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 Gutteridge 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 Gutteridge 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

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Abbreviations used: DAT, dopamine transport system; DME, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethylpyrroline-*N*-oxide; EB, ethidium bromide; ESR, electron spin resonance; FA, fluorescein diacetate; PD, Parkinson's disease; SOD, superoxide dismutase.

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 leukoaminochrome *o*-semiquinone by reducing aminochrome to leukoaminochrome (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 transfering 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 (Ernster 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 leukoaminochrome *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). $^{64}CuSO_4$ 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/cm^2. The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO_2. The cells were grown in Dulbecco's modified Eagle's medium (DME)/nutrient mixture HAM F_{12} (1 : 1), 10% bovine serum, 2.5% fetal bovine serum, and 40 mg/L gentamicine sulfate (Dagnino-Subiabre *et al*. 2000).

Toxicity

For $CuSO_4$ 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 $CuSO_4$, 100 μM dopamine, and 100 μM dicoumarol. The cells were visualized at 100 × magnification in a Nikon Diaphot inverted microscope equipped with phase contrast optics and fluorescence. The $CuSO_4$ 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 $CuSO_4$, 200 μM $CuSO_4$, and 200 μM dopamine; or 200 μM $CuSO_4$, 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 $(dT)_{20}$ 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

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′-CTCAGGAGAGCATTCCATCATTG-3′ (upstream) and 5′-ATCACACCAC AAGCCAAGCG-3′ (downstream) designed from the cDNA sequence of rat CuZn-superoxide dismutase (CuZn-SOD) (Hass *et al*. 1989); 5′-CGCCTTTTTGCTTACCCAGAC-3′ (upstream) and 5′-AGAATGTCCGCACCTGAGTGAC- 3′ (downstream) designed of rat catalase (Furuta *et al*. 1986); 5′-ACAGTC-CACCGTGTATGCCTTC- 3′ (upstream) and 5′-TCTTGCCATTCTCCTGATGTCC-3′ designed of rat glutathione peroxidase (Christensen and Burgener 1992); 5′-AGTCCAAA-AGCAAGGTGCCAACTG-3′ (upstream) and 5′-TCATTCCC-CACTTCTGCCTGTGAC-3′ (downstream) designed for rat Mn-superoxide dismutase (Mn-SOD) (Ho *et al*. 1991); 5′-CAGAAACGACA TCACAGGGGA G-3′ (upstream) and 5′-CAAGCACTCTCTCAAACCAGCC-3′ (downstream) designed of rat DT-diaphorase (Bayney *et al*. 1989). 5′-TTTGTGATGGGTGT-GAACCACGAG-3′ (upstream) and 5′-CCAGCATCAAAGGTG-GAAGAATGG-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, 65°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, 65°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 mM dNTP each, 3 mM $MgCl_2$, 2.5 μM primers, 10 × 5 μL PCR-buffer (GibcoBRL, Rockville, MD, USA), 29 μL H_2O and 2 U Taq polimerasa (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.

$^{64}CuSO_4$ uptake into RCSN-3 cells

The uptake of $^{64}CuSO_4$ into RCSN-3 cells was measured under the same conditions used to study cell viability. $^{64}CuSO_4$ (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 $CuSO_4$ was 1 mM (50 μM $^{64}CuSO_4$) 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 $CuSO_4$ 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 $^{64}CuSO_4$ 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 $^{64}CuSO_4$ 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 mM DMPO before the addition of 100 μM dopamine, 1 mM $CuSO_4$ and 100 μM dicoumarol. The cells were incubated 1 h prior to ESR spectroscopy.

Spectrum Cu-dopamine complex

The spectra of $CuSO_4$-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 $CuSO_4$ (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 $CuSO_4$ 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 menadiol to menadione, and the reduction of cytochrome *c* was followed at 550 nm (extinction coefficient of cytochrome *c* was 18.5 mM/cm). RCSN-3 cells were incubated in the presence and absence of 100 μM dicoumarol 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 $CuSO_4$ and a neuronal cell line (RCSN-3) derived from adult rat substantia nigra. The possibility that $CuSO_4$ catalyzes the oxidation of dopamine was studied *in vitro* by monitoring the spectral changes when dopamine was incubated with $CuSO_4$ under the same conditions used for cell culture. The addition of 1 mM $CuSO_4$ 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.

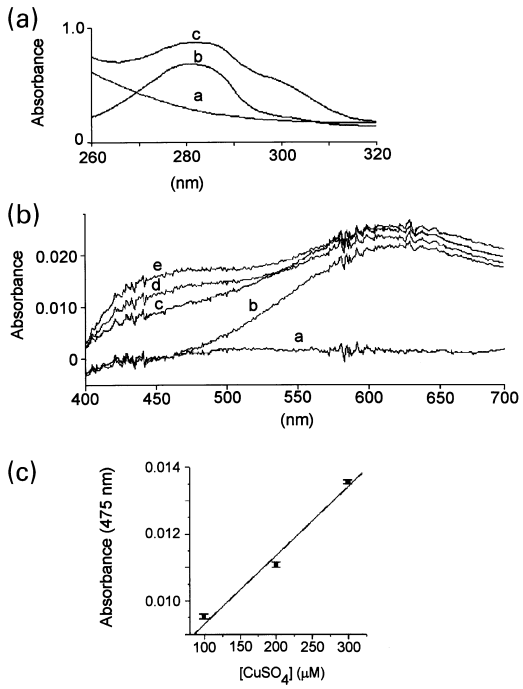

Fig. 1 Cu-dopamine complex spectrum. (a) The spectrum of Cu-dopamine complex in the ultraviolet region. a, 300 μM dopamine with cell culture medium; b, Cu–medium complex after mixing 200 μM $CuSO_4$ with cell culture medium; c, Cu–dopamine complex after mixing 300 μM dopamine and 200 μM $CuSO_4$ with cell culture medium. (b) The spectrum of Cu–dopamine complex at visible region. a, 300-μM dopamine in cell culture medium; b, 300 μM $CuSO_4$ in cell culture medium; c, after addition of 100 μM dopamine; d, after addition of 200 μM dopamine; e, after addition of 300 μM dopamine. (c) $CuSO_4$ dependence of aminochrome formation. The concentration of dopamine was 100 μM. The experimental conditions are described under Materials and methods.

However, a new spectrum appeared when 100 μM dopamine was added to the medium before the addition of $CuSO_4$, 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 $CuSO_4$ (Fig. 1C).

For studying the possible neurotoxic effects of $CuSO_4$ in the RCSN-3 cell line, the cells were treated with 1 mM $CuSO_4$ 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 $CuSO_4$ increased to 23 ng/mg protein determined by both atomic absorption spectrometry and ^{64}Cu 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 $CuSO_4$ 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 $CuSO_4$ 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 $^{64}CuSO_4$

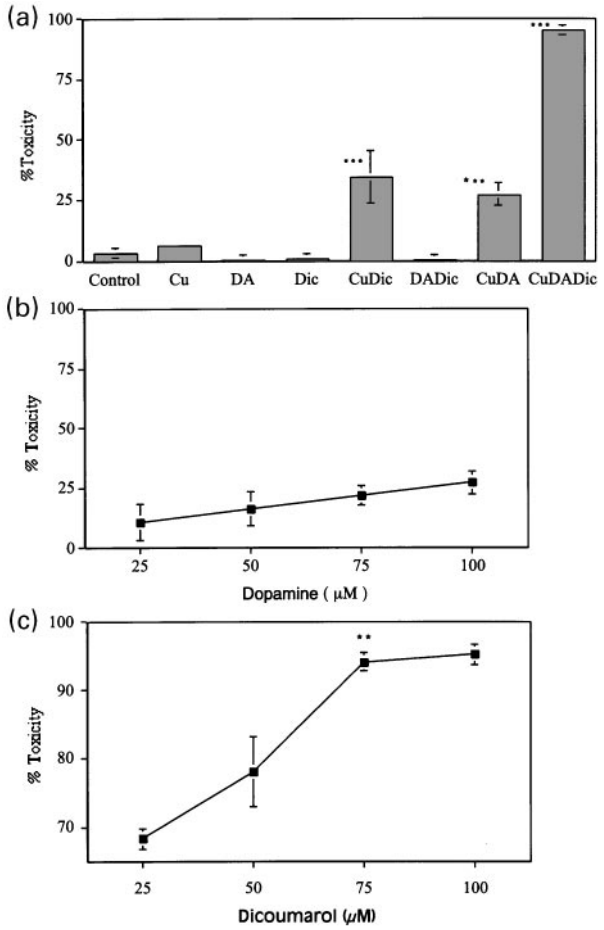

Fig. 2 The effect of $CuSO_4$ on RCSN-3 cell viability. (a) The cells were incubated with 1 mM $CuSO_4$ (Cu); 100 μM dopamine (DA); 100 μM dicoumarol (Dic); 1 mM $CuSO_4$ and 100 μM dicoumarol (CuDic); 1 mM $CuSO_4$ and 100 μM dopamine (CuDA); 1 mM $CuSO_4$, 100 μM dopamine and 100 μM DIC (CuDADic) during 2 h as described under Material and methods. (b) Dopamine-dependent cell survival. The concentration of $CuSO_4$ was 1 mM. (c) Dicoumarol-dependent cell survival. The incubation contained of 1 mM $CuSO_4$ and 100 μM dopamine. The statistical significance was assessed by using unpaired Student's *t*-test (***$p < 0.001$) compared with controls (a) and **$p < 0.01$ compared with 25 and 75 μm dicoumarol (b).

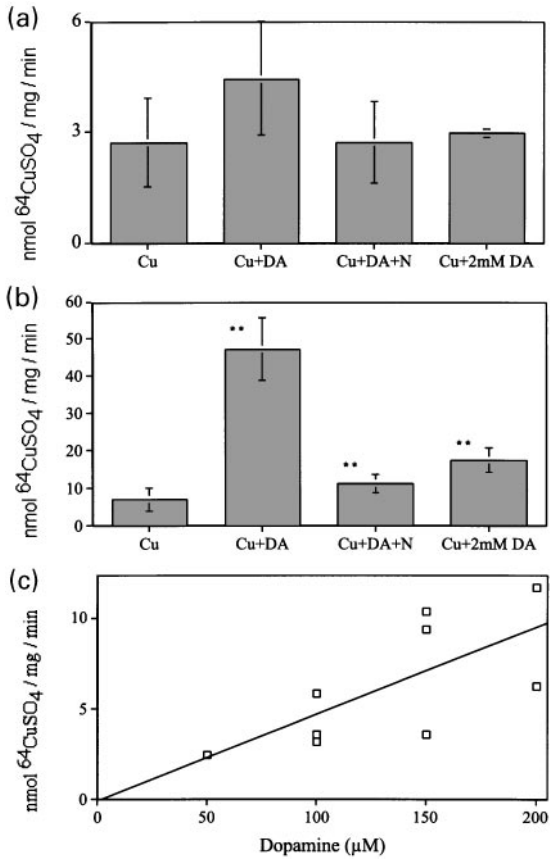

Fig. 3 $^{64}CuSO_4$ uptake into RCSN-3 cells. (a) ^{64}Cu uptake was measured in cell culture medium (a and c) and in Hank's solution (b) as described under Materials and methods. RCSN-3 cells were incubated with $^{64}CuSO_4$ (Cu); $^{64}CuSO_4$ and dopamine (Cu + DA); $^{64}CuSO_4$, dopamine and 2 μM nomifensine (Cu + DA + N); and $^{64}CuSO_4$, 2 mM dopamine (Cu + 2 mM DA). The statistical significance was assessed by using Student's *t*-test (***$p < 0.001$).

with 100 μM dopamine resulted in uptake of the ^{64}Cu–dopamine complex, which was increased in comparison to the ^{64}Cu–medium complex uptake. In addition, incubation of $^{64}CuSO_4$ 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 ^{64}Cu and a clear uptake dependence on the formation of ^{64}Cu–dopamine complex. The uptake was significantly increased by near seven-fold in the presence of dopamine (^{64}Cu–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 ^{64}Cu–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 $CuSO_4$ toxicity (Fig. 1B). We studied the possible involvement of a one-electron reduction of aminochrome in the $CuSO_4$ 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 $CuSO_4$ 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 $CuSO_4$ 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 $CuSO_4$ 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, $CuSO_4$ 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 $CuSO_4$ (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

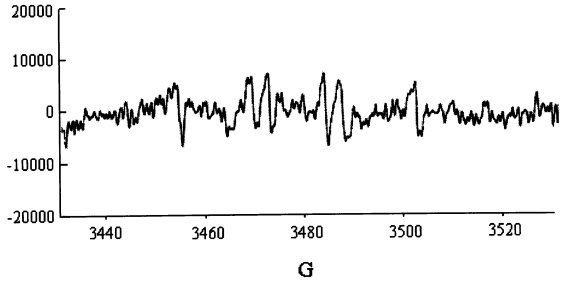

Fig. 4 Determination of intracellular formation of free radicals during incubation of RCSN-3 cells with $CuSO_4$, dopamine and dicoumarol. Intracellular ESR signal of DMPO spin-trapped radical. Spectrometer conditions as described in Material and methods.

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 $CuSO_4$, dopamine, or $CuSO_4$ + 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 $CuSO_4$, dopamine, or $CuSO_4$ + dopamine, respectively. However, the expression of glutathione peroxidase mRNA increased near to the control level when RCSN-3 cells were treated with $CuSO_4$ + dopamine in the presence of 100 μM dicoumarol. The expression of catalase mRNA increased when RCSN-3 cells were treated with $CuSO_4$, 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 $CuSO_4$ + dopamine or

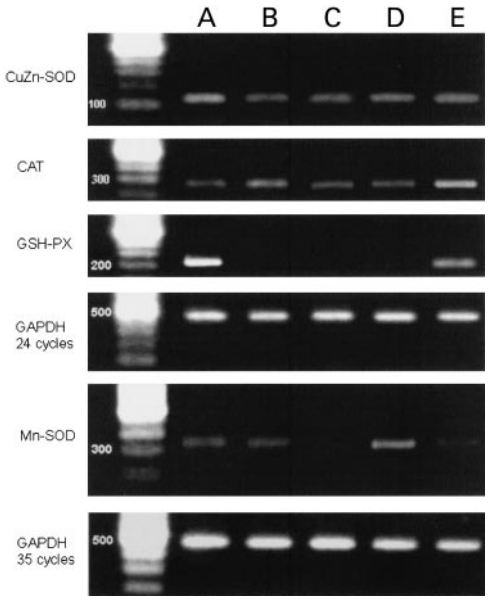

Fig. 5 Expression of antioxidant enzymes mRNA in RCSN-3 cells. The expression of CuZn-SOD, catalase (CAT), glutathione peroxidase (GSH-PX), Mn-SOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured under normal conditions (A) and after treatment with 1 mM $CuSO_4$ (B); 100 μM dopamine (C); 1 mM $CuSO_4$, 100 μM dopamine (D) 1 mM $CuSO_4$, 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.

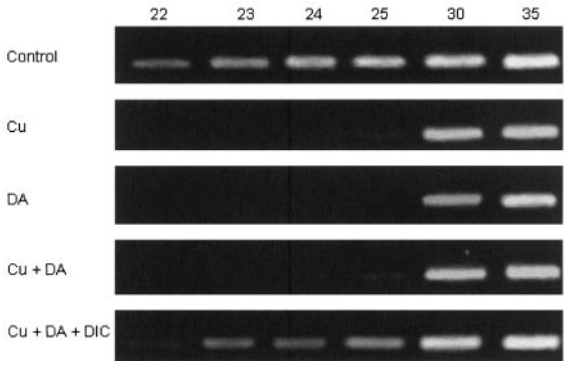

Fig. 6 Expression of glutathione peroxidase mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of glutathione peroxidase of control RCSN-3 cells was compared with cells treated with 1 mM $CuSO_4$ (Cu); 100 μM dopamine (DA); 1 mM $CuSO_4$ and 100 μM dopamine (Cu + DA); 1 mM $CuSO_4$, 100 μM dopamine and 100 μM dicoumarol (Cu + DA + DIC). Experimental procedures are described under Materials and methods.

$CuSO_4$ + dopamine + dicoumarol and at 20 cycles after treatment with $CuSO_4$ (Fig. 7). A decrease in the expression of CuZn-SOD mRNA was observed when the cells were treated with $CuSO_4$ 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 $CuSO_4$, dopamine, $CuSO_4$ + dopamine, and $CuSO_4$ + 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 $CuSO_4$ + 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,

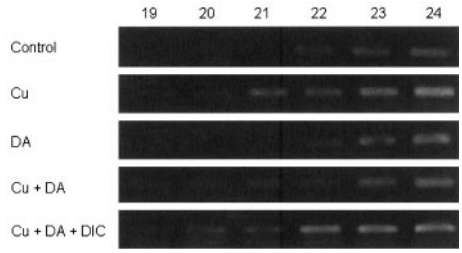

Fig. 7 Expression of catalase mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of catalase in control RCSN-3 cells was compared with cells treated with 1 mM $CuSO_4$ (Cu); 100 μM dopamine (DA); 1 mM $CuSO_4$, 100 μM dopamine (Cu + DA) 1 mM $CuSO_4$, 100 μM dopamine and 100 μM dicoumarol (Cu + DA + DIC). Experimental procedures are described in Materials and methods.

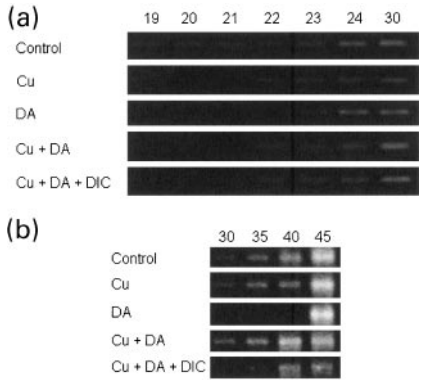

Fig. 8 Expression of CuZn-SOD (a) and Mn-SOD (b) mRNA in RCSN-3 cells with PCR at different number of cycles. The expression of SOD of control RCSN-3 cells was compared after treating cells with 1 mM $CuSO_4$ (Cu); 100 μM dopamine (DA); 1 mM $CuSO_4$ + 100 μM dopamine (Cu + DA); 1 mM $CuSO_4$ + 100 μM 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 $CuSO_4$ 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 aminochrome; and (iii) inhibition of DT-diaphorase. The intracellular mode of action of $CuSO_4$ is supported by the strong effect of dicoumarol in the $CuSO_4$-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 $CuSO_4$ 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. ^{64}Cu 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; Wieczorek and Kruk 1994), inhibited dopamine-mediated uptake of ^{64}Cu into RCSN-3 cells. The present results also suggest the existence of a mechanism of Cu uptake which is not mediated by dopamine, since ^{64}Cu uptake into RCSN-3 was also observed in the absence of dopamine, although this mechanism was only responsible for a portion of the total ^{64}Cu uptake (Fig. 3). The ^{64}Cu uptake into RCSN-3 cells incubated with cell culture medium in the absence of dopamine may be mediated by the formation of ^{64}Cu complex with some amino acids (histidine, lysine, arginine) present in the cell culture medium, which are known to form complex with Cu^{2+} (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 ^{67}Cu 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 ^{64}Cu 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 $CuSO_4$ alone has no effect on cell survival, since the present results demonstrated the uptake of ^{64}Cu 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 $CuSO_4$ 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 $CuSO_4$ (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 Cu^{2+} since the reduction of Cu^{2+} 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 $CuSO_4$ + 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 $CuSO_4$ + 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 $CuSO_4$-induced changes in the regulation of mRNA expression of antioxidant enzymes. Different pathways of regulation can be indicated, since CuZn-SOD and glutathione peroxidase were downregulated while the expression of catalase mRNA was increased when the cells were treated with $CuSO_4$, dopamine, or $CuSO_4$ + dopamine. The downregulation of CuZn-SOD and glutathione peroxidase may explain why the incubation of the RCSN-3 cells with $CuSO_4$ 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 ($CuSO_4$ + 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 $CuSO_4$ + dopamine + dicoumarol. However, no effect was observed in the presence of $CuSO_4$ or $CuSO_4$ + 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 $CuSO_4$ toxicity on RCSN-3 cells is explained in Fig. 9. $CuSO_4$ is dissociated to Cu^{2+} 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 Cu^{2+} is reduced to free Cu^{+} under the catalytic action of Cu^{2+}. Free Cu^{+} may rapidly react with dioxygen to form Cu(I) O_2^{+}, 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

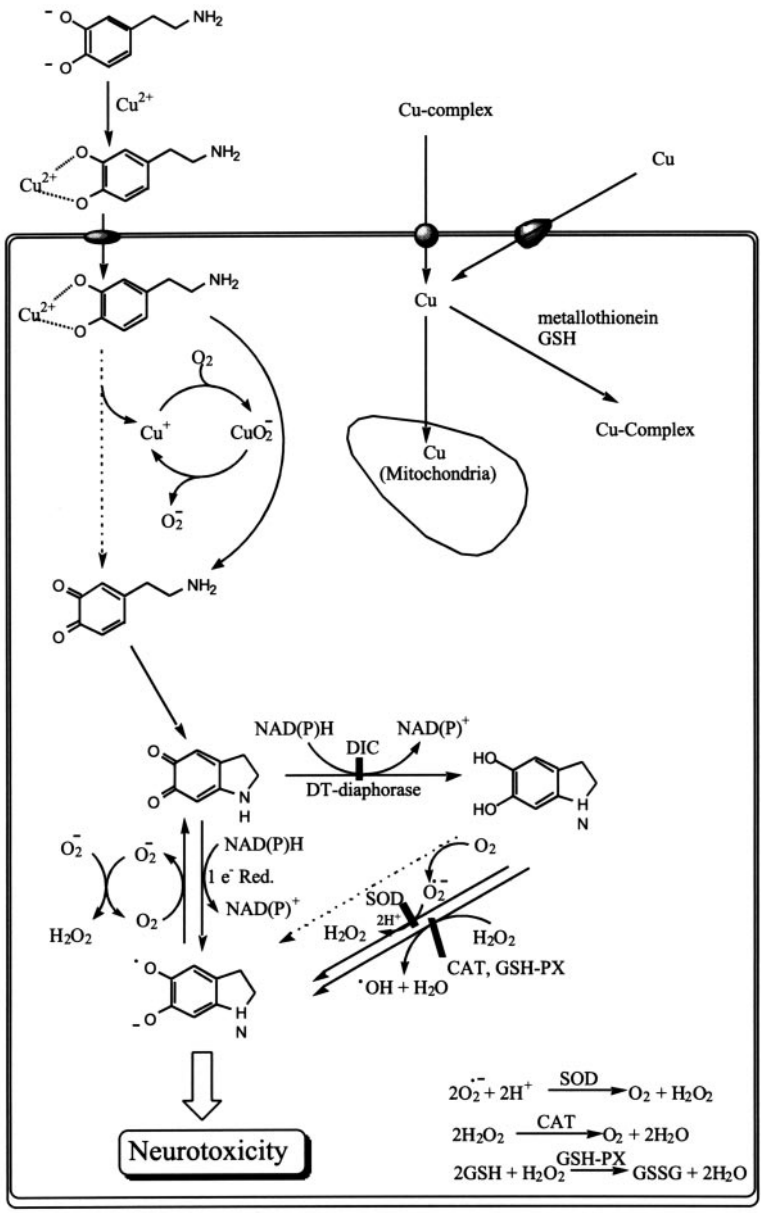

Fig. 9 Possible mechanism of intracellular action of $CuSO_4$ in RCSN-3 cells.

turn react with biomolecules via redox cycling and induce toxicity. The dopamine-dependence of the $CuSO_4$ 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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