Tso et al. 1985). The PCR reaction was performed in four steps: (i) 95°C for 5 min; (ii) 11 cycles at 95°C for 40 s, 56°C (decreasing the temperature one degree per cycle until 55°C) for 40 s, 72°C for 40 s; (iii) 28 cycles at 95°C for 45 s, 60°C for 40 s, 72°C for 50 s; and (iv) one cycle at 72°C for 10 min. The PCR incubation contained 3 μL of RT incubation, 0.4 μm dNTP each, 3 μm MgCl2, 2.5 μm primers, 10 × 5 μL PCR buffer (GibcoBRL, Rockville, MD, USA), 29 μL H2O and 2 μTaq polymerase (GibcoBRL). The region amplified by PCR of CuZn-SOD spanned the region between bases 375–395 and 501–482, resulting in a fragment of 129 bp (Hass et al. 1989); catalase was between bases 1143–1163 and 1422–1401, resulting in a fragment of 280 bp (Furuta et al. 1986); glutathione peroxidase was between bases 381–402 and 610–589, resulting in a fragment of 230 bp (Christensen and Burgener 1992); Mn-SOD was between bases 259–282 and 590–613, resulting in a fragment of 355 bp (Ho et al. 1991); DT-diaphorase was between the bases 156–177 and 364–343, resulting in a fragment of 209 bp (Bayney et al. 1989); and GAPDH was between bases 408–431 and 910–887, resulting in a fragment of 502 bp used as housekeeping gene (Tso et al. 1985). The PCR products were electrophoresed on 2% agarose gels, stained with EB and photographed.

64CuSO4 uptake into RCSN-3 cells

The uptake of 64CuSO4 into RCSN-3 cells was measured under the same conditions used to study cell viability. 64CuSO4 (50 μCi) were added to each dish and the uptake was assessed after an incubation period of 1 min at room temperature in cell culture medium or Hank’s solution. The final concentration of CuSO4 was 1 μM (50 μM 64CuSO4) and 100 or 2000 μM dopamine. To inhibit the dopamine transport system (DAT) a concentration of 2 μM nomifensine was used. In the experiments where the concentration of dopamine was different, the final concentration of CuSO4 was the same as described above. At the end of the uptake period, the extracellular medium was removed and the cells were rapidly washed five times with 2 mL medium or Hank’s solution to remove residual 64CuSO4 tracers. Cell membranes were disrupted with 1 mL of 1% Triton X-100 and after 15 min incubation 900 μL of the cell/Triton X-100 extract were removed and analyzed for 64CuSO4 tracer content by liquid scintillation counting. The remaining 100 μL were used for protein determination by bicinconinic acid method according to Smith et al. (1985) (Pierce, Rockford, IL, USA). The results were expressed in nmol/mg protein/min.

Spectroscopy

ESR spectroscopy

ESR spectra were recorded in X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 KHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The cells were incubated for 5 min with 100 μM DMPO before the addition of 100 μM dopamine, 1 mM CuSO4 and 100 μM dicumarol. The cells were incubated 1 h prior to ESR spectroscopy.

Spectrum Cu-dopamine complex

The spectra of CuSO4-dopamine complex was performed in the same medium used for cell culture.

Atomic absorption

A Perkin-Elmer 3110 atomic absorption spectrometer was used. The standard solutions were freshly prepared by dilution (with distilled water) of CuSO4 (1000 ppm). Standard solutions between 0 and 5 ppm were measured. The procedure was carried out in triplicate with correction for the blank sample. The treated cells were incubated for 1 min with 1 mM CuSO4 in the presence of cell culture medium.

Determination of DT-diaphorase activity

The total DT-diaphorase activity in RCSN-3 cells was measured spectrophotometrically as described previously (Schultzberg et al. 1988) at 30°C with NADH as electron donor and menadione as electron acceptor. Cytochrome c was added for continuous reoxidation of menadione to menadione, and the reduction of cytochrome c was followed at 550 nm (extinction coefficient of cytochrome c was 18.5 mm/m/cm). RCSN-3 cells were incubated in the presence and absence of 100 μM dicumarol for 10 min before measuring the activity.

Data analysis

All data were expressed as mean ± SD values. The statistical significance was calculated using Student’s unpaired t-test, compared with controls.

Results

The possible neurotoxic effects of copper was studied by using CuSO4 and a neuronal cell line (RCSN-3) derived from adult rat substantia nigra. The possibility that CuSO4 catalyzes the oxidation of dopamine was studied in vitro by monitoring the spectral changes when dopamine was incubated with CuSO4 under the same conditions used for cell culture. The addition of 1 mM CuSO4 to the cell culture medium, which contain amino acids, resulted in a spectral change with maximum absorptions at 623 nm (Fig. 1B, scan b), suggesting the formation of a Cu–amino acid complex.
However, a new spectrum appeared when 100 μM dopamine was added to the medium before the addition of CuSO₄, with a maximum absorption at 298 nm (Fig. 1A, scan c) suggesting the formation of a Cu±dopamine complex. The visible region of the Cu±dopamine complex showed the appearance of a new peak at 475 nm (Fig. 1B, scans c, d and e), which suggested dopamine oxidation to aminochrome (Segura-Aguilar and Lind 1989). The formation of the peak at 475 nm was found to be dependent on the concentration of CuSO₄ (Fig. 1C).

For studying the possible neurotoxic effects of CuSO₄ in the RCSN-3 cell line, the cells were treated with 1 mM CuSO₄ due to the chelating capacity of amino acids present in the cell culture medium. The intracellular Cu concentration of untreated cells was 8 ng/mg protein determined by atomic absorption spectrometry. The intracellular copper concentration of cells incubated with 1 mM CuSO₄ increased to 23 ng/mg protein determined by both atomic absorption spectrometry and ⁶⁴Cu uptake data, which yielded comparable data for both techniques, but these conditions did not induce significant cell death after 2 h incubation (6.3%). However, cell death increased significantly after incubation of RCSN-3 cells with 1 mM CuSO₄ and 100 μM dopamine (27%, p < 0.001) (Fig. 2a), where the intracellular concentration of copper increased to 34 ng/mg protein. This increase was also dependent on dopamine concentration (Fig. 2b). The presence of 100 μM dopamine by itself had no effect on cell death (Fig. 2a).

The dopamine dependence of CuSO₄ toxicity on RCSN-3 cells and the formation of a Cu–dopamine complex suggested a possible role of the Cu–dopamine complex in copper uptake into the cell. Hence, incubation of ⁶⁴CuSO₄...
with 100 μM dopamine resulted in uptake of the 64Cu–dopamine complex, which was increased in comparison to the 64Cu–medium complex uptake. In addition, incubation of 64CuSO4 in the presence of 2 mM dopamine and 2 μM nomifensine inhibited the Cu–dopamine complex dependent uptake (Fig. 3a). However, incubation of the cells with Hank’s solution showed a 2.6-fold higher uptake of 64Cu and a clear uptake dependence on the formation of 64Cu–dopamine complex. The uptake was significantly increased by near seven-fold in the presence of dopamine (64Cu–dopamine; p < 0.001) and it was strongly inhibited by a high concentration of dopamine (2 mM) (63% inhibition; p < 0.001) and 2 μM nomifensine (77% inhibition; p < 0.001) (Fig. 3b). It is interesting to note that the increase of 64Cu–dopamine complex uptake was dependent on dopamine concentration (Fig. 3c).

The appearance of a peak at 475 nm during the formation of the Cu–dopamine complex suggested that the oxidation of dopamine to aminochrome may play a role in CuSO4 toxicity (Fig. 1B). We studied the possible involvement of a one-electron reduction of aminochrome in the CuSO4 effect on RCSN-3 cell survival. This was studied by incubating the cells in the presence of dicoumarol, a specific inhibitor of DT-diaphorase, which is the sole enzyme that catalyzes two-electron reduction of quinones. Cell death dramatically increased in the presence of 100 μM dicoumarol, 1 mM CuSO4 and 100 μM dopamine (95%; p < 0.001) (Fig. 2a). The effect of dicoumarol on cell death was concentration dependent (Fig. 2c; p < 0.01 between 25 and 75 μM). A significant increase in cell death was also observed when RCSN-3 cells were incubated with 1 mM CuSO4 and 100 μM dicoumarol (34%; p < 0.001), but dicoumarol alone had no effect on cell death (Fig. 2a). Since dicoumarol is a specific inhibitor of DT-diaphorase, we measured the enzyme activity in RCSN-3 cells in the presence and absence of dicoumarol. The basal DT-diaphorase activity in RCSN-3 cells was found to be 3.29 μmol/min/mg protein and constituted 94% of the total quinone reductase activity in these cells. The DT-diaphorase activity in cells treated with 100 μM dicoumarol was inhibited by 84%.

The intracellular action of CuSO4 should comprise the formation of free radicals such as hydroxyl radicals in a Fenton reaction catalyzed by Cu+ and leukoaminochrome o-semiquinone radicals in one-electron reduction of aminochrome. The spin-trapping agent DMPO was used to detect the intracellular formation of free radicals when the cells were incubated with dopamine, CuSO4 and dicoumarol. A signal of the adducts of spin trap of DMPO with a C-centered radical were observed (Fig. 4). The ESR spectrum was analysed and simulated in terms of two hyperfine constant corresponding to aN = 15.0 G and aH = 18.5 G. No ESR signal was observed when the cells were incubated with dopamine or dopamine and CuSO4 (not shown).

A pro-oxidant role of superoxide dismutase, catalase and glutathione peroxidase during one-electron reduction of aminochrome to leukoaminochrome o-semiquinone has been proposed (Baez et al. 1995). Therefore, the study of
the regulation of superoxide dismutase, catalase and glutathione peroxidase mRNA expression in RCSN-3 cells treated with copper may provide important information to understand the mechanism of copper toxicity. A significant downregulation in the expression of glutathione peroxidase mRNA was observed with RT-PCR when the cells were incubated with CuSO₄, dopamine, or CuSO₄ + dopamine (Fig. 5). To demonstrate the differences in the expression of glutathione peroxidase mRNA in RCSN-3 cells subjected to the treatments mentioned above, we carried out PCR studies at different cycles (Fig. 6). No expression of this enzyme was detected until 25, 30 and 24 cycles were performed, when the cells were treated with CuSO₄, dopamine, or CuSO₄ + dopamine, respectively. However, the expression of glutathione peroxidase mRNA increased near to the control level when RCSN-3 cells were treated with CuSO₄ + dopamine in the presence of 100 μM dicoumarol. The expression of catalase mRNA increased when RCSN-3 cells were treated with CuSO₄, dopamine, or dopamine + dicoumarol (Fig. 5), which is also supported by the data gathered after using PCR at different cycles (Fig. 7). No expression of catalase was observed prior to 22 cycles in RCSN-3 cells under control conditions. However, the expression of the enzyme was observed at 19 cycles when the cells were treated with CuSO₄ + dopamine or CuSO₄ + dopamine + dicoumarol and at 20 cycles after treatment with CuSO₄ (Fig. 7). A decrease in the expression of CuZn-SOD mRNA was observed when the cells were treated with CuSO₄ or dopamine (Fig. 5); this was clearer when PCR was performed at different numbers of cycles (Fig. 8a). The expression of CuZn-SOD mRNA in control RCSN-3 cells became evident at 19 cycles but it was not observed until cycle 22, 23, 22 and 22 when RCSN-3 cells were treated with CuSO₄, dopamine, CuSO₄ + dopamine, and CuSO₄ + dopamine + dicoumarol, respectively (Fig. 8a). We also observed a marked decrease in the expression of Mn-SOD mRNA when the RCSN-3 cells were treated with dopamine, or CuSO₄ + dopamine + dicoumarol (Fig. 5), which is supported by PCR studies carried out at different cycles since the enzyme was not expressed before 45 and 40 cycles, respectively. Conversely, 30 cycles were required to express the enzyme in the control cells (Fig. 8a). The expression of DT-diaphorase mRNA was also studied.

Fig. 5 Expression of antioxidant enzymes mRNA in RCSN-3 cells. The expression of CuZn-SOD, catalase (CAT), glutathione peroxidase (GSH-PX), Mn-SOD and glycereraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured under normal conditions (A) and after treatment with 1 mM CuSO₄ (B); 100 μM dopamine (C); 1 mM CuSO₄, 100 μM dopamine (D) 1 mM CuSO₄, 100 μM dopamine and 100 μM dicoumarol (E). In all conditions noted, the expression was measured at 24 cycles with the exception of Mn-SOD and GAPDH, which was determined at 35 cycles by using RT-PCR.

Fig. 6 Expression of glutathione peroxidase mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of glutathione peroxidase of control RCSN-3 cells was compared with cells treated with 1 mM CuSO₄ (Cu); 100 μM dopamine (DA); 1 mM CuSO₄ and 100 μM dopamine (Cu + DA); 1 mM CuSO₄, 100 μM dopamine and 100 μM dicoumarol (Cu + DA + DIC). Experimental procedures are described under Materials and methods.

Fig. 7 Expression of catalase mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of catalase of control RCSN-3 cells was compared with cells treated with 1 mM CuSO₄ (Cu); 100 μM dopamine (DA); 1 mM CuSO₄, 100 μM dopamine (Cu + DA) 1 mM CuSO₄, 100 μM dopamine and 100 μM dicoumarol (Cu + DA + DIC). Experimental procedures are described in Materials and methods.
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Fig. 8 Expression of CuZn-SOD (a) and Mn-SOD (b) mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of SOD of control RCSN-3 cells was compared after treating cells with 1 mM CuSO₄ (Cu); 100 μM dopamine (DA); 1 mM CuSO₄ + 100 μM dopamine (Cu + DA); 1 mM CuSO₄ + 100 μM dopamine + 100 μM dicoumarol (Cu + DA + DIC). The number of cycles for CuZn-SOD (a) ranged between 19 and 30 while 30–45 cycles were used for Mn-SOD (b). Experimental procedures are described in Materials and methods.

but no changes were observed with the treatments described above (not shown).

Discussion

The present investigation demonstrates that the CuSO₄ toxicity effect on the survival of dopaminergic cells of rat substantia nigra (RCSN-3) is intracellular and is dependent on (i) dopamine-mediated uptake; (ii) formation of amino-chrome; and (iii) inhibition of DT-diaphorase. The intracellular mode of action of CuSO₄ is supported by the strong effect of dicoumarol in the CuSO₄-dependent decrease of cell survival. Dicoumarol, a hydrophobic compound that penetrates membranes, is a specific inhibitor of DT-diaphorase, which is mainly located in the cytosol (95%) and is associated with the endoplasmic reticulum and mitochondria (5%) (Ernster et al. 1962; Ernster 1987).

Intracellular metabolism of CuSO₄ requires its uptake into the cell and several proteins involved in copper uptake into mammalian cells has been reported, such as copper translocating P-type ATPases (Camakaris et al. 1999; Bull et al. 1993; Tumer et al. 1997). The present results suggest a new and specific mechanism of copper uptake in the dopaminergic neurons. ⁶⁴Cu uptake in RCSN-3 cells was mediated by dopamine, forming a complex with Cu, and taken up by the specific dopamine transport system. Thus, the presence of excess of dopamine (2 mM) to saturate the dopamine uptake system or 2 μM nomifensine, a specific inhibitor of dopamine transport system (Meiergerd and Schenk 1994; Wieszorek and Kruk 1994), inhibited dopamine-mediated uptake of ⁶⁴Cu into RCSN-3 cells. The present results also suggest the existence of a mechanism of Cu uptake which is not mediated by dopamine, since ⁶⁴Cu uptake into RCSN-3 was also observed in the absence of dopamine, although this mechanism was only responsible for a portion of the total ⁶⁴Cu uptake (Fig. 3). The ⁶⁴Cu uptake into RCSN-3 cells incubated with cell culture medium in the absence of dopamine may be mediated by the formation of ⁶⁴Cu complex with some amino acids (histidine, lysine, arginine) present in the cell culture medium, which are known to form complex with Cu²⁺ (Richter et al. 1977; Kiss and Gergely 1979; Alberghina et al. 1982; Kiss et al. 1984; Barnea et al. 1989; Aoyagi and Baker 1993; Chikira et al. 1997). The uptake of ⁶⁷Cu complexed to histidine in hypothalamic slices has been reported (Barnea et al. 1989). The formation of Cu–medium complex is showed in Fig. 1. However, a higher uptake of free ⁶⁴Cu in RCSN-3 cells incubated with Hank's solution was observed, which may be explained by the chelating capacity of the histidine, arginine and lysine present in the cell culture medium. In conclusion our results suggest the existence of two different uptake mechanisms in RCSN-3 cells: (i) dopamine-dependent and specific uptake of copper mediated by the dopamine transport system, which is more efficient and responsible for the majority of copper transport into RCSN-3 cells; and (ii) uptake of free copper or copper complexed by histidine through non-dopamine-mediated mechanisms.

It is striking that the exposure of the cells to CuSO₄ alone has no effect on cell survival, since the present results demonstrated the uptake of ⁶⁴Cu when RCSN-3 cells were incubated with copper in the presence of cell culture medium (Fig. 2). Possible explanations for the lack of toxic effects of CuSO₄ are: (i) RCSN-3 cells have a very effective system for chelating intracellular copper, preventing its participation in redox reactions, which catalyze the formation of hydroxyl radicals in the presence of hydrogen peroxide. It seems plausible that the Cu⁺ released from the Cu–dopamine complex after intracellular uptake with concomitant dopamine oxidation to aminochrome is chelated directly by metallothionein or glutathione (Schipper et al. 1998). (ii) The intracellular copper concentration is too low in the cells treated with 1 mM CuSO₄ (23 ng/mg protein) due to the high chelating capacity of the components present in the medium, which include amino acids that are known to form complex with copper (Barnea et al. 1989). (iii) Copper, like iron, may progressively accumulate in mitochondria (Schipper et al. 1998) which is promoted by dopamine (Schipper et al. 1999). However, these explanations do not clarify why copper toxicity in RCSN-3 cells is dependent on dopamine. Indeed, since the uptake is significantly lower in the absence of dopamine, copper may accumulate in the mitochondria (Schipper et al. 1998). One possible explanation of dopamine-dependent copper toxicity may be the formation of a Cu–dopamine complex which can: (i)
mediate the uptake of copper into the cell via dopamine transport system; (ii) change the redox properties of Cu²⁺ since the reduction of Cu²⁺ to Cu⁺ is favoured in the Cu–dopamine complex with the concomitant oxidation of dopamine to aminochrome (Fig. 2); and (iii) perform one-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical, which has been reported to be extremely reactive (Segura-Aguilar et al. 1998).

It is of interest to point out the neuroprotective role of DT-diaphorase since the toxicity induced by CuSO₄ + dopamine was dramatically increased (to 95%, p < 0.001) when DT-diaphorase was inhibited by dicoumarol (Fig. 2a). It should be noted that DT-diaphorase constitutes 94% of total quinone reductase activity in RCSN-3 cells and 97% in rat substantia nigra (Schultzberg et al. 1988). These results support the idea that DT-diaphorase constitutes an important cellular protective mechanism against aminochrome toxicity by preventing the formation of leukoaminochrome o-semiquinone radical (Segura-Aguilar and Lind 1989; Baez et al. 1995; Segura-Aguilar et al. 1998; Segura-Aguilar et al. 2000). Leukoaminochrome o-semiquinone radical has been demonstrated to be extremely reactive with oxygen in ESR experiments (Segura-Aguilar et al. 1998).

ESR studies using spin-trap agents revealed the formation of a radical trapped by DMPO only when the cells were incubated with CuSO₄ + dopamine + dicoumarol (Fig. 4). The spectrum of the trapped radical was different to the spectrum expected for hydroxy radicals, since the hyperfine pattern could be attributed to C-centered radical species. The question about the identity of the C-centered radical that reacted with DMPO remains unclear, but the possible radicals which may be formed under the incubations conditions are: (i) Leucoaminochrome o-semiquinone radical, which can be formed during one-electron reduction of aminochrome in the presence of the specific inhibitor of DT-diaphorase dicoumarol. Indeed, the radical of leucoaminochrome o-semiquinone radical can be delocalized and therefore this radical can be C-centered. (ii) A peptide C-centered radical can be formed when a radical draws a proton from the polypeptide chain of a protein.

The treatment of RCSN-3 cells with CuSO₄-induced changes in the regulation of mRNA expression of antioxidant enzymes. Different pathways of regulation can be indicated, since CuZn-SOD and glutathione peroxidase are downregulated while the expression of catalase mRNA was increased when the cells were treated with CuSO₄, dopamine, or CuSO₄ + dopamine. The downregulation of CuZn-SOD and glutathione peroxidase may explain why the incubation of the RCSN-3 cells with CuSO₄ and dopamine resulted in a 27% decrease of cell survival. Under these conditions DT-diaphorase reduced aminochrome to leukoaminochrome, which can also autoxidize in the presence of oxygen. However, CuZn-SOD and glutathione peroxidase prevented the autoxidation of leukoaminochrome in the cytosol, since the autoxidation of leukoaminochrome is dependent on the presence of superoxide radicals and hydrogen peroxide (Segura-Aguilar and Lind 1989; Baez et al. 1995). The chemistry of the autoxidation of leukoaminochrome was found to be completely different to that of the leukoaminochrome o-semiquinone radical, since superoxide radicals and hydrogen peroxide are mainly responsible for the autoxidation of leukoaminochrome (96% of the total oxygen-dependent autoxidation), while dioxygen is the main cause of leukoaminochrome o-semiquinone autoxidation (Segura-Aguilar and Lind 1989; Baez et al. 1995). Therefore, the presence of SOD and catalase/glutathione peroxidase prevents the autoxidation of leukoaminochrome during aminochrome reduction catalyzed by DT-diaphorase, while these antioxidant enzymes increased the rate of autoxidation of leukoaminochrome o-semiquinone during one-electron reduction of aminochrome (Baez et al. 1995). The expression of glutathione peroxidase, and in part CuZn-SOD, increased almost to control levels, indicating an acute protective action against oxidative stress (CuSO₄ + dopamine + dicoumarol). However, the final effect could be the opposite, since these enzymes reportedly accelerate the autoxidation rate and the formation of reactive oxygen species during one-electron reduction of aminochrome (Baez et al. 1995). These results support the idea that superoxide dismutase and glutathione peroxidase play a pro-oxidant role during one-electron reduction of aminochrome (Baez et al. 1995; Segura-Aguilar et al. 2000). The differences in the regulation of catalase mRNA expression observed may be explained by the fact that catalase is mainly located in peroxisomes, while the activation of aminochrome should occur in the cytoplasm. Interestingly, the regulation of Mn-SOD mRNA was also different in comparison to CuZn-SOD since it decreased when RCSN-3 cells were treated with dopamine or CuSO₄ + dopamine + dicoumarol. However, no effect was observed in the presence of CuSO₄ or CuSO₄ + dopamine. This differential regulation may be explained by the fact that aminochrome is metabolically activated to the leukoaminochrome o-semiquinone radical in the cytosol.

The possible mechanism of action of CuSO₄ toxicity on RCSN-3 cells is explained in Fig. 9. CuSO₄ is dissociated to Cu²⁺ which then complexes with dissociated dopamine, which mediates the copper uptake into the cell via a dopamine transport system. Inside the cell, the dopamine–Cu complex is decomposed during a redox reaction where dopamine is oxidized to dopamine o-quinone and Cu²⁺ is reduced to free Cu⁺ under the catalytic action of Cu²⁺. Free Cu⁺ may rapidly react with dioxygen to form Cu(I) O₂⁺, which is a better oxidizing agent than catechol (Balla et al. 1992). The formed dopamine o-quinone is spontaneously cyclized to form aminochrome. Aminochrome can be reduced by one-electron transfer quinone reductases to the leukoaminochrome o-semiquinone radical, which can in
turn react with biomolecules via redox cycling and induce toxicity. The dopamine-dependence of the CuSO₄ effect on the decreased survival of dopaminergic cells of substantia nigra (RCSN-3), and the finding that this effect is dependent on one-electron reduction of aminochrome suggests that copper may be neurotoxic to dopaminergic systems through aberrant oxidation resulting from nonphysiological levels of copper and inhibition or low levels of expression of DT-diaphorase. Therefore, it may contribute to the death of dopaminergic neurons and this further suggests that oxidation-dependent DNA damage may be the basis for this cell death (Snyeder and Friedman 1998). These findings may explain why copper-workers exposed to high concentrations of copper exhibit degeneration of dopaminergic neuronal systems which leads to parkinsonism.

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References


