Dopamine-Dependent Iron Toxicity in Cells Derived from Rat Hypothalamus

Irmgard Paris,†,‡ Pedro Martinez-Alvarado,†,‡ Sergio Cárdenas,‡ Carolina Perez-Pastene,‡ Rebecca Graumann,‡ Patricio Fuentes,‡ Claudio Olea-Azar,§ Pablo Caviedes,§ and Juan Segura-Aguilar*,‡

Program of Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine, and Department of Biophysics, Faculty of Chemistry and Pharmacy, University of Chile, Casilla 70000, Santiago-7, Chile

We report a new and specific mechanism for iron-mediated neurotoxicity using RCHT cells, which were derived from rat hypothalamus. RCHT cells exhibit immunofluorescent-positive markers for dopamine β-hydroxylase and the norepinephrine transporter, NET. In the present study, we observed that iron-induced neurotoxicity in RCHT cells was dependent on (i) formation of an Fe−−dopamine complex (100 μM FeCl₃:100 μM dopamine); (ii) specific uptake of the Fe−−dopamine complex into RCHT cells via NET (79 ± 2 pmol ⁵⁹Fe/mg/min; P < 0.05), since the uptake of the ⁵⁹Fe−−dopamine complex by the cells was inhibited by 30 μM reboxetine, a specific NET inhibitor (78% inhibition, P < 0.001); and (iii) intracellular oxidation of dopamine present in the Fe−−dopamine complex to aminochrome; (iv) inhibition of DT-diaphorase, since incubation of RCHT cells with 100 μM Fe−−dopamine complex in the presence of 100 μM dicoumarol, an inhibitor of DT-diaphorase, induced significant cell death (51 ± 5%; P < 0.001). However, this cell death was reduced by 75% when the cells were incubated in the presence of 30 μM reboxetine (P < 0.01). No significant cell death was observed when the cells were incubated with 100 μM dopamine, 100 μM Fe−−Dopamine complex, 100 μM dicoumarol, or 100 μM FeCl₃ (8.3 ± 2, 9 ± 4, 8.5 ± 3, or 9.7 ± 2% of control, respectively). ESR studies using the spin trapping agent DMPO showed no formation of hydroxyl radicals when the cells were incubated with 100 μM FeCl₃ alone. However, using the same ESR technique, the formation of hydroxyl radicals and a carbon-centered radical was detected when the cells were incubated with 100 μM Fe−−dopamine complex in the presence of 100 μM dicoumarol. These studies suggest that iron can induce cell toxicity by a mechanism that requires the formation and NET-mediated uptake of an Fe−−dopamine complex, ultimately resulting in the intracellular formation of reactive species.

Introduction

Iron overload in the brain has been associated with several pathologies such as (i) Parkinson’s disease, since a significant increase in iron content has been observed in the substantia nigra (SN)⁴ of both postmortem parkinsonian brains and in live patients using imaging techniques (1−4), and (ii) the hereditary deficiency of ceruloplasmin in aceruloplasminemia, which is associated with both basal ganglia degeneration and iron accumulation in the brain (5, 6). The role of iron in the pathogenesis of Parkinson’s disease has been emphasized because of its ability to enhance the production of oxygen radicals and accelerate neuronal degeneration (1, 7−9). In general, iron toxicity has been associated with the ability of reduced iron(II) to catalyze the formation of hydroxy radicals in the presence of H₂O₂, following the Fenton reaction.

We have now studied an alternative mechanism of iron toxicity. Iron(III) reportedly forms an intermediate 1:1 complex with dopamine, which decomposes releasing Fe−−(II) and oxidizing dopamine to aminochrome (10). Formation of aminochrome seems to be a normal process since this compound is the precursor of neuromelanin, a pigment found in dopaminergic neurons located in the SN. However, certain flavoenzymes can reduce aminochrome with one electron to form leucoaminochrome o-semiquinone radical, which is extremely reactive with oxygen and hence neurotoxic (11−16), and has therefore been proposed as one of the major sources for endogenous generation of reactive species involved in the degenerative process leading to Parkinson’s disease (14, 16−19).
iron-induced neurotoxicity, using cells derived from rat hypothalamus (RCHT), and the possible protective role of DT-diaphorase.

**Experimental Procedures**

**Chemicals.** Dopamine, nomifensine, dicoumarol, FeCl₃, DME/HAM-F12 nutrient mixture (1:1), and 5,5-dimethylpyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis, MO). Calcein AM, ethidium homodimer-1, was from Molecular Probes (Eugene, OR).

**Cell Culture.** The RCHT cell line was derived from the hypothalamus of a 4 month old normal Fisher 344 rat. The RCHT cell line grows in monolayers, with a doubling time of 52 h, a plating efficiency of 21%, and a saturation density of 56.10⁶ cells/cm² when kept in normal growth media composed of DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, and 40 mg/L gentamicine sulfate (20). The cultures were kept in an incubator at 37 °C with 100% humidity and an atmosphere of 10% CO₂.

**Cell Death.** For toxicity experiments, the cells were incubated with cell culture medium without bovine serum and phenol red for 120 min. The cells were visualized at 100× magnification in a Nikon Diaphot inverted microscope equipped with phase contrast and fluorescence optics. The toxicity was measured by counting live and dead cells after staining with 0.5 µM Calcein AM and 5 µM ethidium homodimer-1 for 45 min at room temperature (37°C) as described in Arriagada et al. (14). Calcein is a marker for live cells, and ethidium homodimer-1 intercalates in the DNA of dead cells, giving a green and red fluorescent signal, respectively.

**Fe Uptake.** The uptake of 100 µM FeCl₃ (54 µCi [⁵⁹FeCl₃]) into RCHT cells was measured under the same conditions used to study cell viability. The ⁵⁹Fe–dopamine complex was formed by adding 100 µM dopamine to cell culture medium containing 100 µM FeCl₃ (54 µCi [⁵⁹FeCl₃]) and allowing 2 min for complex formation at room temperature prior to addition to the cells. The uptake was assessed after an incubation period of 1 min at room temperature. Cell membranes were disrupted with 1 mL of 1% Triton X-100 and after 15 min of incubation, and 900 µL of the cell/Triton X-100 extract was removed and analyzed for ⁵⁹Fe tracer content by liquid scintillation counting. The remaining 100 µL was used for protein determination by the bicinonicinic acid method according to Smith et al. (Pierce, Rockford, IL). The results were expressed in pmol/mg protein/min. The ⁵⁹Fe–dopamine complex was also incubated in the presence of 2 µM nomifensine (inhibitor of dopamine transporter, DAT); 30 µM reboxetine (inhibitor of norepinephrine transporter, NET); 100 µM imipramine (inhibitor of serotonin transporter, 5-HTT); and 2 mM dopamine.

**Immunofluorescence Analysis with Confocal Microscopy.** The coverslips containing control RCHT cells at 50% confluence were washed twice with Dulbecco’s PBS, pH 7.4. They were then washed for 30 min with methanol at −20°C. The cells were rinsed twice with PBS and blocked with 1.5% bovine albumin serum diluted in PBS for 40 min. The coverslips were incubated with the primary antibody (rabbit anti-DAT, Sigma Chemical Co.; rabbit anti-NET, Chemicon International; rabbit anti-5HTT, Chemicon International; and rabbit anti-DBH, Chemicon International) at a dilution of 1:1000, 1:1000, 1:500, and 1:1000 in PBS overnight, respectively. After they were washed, the cells were incubated with a secondary antibody [biotinylated anti-rabbit IgG (H + L), Vector Laboratories] diluted 1:250 in PBS for 1 h. The cells were later incubated with Cy-3 conjugated Streptavidin 3 µg/mL (Jackson Immunoresearch Laboratories) for 1 h. The streptavidin solution was then removed, and the cells were washed three times with PBS. Coverslips were mounted onto slides with DAKO fluorescent mounting medium and kept in the dark at 4 °C. For visualization and study, confocal microscopy (Zeiss, model LSM-410 Axiowert-100) was utilized. For NET studies, Z resolution imaging was used, with a thickness of 1.0 µm.

**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. Spectrometer conditions: microwave frequency, 9.68 GHz; microwave power, 20 mW; modulation amplitude, 0.4 G; scan rate, 0.83 G/s; time constant, 0.25 s; and number scans, 10. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The cells were incubated for 35 min with 100 mM DMPO or 100 mM PBN before the addition of (i) FeCl₃, (ii) 100 µM Fe–dopamine complex, (iii) 100 mM Fe–dopamine complex, and 100 µM dicoumarol and incubation during 2 h.

**Data Analysis.** All data were expressed as means ± SD values. The statistical significance was calculated using Student’s unpaired t-test, as compared to controls.

**Results**

The incubation of RCHT cells with 100 µM FeCl₃ for 2 h did not induce significant toxicity (9.7 ± 2% as compared to 3.3 ± 3% of control cells). However, incubation of RCHT cells with 100 µM Fe–dopamine complex in the presence of 100 µM dicoumarol, an inhibitor of DT-diaphorase, induced significant cell death (51 ± 5%; P < 0.001). No significant cell death was observed when the cells were incubated with 100 µM dopamine, 100 µM Fe–dopamine complex, or 100 µM dicoumarol (8.3 ± 2, 9 ± 4, and 8.5 ± 3% of control, respectively). The toxic effect of 100 µM Fe–dopamine complex in the presence of 100 µM dicoumarol was strongly inhibited by 30 µM reboxetine (Figure 1A), contrasting with the lack of effect of 2 µM nomifensine, an inhibitor of DAT (not shown). The toxic effects of 100 µM Fe–dopamine complex in the presence of 100 µM dicoumarol induced strong morphological changes, including membrane disruption (Figure 1B–D). This contrasted with the lack of morphological changes observed in RCHT cells treated solely with 100 µM FeCl₃ or with 100 µM Fe–dopamine complex (Figure 1B and C, respectively).

To determine the formation of free radicals, we used ESR with DMPO as a trapping agent. No ESR signal was observed when RCHT cells were treated with 100 µM FeCl₃. However, when the cells were incubated with 100 µM Fe–dopamine complex, a well-resolved ESR spectrum appeared. The ESR signal intensity was consistent with the trapping of both the hydroxyl radical and an unknown radical probably centered in a C atom (DMPO–OH spin adduct: a N = 14.78 G; DMPO–C centered radical adduct: a N = 15.21 G, a H = 23.48 G; not shown). These hyperfine constants are in agreement with the splitting constants of other DMPO–OH adducts and carbon-centered radicals trapped by DMPO. A similar hyperfine pattern was observed when the cells were incubated with 100 µM Fe–dopamine complex together with 100 µM dicoumarol. However, in the latter condition, the intensity of the signal attributed to the DMPO–OH adducts increased by 197% and the DMPO–C adduct increased by 97% as compared to the spectra of the Fe–dopamine complex (not shown).

The uptake of ⁵⁹FeCl₃ into HT cells was 45 ± 12 pmol/mg/min, but the uptake increased when the cells were incubated with ⁵⁹Fe–dopamine (79 ± 2 pmol/mg/min; P < 0.05 as compared to FeCl₃). Interestingly, the uptake of the ⁵⁹Fe–dopamine complex was inhibited by 30 µM reboxetine (78% inhibition, P < 0.001) and 100 µM imipramine (46% inhibition; P < 0.05). Two millimolar dopamine also inhibited the uptake of the ⁵⁹Fe–dopamine complex (59% inhibition; P < 0.001). No significant effect
serotonin, was evident in RCHT cells (not shown). Other monoamine transporters, such as dopamine or its metabolites, were also observed (Figure 2). No immunoreactivity against NET was localized in the membrane of RCHT cells, indicating its absence and presence of 100 μM dicoumarol (FeDADR). For control conditions, the cells were incubated with FeCl₃, or 100 μM imipramine (FeDAI), 30 μM nomifensine (FeDAN), or 30 μM reboxetine (FeDAR). The statistical significance was assessed using ANOVA for multiple comparisons and Student’s t-test (*P < 0.05; **P < 0.001). The significance for FeDA2, FeDAR, and FeDAI was obtained by comparison with FeDA. The results represent means ± SD (n = 3).

Discussion

A pivotal role of iron in the pathogenesis of Parkinson’s disease has been emphasized because of its ability to enhance the production of oxygen radicals and accelerate neuronal degeneration (1, 7–9). However, and until now, the neurotoxic action of iron has been restricted to its ability to catalyze the formation of hydroxyl radicals via the Fenton reaction. In this work, we propose a different and more specific neurotoxic action of iron in catecholaminergic neurons, since FeCl₃ alone is not toxic in RCHT cells derived from rat hypothalamus after 2 h of incubation periods, despite the observed uptake of ⁵⁹FeCl₃ into RCHT cells. Interestingly, no ESR signal for DMPO–hydroxyl radical adduct was detected when RCHT cells were incubated with FeCl₃, suggesting that iron was chelated inside the cell, thus preventing its possible toxic action by catalyzing the Fenton reaction. The observed toxic action of iron in RCHT cells was dependent on (i) formation of the Fe–dopamine complex, (ii) specific intracellular uptake by NET, (iii) oxidation of dopamine to aminochrome by Fe(III) (10), and (iv) one-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical by inhibiting DT-diaphorase with dicoumarol. The formation of hydroxyl radical with concomitant formation of a radical centered in a carbon was determined in RCHT cells incubated with FeCl₃. The identity of this radical is unknown, but it seems likely that the ESR spectra of this radical correspond to leukoaminochrome o-semiquinone radical, with the radical delocalized into a carbon. The formation of hydroxyl radicals and C-centered radicals when RCHT cells were incubated with the Fe–dopamine complex alone may be explained by the concentration of the Fe–dopamine complex achieved, which probably surpassed DT-diaphorase capacity to prevent one-electron reduction of aminochrome.

This neurotoxic action of iron, via formation of a complex with dopamine, is specific for cells expressing NETs. Indeed, reboxetine, an inhibitor of NET-mediated transport, reduced both Fe–dopamine complex uptake into RCHT cells and toxicity in RCHT-treated dicoumarol. This may be explained by the fact that NET is not absolutely specific for norepinephrine (Kᵣ = 640 nM) and that this transporter exhibits an affinity for dopamine (Kᵣ = 290 nM) (21). It then seems plausible that this specific
neurotoxic action is not solely restricted to adrenergic cells, such as the RCHT cell, which expresses dopamine β-hydroxylase and NET, but it is also valid for catecholaminergic cells, which exhibit dopamine uptake.

Our results suggest the existence of two separate mechanisms of iron uptake into RCHT cells: (i) uptake of iron alone and (ii) Fe–dopamine complex uptake through NET (Scheme 1). However, in the presence of iron and dopamine at similar concentrations, the high efficiency of the Fe–dopamine complex formation (about 90% determined by HPLC) shifts the iron uptake through NET-dependent uptake of the Fe–dopamine complex.

This idea is supported by the high inhibition of the Fe–dopamine complex uptake observed in the presence of reboxetine (79%; Figure 2).

One important feature of iron toxicity in cells expressing NET is dopamine oxidation to aminochrome (Scheme 1). Aminochrome formation and its polymerization to

---

**Figure 3.** Confocal microscopy images of NET immunofluorescence in Z sections in RCHT cells. (A) Immunostaining images of RCHT cells using confocal microscopy. Cells were incubated with anti-NET antisera, and Z sections images were integrated into one picture. (B) One micrometer thick Z sections arranged from top to bottom. RCHT cells incubated with anti-NET antisera, as described under the Experimental Procedures.

**Figure 4.** Immunofluorescence confocal microscopy images of dopamine β-hydroxylase in RCHT cells. The image shows a cytoplasmic labeling pattern of dopamine β-hydroxylase immunofluorescence, which was determined as described under the Experimental Procedures.

**Scheme 1. Possible Mechanism of Dopamine-Dependent Iron Toxicity in RCHT Cells**

\[ \text{Fe}^3+ \text{can form complexes with both dopamine (DA) and iron (Fe). The uptake of Fe–dopamine complex (FeDA) is mediated by NET. Complexed dopamine is oxidized to aminochrome, and Fe}^3+ \text{is reduced to free Fe}^{2+}. \text{Aminochrome then has two alternatives: (i) two-electron reduction by DT-diaphorase (neuroprotection) or (ii) one-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical when DT-diaphorase is inhibited by dicoumarol, thus leading to neurotoxicity.} \]
neuromelanin (22, 23) seem to be a normal process occurring when the cellular capacity to package free dopamine into vesicles for neurotransmission and degredation with MAO is exceeded. Recently, it has been reported that the biosynthesis of neuromelanin is driven by excess cytosolic dopamine, since adenosinergic-mediated overexpression of the synaptic vesicle monoamine transporter VMAT2 inhibited neuromelanin formation (24). Dopamine oxidation to aminochrome and its subsequent polymerization to neuromelanin are not neurotoxic due to the existence of DT-diaphorase, which prevents one-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical (13–19, 25). One-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical 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 (11–16). Leukoaminochrome o-semiquinone radical is extremely reactive with oxygen and generates a redox cycling with concomitant formation of superoxide radicals (11, 12), which generate hydrogen peroxide. In turn, hydrogen peroxide is the precursor of hydroxyl radical, one of the most harmful free radicals. The results presented in this work also support the idea that DT-diaphorase is a neuroprotective enzyme, which prevents aminochrome-dependent toxicity during its one-electron reduction to leukoaminochrome o-semiquinone radical (11–16).

Acknowledgment. This work was supported by FONDECYT Grant 1020672 (Chile).

Note Added after ASAP Publication. An arrow and some text were inadvertently omitted from Scheme 1 in the version published ASAP January 28, 2005; the corrected version was published ASAP February 7, 2005.

References