On the neurotoxicity mechanism of leukoaminochrome *o*-semiquinone radical derived from dopamine oxidation: mitochondria damage, necrosis, and hydroxyl radical formation

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Leukoaminochrome *o*-semiquinone radical is generated during one-electron reduction of dopamine oxidation product aminochrome when DT-diaphorase is inhibited. Incubation of 100 μM aminochrome with 100 μM dicoumarol, an inhibitor of DT-diaphorase during 2 h, induces 56% cell death ($P < 0.001$) with concomitant formation of (i) intracellular hydroperoxides (4.2-fold increase compared to control; $P < 0.001$); (ii) hydroxyl radicals, detected with ESR and spin trapping agents (2.4-fold increase when cells were incubated with aminochrome in the presence of dicoumarol compared to aminochrome alone); (iii) intracellular edema, and cell membrane deterioration determined by transmission electron microscopy; (iv) absence of apoptosis, supported by using anexin-V with flow cytometry; (v) a strong decrease of mitochondrial membrane potential determined by the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide ($P < 0.01$); (vi) swelling and disruption of outer and inner mitochondrial membranes determined by transmission electron microscopy. These results support the proposed role of leukoaminochrome *o*-semiquinone radical as neurotoxin in Parkinson's disease neurodegeneration and DT-diaphorase as neuroprotective enzyme.

Keywords: Neurodegeneration; Dopamine; Neurotoxicity; DT-diaphorase; Parkinson's disease; Mitochondria; Necrosis; Free radicals

Abbreviations: Mn^{3+}, Manganese pyrophosphate complex; MAO, monoamine oxidase; SOD, superoxide dismutase; VMAT, Vesicular monoamine transporter; DAT, dopamine transporter.

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Introduction

Parkinson's disease is a neurodegenerative disease characterized by degeneration of the nigro-striatal dopaminergic system. Although it is generally accepted that free radicals are involved in the neurodegenerative process affecting the nigro-striatal system of Parkinson's patients, the exact mechanism of neurodegeneration in vivo is still unknown (Facchinetti et al., 1998; Foley and Riederer, 2000; Gassen and Youdim, 1999; Ilic et al., 1999; Jenner, 1998; Selley, 1998; Venarucci et al., 1999). One possible endogenous source of free radicals in the degenerative processes underlying Parkinson's disease may involve the oxidation of dopamine to the *o*-quinone aminochrome (Foppoli et al., 1997; Galzigna et al., 2000; Graham, 1978; Hastings, 1995; Hawley et al., 1967; Paris et al., 2001; Rabinovic et al., 2000; Segura-Aguilar, 1996; Segura-Aguilar and Lind, 1989; Smythies et al., 2002; Thompson et al., 2000) and the formation of reactive oxygen species during one-electron reduction of aminochrome. One-electron reduction of aminochrome has been proposed to be one of the major sources for endogenous generation of reactive species involved in the degenerative process leading to Parkinson's disease (Baez et al., 1995; Paris et al., 2001; Segura-Aguilar et al., 1998, 2001). In dopaminergic neurons, intracellular dopamine is efficiently incorporated into vesicles for neuronal transmission. The low pH inside of the vesicles prevents oxidation of the catechol structure of dopamine to the *o*-quinone aminochrome. Saturation of dopamine uptake into vesicles and monoamine oxidase (MAO) metabolism allows intracellular dopamine autoxidation to aminochrome, which in turn is the precursor of neuromelanin in dopaminergic neurons. The formation of the latter is a normal process while we can prevent one-electron reduction of aminochrome. DT-diaphorase (EC.1.6.99.2) has been reported to prevent one-electron reduction of aminochrome to

leukoaminochrome *o*-semiquinone radical by reducing aminochrome with two-electrons to leukoaminochrome (Segura-Aguilar and Lind, 1989). This enzyme is localized both in dopaminergic neurons and glia cells (Schultzberg et al., 1988) and prevents toxic effects of $CuSO_4$ in RCSN-3 cells (Paris et al., 2001). Inhibition of DT-diaphorase by dicoumarol induces a 6-OH-dopamine like contralateral rotation and an extensive loss of tyrosine hydroxylase staining in rats injected intracerebrally with manganese^{3+} pyrophosphate (Mn^{3+}) into medial forebrain bundle and substantia nigra, respectively (Díaz-Véliz et al., 2004; Segura-Aguilar et al., 2002).

In the present work, we investigated the mechanism of neurotoxic effects of leukoaminochrome *o*-semiquinone radical generated during one-electron reduction of aminochrome in cells derived from rat substantia nigra (RCSN-3) and the possible protective role of DT-diaphorase. To oxidize dopamine to aminochrome, we have used Mn^{3+} as an oxidizing agent (Segura-Aguilar and Lind, 1989), since manganese is an essential element for brain development and other biological functions (Hurley, 1981; Rogers et al., 1985) but it can also induce parkinsonism in subjects exposed to high concentrations of this transition metal (Juncos et al., 1968; Pal et al., 2001).

Materials and methods

Chemicals

Dopamine, nomifensine, dicoumarol, DME/HAM-F12 nutrient mixture (1:1), terminal transferase, and 5,5-dimethylpyrroline-*N*-oxide (DMPO) were purchased from Sigma Co. (St. Louis, MO, USA). Calcein AM, ethidium homodimer-1, diphenyl-1-pyrenylphosphide, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide, Annexin V kit were from Molecular Probes (Eugene, OR, USA). Thermoscript RT-PCR system and Taq DNA polymerase were obtained from Life Technologies (California, USA). RNeasy MIDI system was obtained from QIAGEN (Hilden, Germany). The primers were obtained from T-A-G-Copenhagen A/S (Copenhagen, Denmark). Manganese^{3+}-pyrophosphate complex (Mn^{3+}) was prepared according to standard procedures (Archibald and Fridovich, 1982). Aminochrome was prepared directly in the cell culture medium immediately before addition to the cells by adding 100 μM dopamine and excess of Mn^{3+}. Despite the fact that 400 μM Mn^{3+} is required to oxidize 100 μM dopamine in water, we have used 1 mM Mn^{3+} to be sure that all dopamine is converted in the cell culture medium to aminochrome. The reaction was allowed to proceed for 2 min (Segura-Aguilar and Lind, 1989).

Cell culture

The RCSN-3 cell line was derived from the substantia nigra of a 4-month-old normal Fisher 344 rat. The RCSN-3 cell line grows in monolayers, with a doubling time of 52 h, a plating efficiency of 21%, and a saturation density of 56.000 cells/cm^2 when kept in normal growth media composed of: DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, 40 mg/l gentamicine sulphate (Dagnino-Subiabre et al., 2000; Paris et al., 2001). The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO_2.

Aminochrome uptake

The uptake of ^{3}H-aminochrome into RCSN-3 cells was measured under the same conditions used to study cell viability. ^{3}H-aminochrome was formed by adding 100 μM dopamine (1 μCi ^{3}H-dopamine) to cell culture medium containing 1 mM Mn^{3+}, and allowing 2 min for dopamine oxidation to proceed at room temperature. ^{3}H-aminochrome was added to each dish and the uptake was assessed after an incubation period of 1 min at room temperature. At the end of the uptake period, the extracellular medium was removed and the cells were rapidly washed five times with 2-ml medium to remove residual ^{3}H-aminochrome-tracers. Afterwards, cell membranes were disrupted by adding 1 ml of 1% Triton X-100. After 15 min of incubation, 900 μl of the cell/Triton X-100 extract was removed and analyzed for ^{3}H-aminochrome-tracer content by liquid scintillation counting. The remaining 100 μl were used for protein determination by bicinchoninic acid method (Pierce, Rockford, IL, USA). The results were expressed in nmol/mg protein/min. ^{3}H-aminochrome was also incubated in the presence of dopamine transport (DAT) inhibitor 2 μM nomifensine.

Cell death

For aminochrome toxicity experiments, the cells were incubated with cell culture medium but in the absence of bovine serum and phenol red for 120 min. The concentration used for toxicity experiments was 100-μM aminochrome in the presence and absence of 100-μM dicoumarol. For control conditions, we used 100-μM dopamine, 1 mM Mn^{3+}, or 100 μM dicoumarol incubated alone. The cells were visualized at 100× magnification in a Nikon Diaphot inverted microscope equipped with phase contrast and fluorescence optics. Manganese toxicity was measured by counting live and dead cells after staining with 120 μM Calcein AM for 4 min at 37°C, followed by incubation with 25 μM ethidium heterodimer-1 for 1 min at 37°C. Calceine is a marker for live cells, and ethidium heterodimer-1 intercalates in the DNA of dead cells. The cells were counted in a phase contrast microscope equipped with fluorescence, using the following filters: calcein AM, 510–560 nm (excitation) and LP-590 nm (emission); ethidium heterodimer-1, 450–490 nm (excitation), and 515–565 nm (emission).

Hydroperoxide formation

DPPP was used as a fluorescent probe that reacts specifically with hydroperoxides and becomes highly fluorescent when oxidized. The cells were washed twice with PBS and placed in a standard quartz cuvette (10 × 10 mm) containing 50 μM DPPP in PBS. After incubation for 10 min at 37°C, the cells were washed twice with fresh PBS and the increase in fluorescence (excitation, 351 nm; emission 380 nm; excitation slit, 1 nm; emission slit, 3 nm) was measured.

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 5 min with 100 mM DMPO before the addition of (i) 1 mM

Mn^{3+}; (ii) 100 μM aminochrome; (iii) 100 μM aminochrome 100 μM dicoumarol. The cells were incubated 1 h before ESR spectroscopy.

Transmission electron microscopy

Cells were grown and suspended in culture medium before the application of experimental conditions. Cells, 1×10^6, in a final volume of 1 ml were used in the different conditions studied. After the treatments, cells were pelleted and fixed in 3% glutaraldehyde for 60 min, washed three times and post-fixed in osmium tetroxide 1% for 60 min. The cells were dehydrated in an ascending ethanol battery ranging from 20% to 100% and were later placed in acetone for final embedding in epon-812 resin. Ultrathin sections were made and impregnated with 1% uranyl acetate and Reynold's lead citrate. The sections were visualized in a Zeiss EM-900 transmission electron microscope at 50 kV, photographed, the negatives were scanned at 600 × 600 ppi resolution, and the images obtained were analyzed later in a PC-compatible computer using a customized software.

Annexin V staining and flow cytometry

RCSN-3 cells were treated with 100 μM dopamine, 100 μM dicoumarol, 1 mM Mn^{3+}, 100 μM aminochrome, or 100 μM aminochrome together with 100 μM dicoumarol for 2 h at 37°C. Negative controls were carried out by incubating the cells in cell culture medium, and positive controls were established by incubating the cells with 100 μM menadione for 4 h. Apoptosis was determined by staining with Alexa Fluor 488-labeled annexin V (Vybrant™ Apoptosis Assay Kit #2, Molecular Probes). After the incubations, floating as well as adherent cells that were later trypsinized were pooled and centrifuged for 5 min at 1000 × *g*. Pelleted cells were washed in cold phosphate-buffered saline (PBS). Thereafter, cells were centrifuged again for 5 min at 1000 × *g* and resuspended in 100 μl 1× Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ pH 7.4) yielding a cell density of 1 10^6 cells/ml. Five microliters of annexin V conjugate and 1 μl of 100 μg/ml PI working reagent were added to each 100 μl of cell suspension. The cells were incubated at room temperature for 15 min. After the incubation period, 400 μl of 1× Annexin-Binding Buffer was added and mixed gently, and the samples were kept on ice. The stained cells were analyzed by flow cytometry, where the fluorescence emission was measured at 530 nm (Alexa Fluor 488). The percentage of cells was calculated using the Cellquest software (BD Biosciences, Franklin Lakes, NJ).

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was assessed in RCSN-3 cells using the JC-1 probe, a cationic chemical that exists as a green-fluorescing monomer at low membrane potentials (<120 mV) and as a red-fluorescing dimer at membrane potentials greater than 180 mV (Reers et al., 1995). The cells were treated with 100 μM dopamine, 100 μM dicoumarol, 1 mM Mn^{3+}, 100 μM aminochrome, or 100 μM aminochrome together with 100 μM dicoumarol. Following excitation at 488 nm, the ratio of red (595-nm emission) to green (525-nm emission) fluorescence determines the ratio of high-to-low mitochondrial membrane potential (Reers et al., 1995). JC-1 (5.0 μM) was added to the cells and they were incubated for 30 min at room temperature in the dark with gentle agitation. Cells were then washed twice with fresh media and resuspended in 0.5 ml culture media without phenol red.

RT-PCR

The mRNA expression of Cu/Zn-SOD; Mn-SOD, catalase, glutathione peroxidase, DT-diaphorase were studied in RCSN-3 cells by using the RT-PCR technique as we described before (Paris et al., 2001). The cells were incubated with (i) cell culture medium alone (ii) 1 mM Mn^{3+}; (iii) 100 μM aminochrome; or (iv) 100 μM aminochrome and 100 μM dicoumarol for 10 min before the extraction of total RNA. The region of Mn-SOD amplified by PCR spanned between bases 259–282 and 590–613, which resulted in a fragment of 355 bp. Actin was amplified between bases 1725–1745 (5′-TTGGGTCAACTTCTCAGCACG-3′) and 1996–2017 (5′-AGGACAGGGGCTCCATTTAGAC-3′), which resulted in a fragment of 292 bp used as housekeeping gene.

Data analysis

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

Results

Aminochrome was formed in the medium immediately prior addition to the cells by oxidizing dopamine with Mn^{3+}. To determine whether the action of aminochrome formed in the cell culture medium is extracellular or intracellular, we studied aminochrome uptake into RCSN-3 cell by using ^{3}H-aminochrome (1 μCi). The uptake of 100 μM aminochrome (1 μCi ^{3}H-aminochrome) is higher than 100 μM dopamine alone (1 μCi ^{3}H-dopamine) but not statistically significant. Incubation of RCSN-3 cells with 2 μM nomifensine, a specific DAT inhibitor, reduced aminochrome uptake by 80% ($P < 0.001$) (Fig. 1). To study

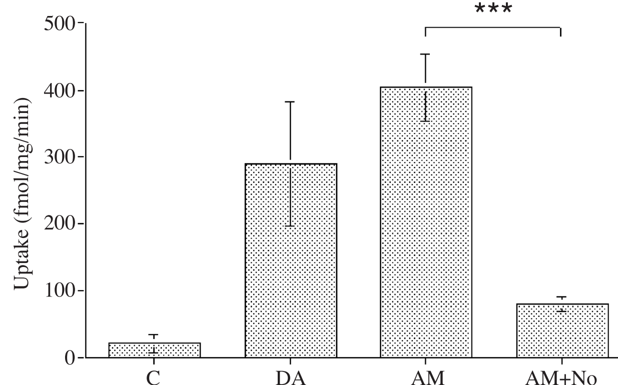

Fig. 1. Aminochrome uptake into RCSN-3 cells. The uptake of aminochrome into RCSN-3 cells was measured in cells kept in cell culture medium without bovine serum, as described under Materials and methods. RCSN-3 cells were incubated with ^{3}H-aminochrome (AM) or ^{3}H-dopamine (DA). The inhibition of AM uptake in the presence of nomifensine (AM+No) was statistically significant compared to AM (***$P < 0.001$). The results represent means ± SD ($n = 3$) and are expressed as fmol/mg/min. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test.

possible toxic effects of one-electron reduction of aminochrome in RCSN-3 cells, we have done incubations during 2 h since the cells do not survive longer incubation when DT-diaphorase was inhibited with dicoumarol. Incubation of RCSN-3 cells with 100 μM aminochrome induced 8.3 ± 4% cell death. However, incubation with 100 μM aminochrome together with 100 μM of the DT-diaphorase inhibitor dicoumarol, induced a significant increase in cell death (56 ± 7%; $P < 0.001$ and $P < 0.001$, compared to the control and aminochrome alone, respectively) (Fig. 2A). No significant toxic effects were observed when RCSN-3 cells were incubated solely with 100 μM dicoumarol, 100 μM dopamine or 1 mM Mn^{3+} (1.8 ± 1%, 5.8 ± 2%, and 4.4 ± 2% cell death, respectively). We tested the ability of antioxidants to prevent the cell death observed when the cells were incubated 100 μM aminochrome plus 100 μM dicoumarol by incubating the cells in the presence of 500 μM ascorbic acid or 1 mM GSH. The toxic effects observed when the cells were incubated with 100 μM aminochrome and 100 μM dicoumarol in RCSN-3 cells decreased to 26 ± 8% ($P < 0.001$) when the cells were incubated with ascorbic acid. However, as we expected GSH has no inhibitory effect on aminochrome plus dicoumarol-dependent cell death (not shown).

The toxic effects observed in RCSN-3 cells after incubation with aminochrome and dicoumarol may be explained by the formation of reactive oxygen species, like hydrogen peroxide and hydroxyl radicals because of the autoxidation of leukoaminochrome *o*-semiquinone radical formed during one-electron reduction of aminochrome (Segura-Aguilar et al., 1998). We determined the formation of hydroperoxides in RCSN-3 cells, using diphenyl-1-pyrenylphosphide (DPPP), which forms a fluorescent phosphide oxide when reacting with intracellular hydroperoxides, including those contained in membranes. A significant increase of 4.2-fold was observed in the formation of hydroperoxides when RCSN-3 cells were incubated with aminochrome and dicoumarol, compared to control conditions (31.6 ± 2.4 relative fluorescence/1000 cells, $P < 0.001$; Fig. 3). A non-significant increase was also observed when the cells were incubated with aminochrome alone (14.1 ± 0.4 relative fluorescence/1000 cells). The formation of hydroxyl radicals during aminochrome incubation (2 h) in RCSN-3 cells was determined by using ESR with the spin-trapping agent DMPO. An ESR signal corresponding to DMPO-hydroxyl radical was observed when the cells were incubated with 100 μM aminochrome in the

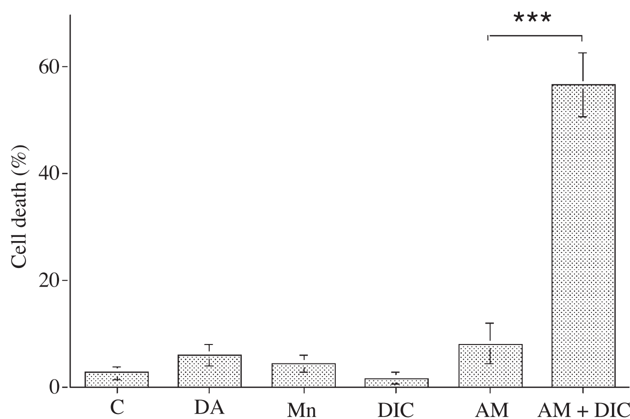

Fig. 2. Neurotoxic effects of aminochrome on RCSN-3 cells. RCSN-3 cells were treated with 100 μM aminochrome formed in the cell culture medium prior addition to the cells by oxidizing dopamine with excess Mn^{3+} in the absence and presence of 100 μM dicoumarol. For control conditions, the cells were incubated with 100 μM dopamine, 1 mM Mn^{3+}, or 100 μM dicoumarol alone. The experimental conditions are described under Materials and methods. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test. (***$P < 0.001$) $n = 5$.

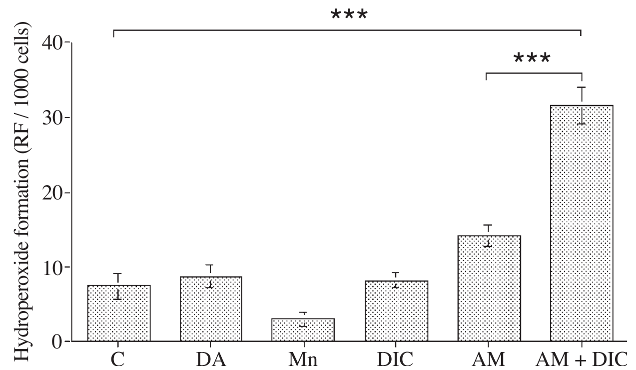

Fig. 3. Determination of hydroperoxide formation in RCSN-3. The intracellular formation of hydroperoxides was measured by using DPPP as described under Materials and methods. The results represent means ± SD ($n = 3$), expressed as relative fluorescence (RF) per 1000 cells. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test. (***$P < 0.001$.)

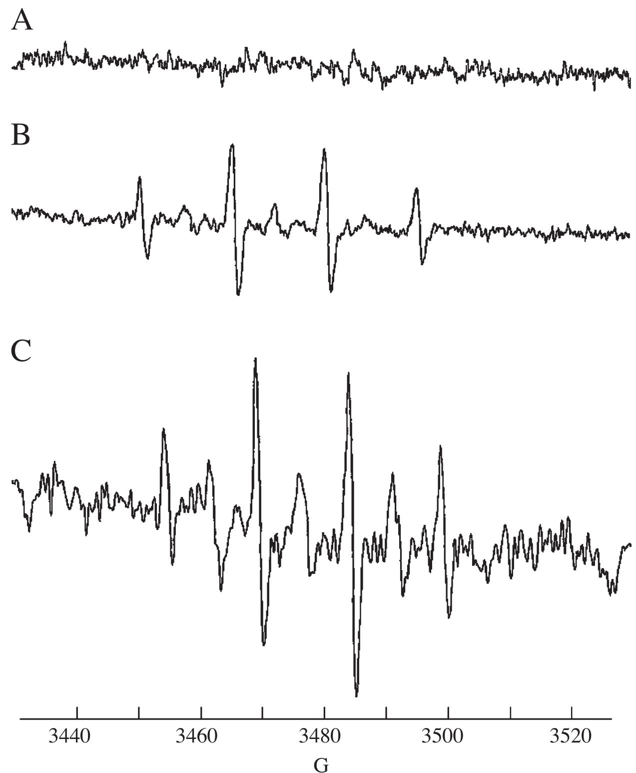

Fig. 4. Determination of hydroxyl radical-DMPO ESR signal during incubation of RCSN-3 cells. The formation of hydroxyl radicals was determined by using the spin trapping agent DMPO when RCSN-3 cells were incubated with 1 mM Mn^{3+} (A), 100 μM aminochrome (B) or 100 μM aminochrome together with 100 μM dicoumarol (C). Spectrometer conditions are described in Materials and methods.

presence and absence of 100 μM dicoumarol (Figs. 4B and C, respectively). However, the ESR signal of DMPO-OH-radicals was 2.4-fold higher ($P < 0.01$) when the cells were incubated with 100 μM aminochrome in the presence of 100 μM dicoumarol, compared to 100 μM aminochrome alone. No ESR signal was observed when the cells were incubated solely with Mn^{3+} (Fig. 4A).

To study morphological changes of RCSN-3 cells as a consequence of aminochrome toxicity, we used transmission electron microscopy after incubation times of 30 min. Cells treated with 100 μM dopamine (B), 1 mM manganese, 100 μM dicoumarol (E), and aminochrome (G) maintained traits similar to those of control conditions, namely expressed in the presence of condensed perinuclear chromatin. A fine granular cytoplasm is evident in cells treated with dicoumarol, similar to that of controls (Figs. 5E and A, respectively). Dopamine, manganese, and aminochrome (Figs. 5B, D, G, respectively) induced an increase in vacuole-containing material of undetermined origin. Also, a stronger cytosolic electron density due to an increase in the amount of free ribosomes was observed, probably due to oxidative stress produced by the aforementioned conditions. In cells treated with aminochrome, some cells exhibited apoptotic features such as blebbing of the plasma membrane (Fig. 5H). In cells treated with aminochrome plus dicoumarol, cytoarchitectural changes were observed, and intracellular edema was also present, probably due to the disruption of plasma membrane because of intense oxidative stress (Figs. 5J, K).

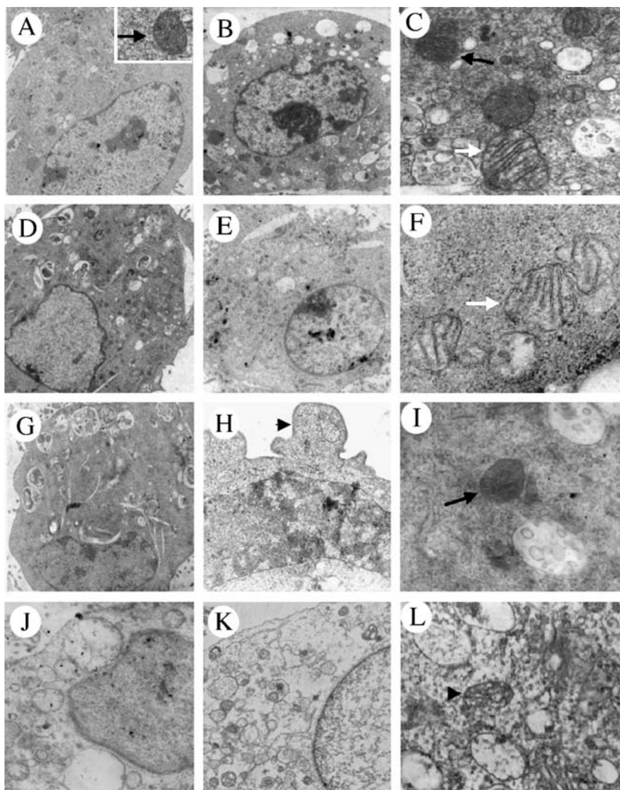

Fig. 5. Transmission electron microscopy of RCSN-3 cells treated with aminochrome. Ultrastructural changes in RCSN-3 cell line treated with aminochrome plus dicoumarol. RCSN-3 cells were incubated for 30 min with 100 μM dopamine (B), 1 mM Mn^{3+} (D), 100 μM dicoumarol (E), 100 μM aminochrome (G and H), 100 μM aminochrome plus 100 μM dicoumarol (J and K). The control was incubated with cell culture medium in the absence of bovine serum and phenol red and the inset shows a mitochondria (A). Black arrowhead: plasma membrane blebbing observed in some cells treated with aminochrome. Magnification: A, B, C, D, E and G: 7000×, F and H: 12 000×. The mitochondria are shown in RCSN-3 cells treated with 100 μM Dopamine (C), 1 mM manganese (F), 100 μM aminochrome (I): 100 μM aminochrome plus 100 μM dicoumarol (L). Black arrow: normal mitochondria, white arrow: edematic mitochondria, black arrowhead: damaged mitochondria. Magnification: 20 000×.

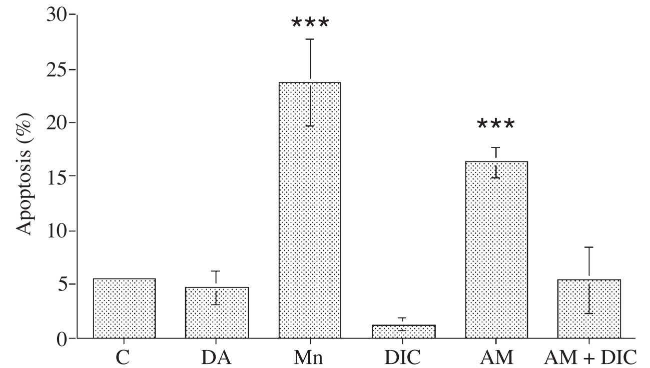

Fig. 6. Determination of apoptosis with annexin V staining using flow cytometry. RCSN-3 cells were treated with 100 μM aminochrome (AM) in the absence and presence of 100 μM dicoumarol (AM + DIC). As control, the cells were incubated with culture medium (C) 100 μM dopamine (DA), 100 μM dicoumarol (DIC) and 1 mM manganese^{3+} (Mn). Experimental procedures are described under Materials and methods. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's t test. (***$P < 0.001$); $n = 3$.

The presence of blebbing in cells incubated with aminochrome suggests a possible involvement of apoptotic processes in leukoaminochrome *o*-semiquinone toxicity. To test this hypothesis, we used Alexa Fluor 488-labeled annexin V with flow cytometry in RCSN-3 cells incubated for 2 h in experimental conditions (Fig. 6). No signal of apoptosis was observed when the cells were incubated with 100 μM aminochrome in the presence of 100 μM dicoumarol, which values were similar to those of control cells. However, 24% of cells underwent apoptosis when incubated with 1 mM Mn^{3+}.

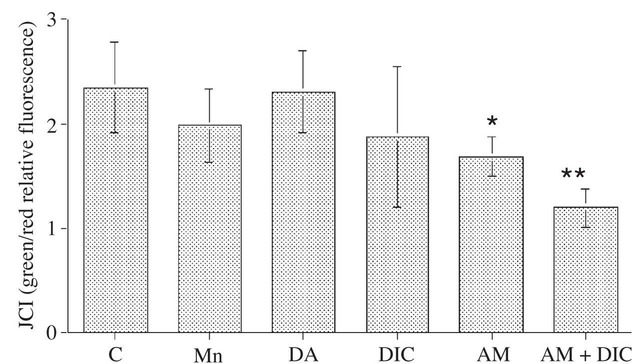

Fig. 7. Determination of changes in mitochondrial membrane potential. Changes in mitochondrial membrane potential was measured using JC-1 staining in RCSN-3 cell incubated with 1 mM Mn^{3+} (Mn); 100 μM dopamine (DA); 100 μM dicoumarol (DIC); 100 μM aminochrome (AM); 100 μM aminochrome plus 100 μM dicoumarol (AM + DIC). The values are the ratio between green/red fluorescence of JC-1. Experimental procedures are described under Materials and methods. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's t test. (*$P < 0.05$; **$P < 0.01$); $n = 7$.

Interestingly, incubation of cells with 100-μM aminochrome induced 16% apoptosis. No apoptosis was observed when the cells were incubated with 100 μM dicoumarol or 100 μM dopamine.

One possible mechanism of action in aminochrome toxicity during one-electron reduction is mitochondrial damage. To determine possible changes in the membrane potential of active mitochondria, we used the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide to measure changes in mitochondrial membrane potential. A strong reduction in mitochondrial membrane potential was observed in RCSN-3 cells treated with aminochrome in the presence of dicoumarol compared to control cells (1.2 ± 0.2, $P < 0.01$ and 2.4 ± 0.4 RF (JC-1 green/red relative fluorescence), respectively; Fig. 7). A decrease in the membrane potential was also observed when the cells were incubated with 100 μM aminochrome; 100 μM dicoumarol; or 100 μM dopamine alone; (1.7 ± 0.2, $P < 0.05$; 1.9 ± 0.7; and 2.0 ± 0.4 RF, respectively; Fig. 7), while 1 mM Mn^{3+} have no effect on membrane potential (2.3 ± 0.4 RF (Fig. 7B). The strong dissipation in mitochondrial membrane potential suggests a possible disruption of mitochondrial membrane when the cells are treated with aminochrome and dicoumarol. Transmission electron microscopy studies showed mitochondrial changes in cells treated with dopamine (Fig. 5B) or manganese (Fig. 5D). These changes include mitochondrial edema, evidenced as a loss of electron density of the mitochondrial matrix (Figs. 5A inset, B and D). Cells treated with aminochrome did not present significant changes compared to control conditions (Fig. 5K). However, when dicoumarol was added to the cells treated with aminochrome, they underwent intense mitochondrial membrane disruption (Fig. 5L).

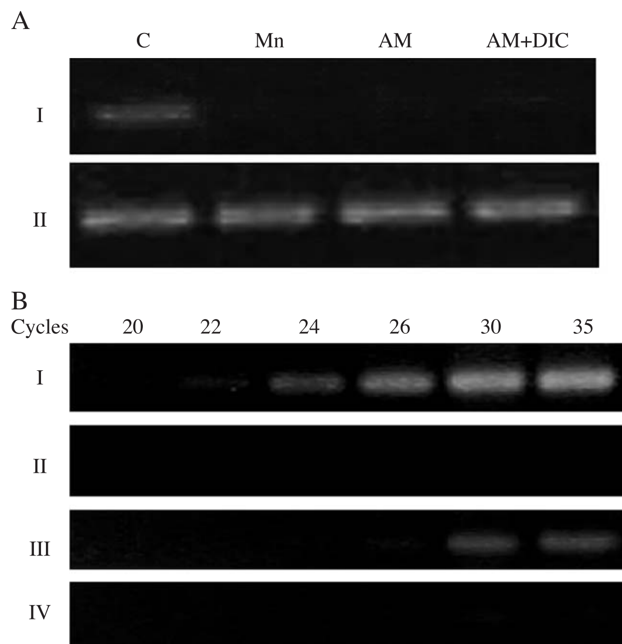

Fig. 8. Mn-SOD expression determined with semi-quantitative RT-PCR. (A.I) RCSN-3 control cells were compared to cells treated with 1 mM Mn^{3+}, 100 μM aminochrome and 100 μM aminochrome together with 100 μM dicoumarol. (A.II) The level of expression of actin. (B) Comparison of Mn-SOD expression at different cycles in RCSN-3 cells treated with 1 mM Mn^{3+} (II); 100 μM aminochrome (III); 100 μM aminochrome together with 100 μM dicoumarol (IV); and control cells (I). The RT-PRC was described under Materials and methods.

We also studied possible changes in the mRNA expression of Cu-ZN-SOD, Mn-SOD, glutathione peroxidase, DT-diaphorase, and catalase, in response to aminochrome using semi quantitative RT-PCR. However, no significant changes in the expression of these enzymes (not shown) was observed in RCSN-3 cells when they were incubated under the same conditions used to determine leukoaminochrome *o*-semiquinone toxicity with the exception of Mn-SOD (Fig. 8A.I). The expression of Mn-SOD was strongly affected when the cells were incubated with 1 mM Mn^{3+}, 100 μM aminochrome, or 100 μM aminochrome together with 100 μM dicoumarol (Fig. 8A.I). No changes in the expression of actin mRNA under all conditions were observed (Fig. 8.II). To demonstrate the changes in the expression of Mn-SOD mRNA we have compared at different number of cycles. A complete downregulation of Mn-SOD was observed when the cells were treated with 1 mM Mn^{3+} (Fig. 8B.II). Incubation of the cells with 100 μM aminochrome together with 100 μM dicoumarol also exerted a strong decrease in Mn-SOD mRNA expression but a very weak band was observed at 30 and 35 cycles which is difficult to observe in the picture (Fig. 8B.IV). A decrease was also observed when the cells were treated with 100 μM aminochrome compared to the control but a weak band appears in 26 cycles (Fig. 8B.III).

Discussion

The chemistry of dopamine allows this molecule to be both an essential neurotransmitter and a toxic substance that determines toxicity and apoptosis in different cell lines (Cadet and Brannock, 1998; Cheng et al., 1996; Emdadul Haque et al., 2003; Jacobsson and Fowler, 1999; Lai and Yu, 1997; Masserano et al., 1996; Offen et al., 1995; Simantov et al., 1996; Stokes et al., 2002; Velez-Pardo et al., 1997; Ziv et al., 1994). One possible explanation for the ability of dopamine to act as a neurotoxin is its capacity to induce the formation of reactive species during dopamine oxidation to aminochrome and its subsequent one-electron reductive metabolism. Dopamine oxidizes to dopamine *o*-quinone, which automatically cyclizes in several steps to aminochrome at physiological pH. The evidence that oxidation of dopamine to the *o*-quinone aminochrome indeed occurs in the brain in vivo are: (i) the existence of neuromelanin in the substantia nigra, for which aminochrome is a precursor, and which also accumulates with age (Zecca et al., 2002) since aminochrome is the precursor of this pigment in these cells; (ii) the finding that cysteinyl adducts, such as 5-cysteinyl-dopamine and quinone adducts, have been encountered in rat, guinea pig, and human brain (Carlsson and Fornstedt, 1991; Forstedt et al., 1990). There are two important mechanisms that prevent dopamine oxidation: (i) incorporation of dopamine into monoamine transporter vesicles (VMAT). The low pH inside of VMAT prevents dopamine oxidation to aminochrome, as the hydroxyl groups in the molecule are well protonated, yielding oxidation or polymerization very unlikely. It has been reported that neuromelanin synthesis is abolished by adenoviral-mediated overexpression of the synaptic vesicle catecholamine transporter VMAT2, which would decrease cytosolic dopamine by increasing vesicular accumulation of the neurotransmitter (Sulzer et al., 2000); and (ii) degradation of dopamine with MAO. In this regard, overexpression of monoamine oxidase A induces protection against intracellular L-DOPA toxicity (Weingarten and Zhou, 2001). However, at physiological pH in the cytosol, the hydroxyl groups in dopamine are dissociated, thus favoring dopamine

autoxidation (Forstedt et al., 1990). The formed aminochrome is then confronted with two possible fates (i) polymerization to neuromelanin or (ii) processing by reductive metabolism. The possibility that oxidative metabolism of dopamine to aminochrome does not result in the formation of neuromelanin, and in turn induces toxic effects under reductive metabolism was studied in the present work by exposing a cell line derived from rat substantia nigra (RCSN-3) to aminochrome in the absence and presence of dicoumarol. The results presented in this work suggest that aminochrome itself is not neurotoxic but the one-electron reduction of aminochrome induces neurotoxicity by generating leukoaminochrome *o*-semiquinone radical, which is an endogenous neurotoxin. The potency of this neurotoxin is probably depending on the high reactivity of leukoaminochrome *o*-semiquinone radical with oxygen (Segura-Aguilar et al., 1998), which generate a redox cycling (Fig. 9, reactions 3 and 4) with concomitant formation of intracellular reactive oxygen species. The presence of ascorbic acid inhibited in 67% of the cell death when the cells were incubated with aminochrome and dicoumarol. However, GSH has no effect on aminochrome plus dicoumarol-dependent cell death because this compound is not able to cross over the cell membrane.

Our results support the proposed hypothesis that DT-diaphorase plays a neuroprotective role in dopaminergic neurons by preventing one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical (Baez et al., 1995; Paris et al., 2001; Segura-Aguilar et al., 2001; Segura-Aguilar et al., 1998). A significant increase in cell death was observed when RCSN-3 cells were incubated with aminochrome (100 μM) together with the DT-diaphorase inhibitor dicoumarol (100 μM) in comparison to aminochrome alone (100 μM) Fig. 2). All flavoenzymes may act as quinone reductases, catalyzing one-electron reduction of quinones to semiquinones radicals. However, only DT-diaphorase catalyzes

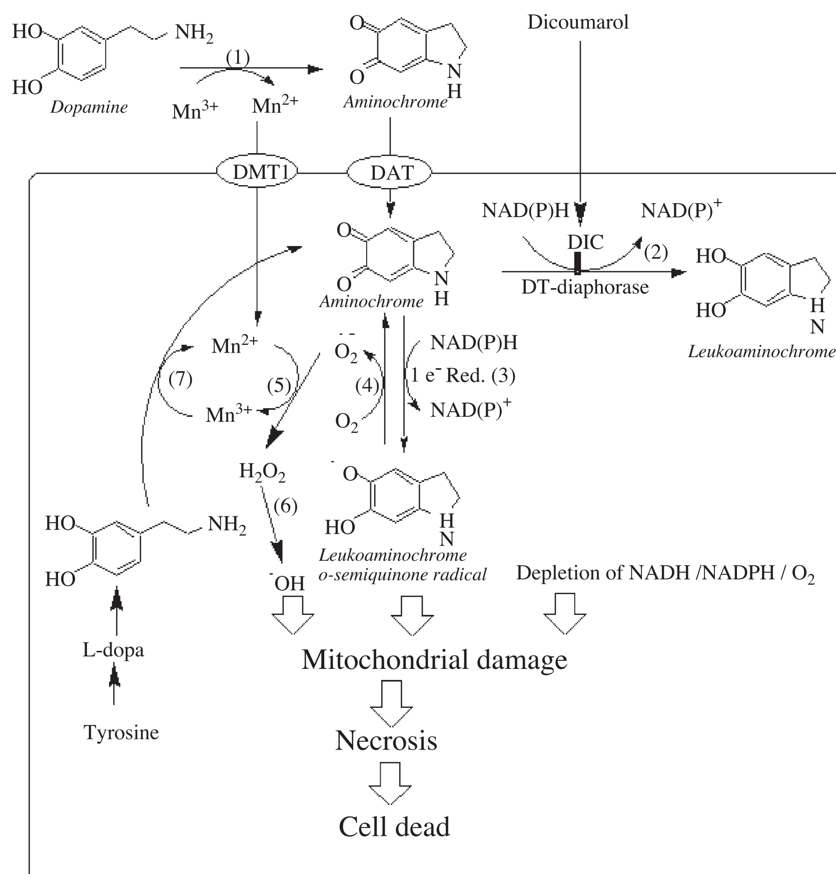

Fig. 9. Possible mechanism of the toxic effects of aminochrome observed in RCSN-3 cells. Dopamine was oxidized by Mn^{3+} to aminochrome in cell culture medium prior addition to the cells, reducing Mn^{3+} to Mn^{2+} (reaction 1). The uptake of aminochrome into the cells was via DAT since it was inhibited by nomifensine. It is possible that the manganese present as Mn^{2+} and Mn^{3+} may stay in the cell culture medium but the divalent metal transporter (DMT1) may facilitate the uptake of Mn^{2+} into the cell. Aminochrome inside the cell has two alternatives (i) to be reduced by two electrons to leukoaminochrome catalyzed by DT-diaphorase (reaction 2); or (ii) to be reduced by one-electron to leukoaminochrome *o*-semiquinone radical by flavoenzymes, which use NADH or NADPH (reaction 3). However, 94% of the total quinone reductase activity in RCSN-3 cells is DT-diaphorase and therefore, the only way to allow this reaction to occur is by inhibiting DT-diaphorase with dicoumarol (DIC), which crosses over the cell membrane due to its hydrophobicity. In the presence of dicoumarol aminochrome is reduced to leukoaminochrome *o*-semiquinone radical which immediately autoxidizes to aminochrome (reaction 4) with the concomitant reduction of oxygen to superoxide radicals, generating a redox cycling (reactions 3 and 4). Two molecules of superoxide radicals will dismutate spontaneously or enzymatically by superoxide dismutase to form hydrogen peroxide, but superoxide radical also catalyzes the oxidation of Mn^{2+} to Mn^{3+} with concomitant formation of hydrogen peroxide (reaction 5) (Segura-Aguilar and Lind, 1989). Hydrogen peroxide is the precursor of hydroxyl radicals, which we detected by ESR and they are probably produced by Fenton reaction (reaction 6). Intracellular Mn^{3+} may catalyze oxidation of intracellular dopamine to aminochrome (reaction 7). It seems plausible that the induced cell death in RCSN-3 cells by leukoaminochrome *o*-semiquinone radical involves mitochondrial damage and necrosis because of (i) hydroxyl radical formation; (ii) leukoaminochrome *o*-semiquinone radical formation; and (iii) depletion of NADH and NADPH.

two-electron reduction of quinones to hydroquinones (Iyanagai and Yamazaki, 1970) and constitutes the 97% of total quinone reductase activity in rat substantia nigra (Schultzberg et al., 1988). Inhibition of DT-diaphorase by dicoumarol allows one-electron reduction of aminochrome, with the formation of leukoaminochrome *o*-semiquinone radical, which is a very reactive species that autoxidizes by reducing oxygen to superoxide radicals, thus initiating a redox cycling process (Baez et al., 1995; Segura-Aguilar et al., 1998). Superoxide radicals generated spontaneously or catalyzed by SOD generate hydrogen peroxide, which is the precursor of hydroxyl radical, one of the most harmful free radical. We have detected a significant increase in the formation of hydroperoxide and hydroxyl radicals in RCSN-3 cells after incubating with aminochrome in the presence of dicoumarol (2.3- and 2.4-fold compared aminochrome alone, respectively; Figs. 3 and 4). Further, a strong decrease in mitochondrial membrane potential was also observed when RCSN-3 cells were incubated with aminochrome in the presence of dicoumarol (Fig. 7). Our results are in agreement with other reports, which indicate that the inhibition of DT-diaphorase with dicoumarol during oxidation of dopamine to aminochrome induces neurotoxicity (Paris et al., 2001). Intracerebral manganese administration together with dicoumarol into the left medial forebrain bundle produced a contralateral behavior when the rats were stimulated with apomorphine, in a manner similar to that when administered to unilaterally 6-hydroxy-dopamine-lesioned animals (Segura-Aguilar et al., 2002). Intranigral injection of Mn^{+3} plus dicumarol produced significant reduction in tyrosine hydroxylase-positive fiber density (Díaz-Véliz et al., 2004).

The strong reduction in mitochondrial membrane potential observed using JC-1 when the cells were incubated with aminochrome together with dicoumarol opened the question about the possible involvement of apoptosis in leukoaminochrome *o*-semiquinone toxicity. In addition, similar concentration of dopamine has been reported to induce apoptosis (Simantov et al., 1996), although in this study all dopamine was oxidized to aminochrome. However, experiments with flow cytometry using annexin V staining showed that an apoptotic process was not involved. Apoptosis is a process which includes several phases (initiation, effector, and degradation) (Mattson, 2000). The effector phase involves also the formation of permeability transition pores in the mitochondrial membrane, and subsequent release of cytochrome *c* into the cytosol. However, transmission electron microscopy revealed that mitochondrial membrane was disrupted (Fig. 5L) supporting the idea that apoptosis was not involved since it is in generally accepted that mitochondrial integrity is required for apoptotic processes (Mattson, 2000). In addition, blebbing formation and nuclear chromatin condensation were not evident, and a large number of vacuoles in the cytosol were present under the aforementioned conditions. A possible explanation for these observations is the generation of the redox cycling during one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical. This redox cycling (Fig. 9, reactions 3 and 4) between aminochrome and leukoaminochrome *o*-semiquinone radical, catalyzed by flavoenzymes using NADH or NADPH, is extremely rapid and potent, producing acute toxicity. The potency of this acute toxicity may be dependent on (i) depletion of NADH required to produce energy; (ii) depletion of NADPH required to keep GSH in the reduced state necessary to exert its antioxidant action; (iii) depletion of oxygen, required for ATP synthesis in the mitochondria; (iv) formation of superoxide radicals, which spontaneously or enzymatically generate hydrogen peroxide, the precursor of hydroxyl radicals. Apoptosis in RCSN-3 cells was observed using annexin V staining when the cells were incubated with Mn^{3+} alone, which is in agreement with other studies which showed that this metal can indeed induce apoptosis (Hirata, 2002). The apoptotic effect of Mn^{3+} is accompanied by a strong decrease in Mn-SOD expression, suggesting the existence of a downregulation mechanism of Mn-SOD in the presence of high concentration of manganese. In our experiments, excess Mn^{3+} was used to oxidize dopamine in the cell culture medium before addition to the cells, and therefore, in the cell culture medium, we will have non-reactive Mn^{3+} and Mn^{2+} formed during dopamine oxidation (Fig. 9, reactions 1, 5, and 7). Therefore, it is possible that the apoptosis observed when RCSN-3 cells were incubated with aminochrome is dependent on the intrinsic apoptotic action of manganese. However, it is also possible that aminochrome-induced apoptosis has a similar mechanism to dopamine-induced apoptosis (Simantov et al., 1996) where DT-diaphorase is not inhibited.

Aminochrome itself does not seem to be neurotoxic under the conditions we used in this study but the formation of hydroxyl radicals was observed when the RCSN-3 cells were exposed to 100 μM aminochrome. One possible explanation is that the concentration of 100 μM aminochrome may surpass DT-diaphorase capacity in RCSN-3 cells to prevent one-electron reduction of aminochrome toxicity although DT-diaphorase constitutes the 94% of the total quinone reductase activity in RCSN-3 cells (Paris et al., 2001). It seems plausible that under physiological conditions, the concentration of aminochrome is much lower than what we used in this study, and under these conditions, hydroxyl radicals are not formed since in rat substantia nigra, DT-diaphorase constitutes 97% of the total quinone reductase activity (Schultzberg et al., 1988).

The neurotoxin(s) involved in the degeneration of dopaminergic neurons in idiopathic Parkinson's disease is still an open question. All model compounds used to study the mechanisms of degeneration in Parkinson's disease such as 6-OH dopamine, MPTP, rotenone are exogenous neurotoxins, which are not present in human dopaminergic neurons. In fact, parkinsonism related to exogenous neurotoxins (MPTP) was identified in young patients, whereas in idiopathic Parkinson's disease, the symptoms appear after 60–65 years of age and when 80% of the substantia nigra dopaminergic neurons are lost. It seems plausible that oxidation of dopamine to aminochrome and subsequent polymerization to neuromelanin is a normal process due to the existence of the catalytic activity of DT-diaphorase, which prevents aminochrome conversion to a neurotoxic substance by one-electron reduction. The results presented in this work support the idea that one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical generates a potent neurotoxin in dopaminergic cells and that DT-diaphorase is a neuroprotective enzyme that prevents one-electron reduction of aminochrome. It seems to be plausible that leukoaminochrome *o*-semiquinone radical may play a role in the degeneration of nigro-striatal dopaminergic system in Parkinson's disease since (i) aminochrome formation is a normal process resulting in the formation of neuromelanin; and (ii) one-electron reduction of aminochrome is possible whenever DT-diaphorase is inhibited, has low expression, or the concentration of aminochrome surpassed the enzyme capacity to prevent one-electron reduction. Interestingly, the existence of a polymorphism in Parkinson patients in the base 609 (C→T) of DT-diaphorase cDNA, yielding a non-active enzyme, results in a 3.8-fold increase

in the risk of developing this disease (Shao et al., 2001). Therefore, conditions that compromise the expression and/or function of this enzyme may result in a disruption of dopamine metabolism, yielding increased free radical formation and neurotoxicity.

Acknowledgments

This work was supported by Fondecyt grants No. 1020672 (Chile), DID TNAC 10-02/01 (University of Chile) and SAF 2001-0479, *Fundación Séneca* PI 021335, Ferrer *Internacional*.

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