# Dopamine-Dependent Iron Toxicity in Cells Derived from Rat Hypothalamus

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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 $\beta$-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 $\mu$M FeCl$_3$:100 $\mu$M dopamine); (ii) specific uptake of the Fe−dopamine complex into RCHT cells via NET (79 $\pm$ 2 pmol $^{59}$Fe/mg/min; $P$ < 0.05), since the uptake of the $^{59}$Fe−dopamine complex by the cells was inhibited by 30 $\mu$M 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 $\mu$M Fe−dopamine complex in the presence of 100 $\mu$M dicoumarol, an inhibitor of DT-diaphorase, induced significant cell death (51 $\pm$ 5%; $P$ < 0.001). However, this cell death was reduced by 75% when the cells were incubated in the presence of 30 $\mu$M reboxetine ($P$ < 0.01). No significant cell death was observed when the cells were incubated with 100 $\mu$M dopamine, 100 $\mu$M Fe−Dopamine complex, 100 $\mu$M dicoumarol, or 100 $\mu$M FeCl$_3$ (8.3 $\pm$ 2, 9 $\pm$ 4, 8.5 $\pm$ 3, or 9.7 $\pm$ 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 $\mu$M FeCl$_3$ 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 $\mu$M Fe−dopamine complex in the presence of 100 $\mu$M 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)[1] 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 gen-

eral, iron toxicity has been associated with the ability of reduced iron(II) to catalyze the formation of hydroxyl radicals in the presence of H$_2$O$_2$, 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 leukoaminochrome *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*). In the present work, we investigated the possible role of dopamine and aminochrome in a novel mechanism of

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[1] Abbreviations: NET, norepinephrine transporter; DAT, dopamine transporter; 5-HTT, serotonin transporter; SN, substantia nigra.

iron-induced neurotoxicity, using cells derived from rat hypothalamus (RCHT), and the possible protective role of DT-diaphorase.

## Experimental Procedures

**Chemicals.** Dopamine, nomifensine, dicoumarol, $FeCl_3$, 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 56.000 cells/cm$^2$ when kept in normal growth media composed of DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, 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% $CO_2$.

**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 $\mu$M Calcein AM and 5 $\mu$M ethidium heterodimer-1 for 45 min at room temperature (37 °C) as described in Arriagada et al. (*14*). Calceine is a marker for live cells, and ethidium heterodimer-1 intercalates in the DNA of dead cells, giving a green and red fluorescent signal, respectively.

**Fe Uptake.** The uptake of 100 $\mu$M $FeCl_3$ (54 $\mu$Ci $^{59}FeCl_3$) into RCHT cells was measured under the same conditions used to study cell viability. The $^{59}$Fe−dopamine complex was formed by adding 100 $\mu$M dopamine to cell culture medium containing 100 $\mu$M $FeCl_3$ (54 $\mu$Ci $^{59}FeCl_3$) 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 $\mu$L of the cell/Triton X-100 extract was removed and analyzed for $^{59}$Fe tracer content by liquid scintillation counting. The remaining 100 $\mu$L was used for protein determination by the bicinconinic acid method according to Smith et al. (Pierce, Rockford, IL). The results were expressed in pmol/mg protein/min. The $^{59}$Fe−dopamine complex was also incubated in the presence of 2 $\mu$M nomifensine (inhibitor of dopamine transporter, DAT); 30 $\mu$M reboxetine (inhibitor of norepinephrine transporter, NET); 100 $\mu$M 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 fixed 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 $\mu$g/mL (Jackson Inmunoresearch 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 Axiovert-100) was utilized. For NET studies, Z resolution imaging was used, with a thickness of 1.0 $\mu$m.

**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) $FeCl_3$, (ii)100 $\mu$M Fe−dopamine complex, (iii) 100 $\mu$M Fe−dopamine complex, and 100 $\mu$M dicoumarol and incubation during 2 h.

**Data Analysis.** All data were expressed as means $\pm$ 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 $\mu$M $FeCl_3$ for 2 h did not induce significant toxicity (9.7 $\pm$ 2% as compared to 3.3 $\pm$ 3% of control cells). However, incubation of RCHT cells with 100 $\mu$M Fe−dopamine complex in the presence of 100 $\mu$M dicoumarol, an inhibitor of DT-diaphorase, induced significant cell death (51 $\pm$ 5%; $P < 0.001$). No significant cell death was observed when the cells were incubated with 100 $\mu$M dopamine, 100 $\mu$M Fe−dopamine complex, or 100 $\mu$M dicoumarol (8.3 $\pm$ 2, 9 $\pm$ 4, and 8.5 $\pm$ 3% of control, respectively). The toxic effect of 100 $\mu$M Fe−dopamine complex in the presence of 100 $\mu$M dicoumarol was strongly inhibited by 30 $\mu$M reboxetine (Figure 1A), contrasting with the lack of effect of 2 $\mu$M nomifensine, an inhibitor of DAT (not shown). The toxic effects of 100 $\mu$M Fe−dopamine complex in the presence of 100 $\mu$M 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 $\mu$M $FeCl_3$ or with 100 $\mu$M 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 $\mu$M $FeCl_3$. However, when the cells were incubated with 100 $\mu$M 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: $a_N = a_H = 14.78$ 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 $\mu$M Fe−dopamine complex together with 100 $\mu$M 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 $^{59}FeCl_3$ into HT cells was 45 $\pm$ 12 pmol/mg /min, but the uptake increased when the cells were incubated with $^{59}$Fe−dopamine (79 $\pm$ 2 pmol/mg/min; $P < 0.05$ as compared to $FeCl_3$). Interestingly, the uptake of the $^{59}$Fe−dopamine complex was inhibited by 30 $\mu$M reboxetine (78% inhibition, $P < 0.001$) and 100 $\mu$M imipramine (46% inhibition; $P < 0.05$). Two millimolar dopamine also inhibited the uptake of the $^{59}$Fe−dopamine complex (59% inhibition; $P < 0.001$). No significant effect
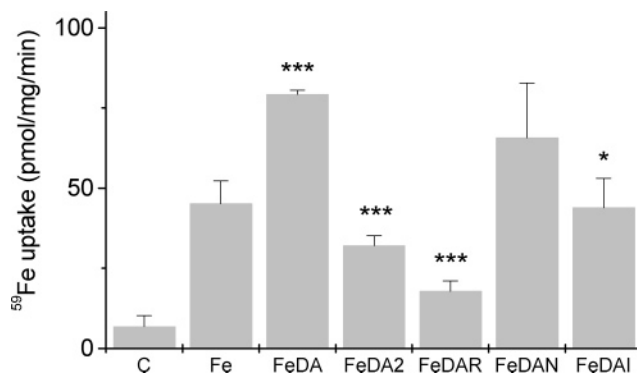
A



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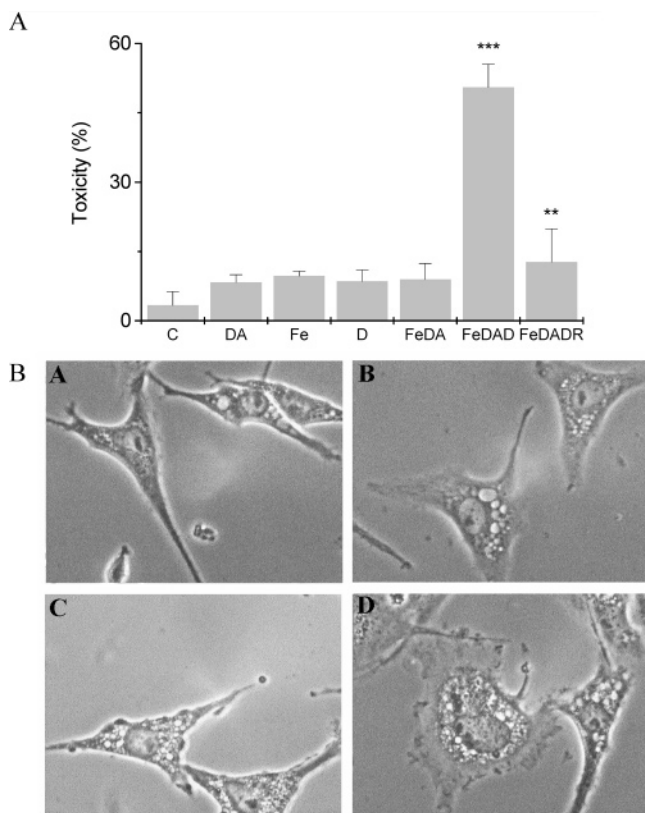


**Figure 1.** Effect of the Fe−dopamine complex on RCHT cell death in the absence and presence of dicoumarol. (A) RCHT cells were treated with 100 $\mu$M Fe−dopamine complex (FeDA) formed in the cell culture medium prior to addition to the cells, both in the absence and in the presence of 100 $\mu$M dicoumarol (FeDAD). The NET inhibitor reboxetine (R, 30 $\mu$M) was incubated with 100 $\mu$M Fe−dopamine complex plus 100 $\mu$M dicoumarol (FeDADR). For control conditions, the cells were incubated with cell culture medium (C), 100 $\mu$M dopamine (DA), 100 $\mu$M FeCl$_3$ (Fe), or 100 $\mu$M dicoumarol (D) alone. The experimental conditions are described under the Experimental Procedures. The statistical significance was assessed using ANOVA for multiple comparisons and Student's $t$-test (**$P < 0.01$; ***$P < 0.001$). (B) RCHT cells kept in culture medium (A) were treated with 100 $\mu$M FeCl$_3$ (B), 100 $\mu$M Fe−dopamine complex (C) formed in the cell culture medium prior to addition to the cells, and in the absence and presence of 100 $\mu$M dicoumarol (D).



**Figure 2.** Uptake of $^{59}$Fe−dopamine complex into RCHT cells. The uptake of $^{59}$Fe−dopamine complex (FeDA) into RCHT cells was measured in cells kept in cell culture medium without bovine serum, as described under the Experimental Procedures. RCHT cells were incubated with $^{59}$Fe−dopamine complex in the presence of 2 mM dopamine (FeDA2), 2 $\mu$M nomifensine (FeDAN), 100 $\mu$M imipramine (FeDAI), or 30 $\mu$M 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 $\pm$ SD ($n = 3$).

of 2 $\mu$M nomifensine on $^{59}$Fe−dopamine complex was observed (Figure 2).

These results suggest that the uptake of the Fe−dopamine complex into RCHT cells is mediated by the NET, since it was strongly reduced by reboxetine, a specific NET inhibitor. To establish the presence of NET in RCHT cells, we used immunofluorescence techniques with confocal microscopy, which confirmed the presence of the transporter (Figure 3A). To determine whether NET was localized in the membrane of RCHT cells, we carried out 1 $\mu$m thick Z sections from the top to the bottom of the cells, using confocal microcopy. In Z sections 1−9 $\mu$m, we observed that NET is mainly localized in the cell membrane (Figure 3B). No immunoreactivity against other monoamine transporters, such as dopamine or serotonine, was evident in RCHT cells (not shown). Finally, RCHT cells were also found to express dopamine $\beta$-hydroxylase (Figure 4).

## 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 FeCl$_3$ alone is not toxic in RCHT cells derived from rat hypothalamus after 2 h of incubation periods, despite the observed uptake of $^{59}$FeCl$_3$ into RCHT cells. Interestingly, no ESR signal for DMPO−hydroxyl radical adduct was detected when RCHT cells were incubated with FeCl$_3$, 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 the Fe−dopamine complex plus dicoumarol. 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.
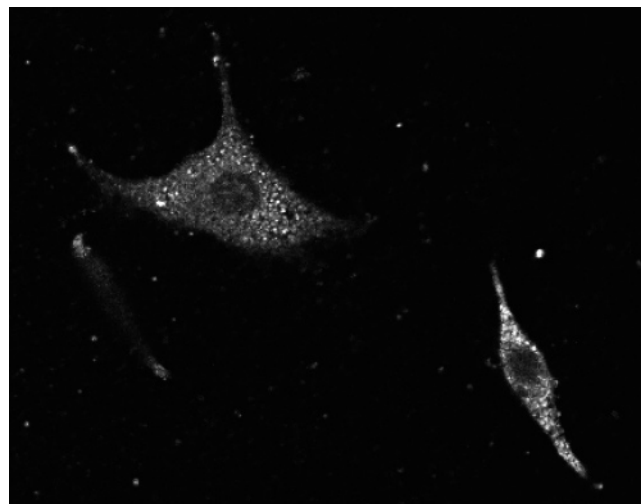
This neurotoxic action of iron, via formation of a complex with dopamine, is specific for cells expressing NETs. Indeed, reboxetine, an inhibitor of NET-mediated transport, reduced both Fe−dopamine complex uptake cells and toxicity in RCHT-treated dicoumarol. This may be explained by the fact that NET is not absolutely specific for norepinephrine ($K_M = 640$ nM) and that this transporter exhibits an affinity for dopamine ($K_m = 290$ nM) (*21*). It then seems plausible that this specific
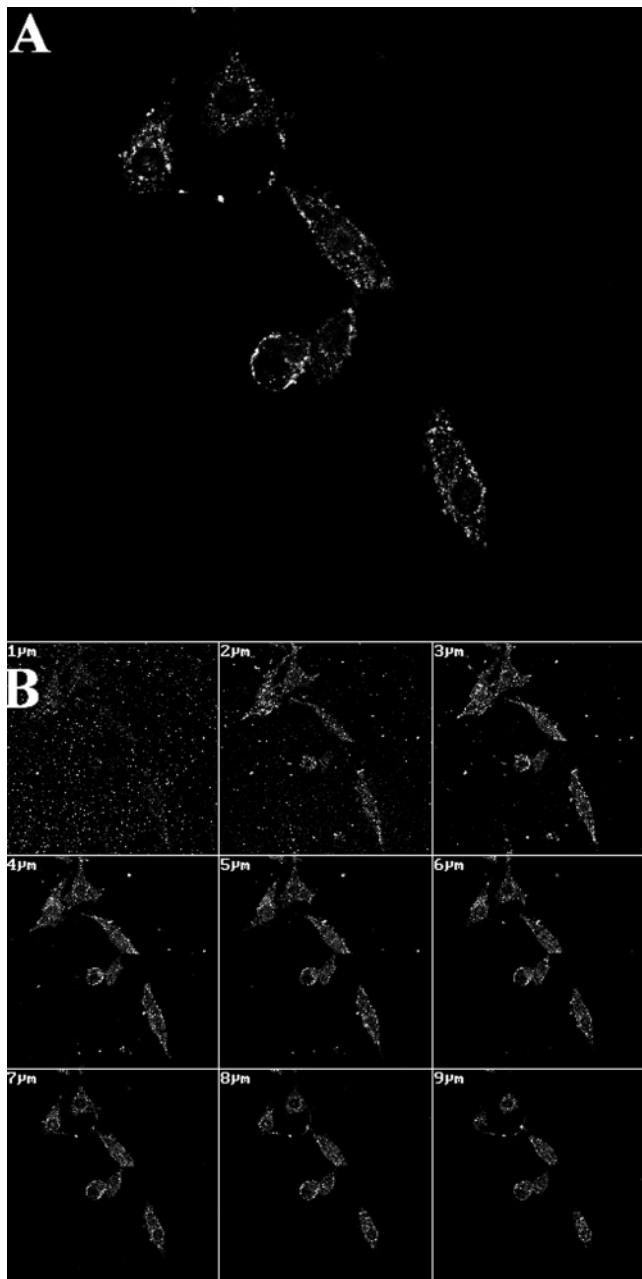
**Figure 3.** Confocal microscopy images of NET immunofluorescence in Z sections in RCHT cells. (A) Immunostaining images of RCHT cells using confocal microscopy. Cells were incubated with anti-NET antisera, and Z sections images were integrated into one picture. (B) One micrometer thick Z sections arranged from top to bottom. RCHT cells incubated with anti-NET antisera, as described under the Experimental Procedures.
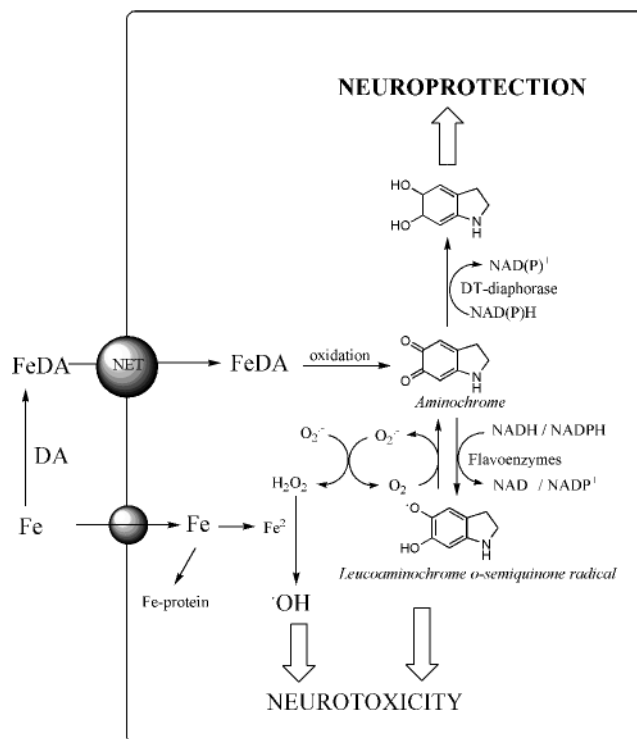


**Figure 4.** Immunofluorescence confocal microscopy images of dopamine β-hydroxylase in RCHT cells. The image shows a cytoplasmic labeling pattern of dopamine β-hydroxylase immunofluorescence, which was determined as described under the Experimental Procedures.

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



[a] $Fe^{3+}$ can form complexes with both dopamine (DA) and iron (Fe). The uptake of Fe−dopamine complex (FeDA) is mediated by he NET. Complexed dopamine is oxidized to aminochrome, and $Fe^{3+}$ is reduced to free $Fe^{2+}$. Aminochrome then has two alternatives: (i) two-electron reduction by DT-diaphorase (neuroprotecion) or (ii) one-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical when DT-diaphorase is inhibited by dicoumarol, thus leading to neurotoxicity.

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

neuromelanin (*22, 23*) seem to be a normal process occurring when the cellular capacity to package free dopamine into vesicles for neurotransmission and degradation with MAO is exceeded. Recently, it has been reported that the biosynthesis of neuromelanin is driven by excess cytosolic dopamine, since adenoviral-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*).

**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.

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