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Dopamine and Iron Induce Apoptosis in PC12 Cells

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Abstract: Recent studies have shown that Fe^{2+} increases the oxidation of monoamines such as serotonin, dopamine and related toxins and that the formed oxidation products can undergo co-valent binding to free sulphydryl groups of proteins such as actin and "serotonin binding proteins" which are present in soluble brain extracts. Here we have tested the ability of ferrous iron to induce [^{3}H]dopamine association to cytoplasmic proteins and we have established that a similar oxidation mechanism evidenced *in vitro* studies could be applied in cell culture. When PC12 cells were incubated with ferrous iron (ferrocene), the binding of [^{3}H]dopamine to proteins was found to be two fold increased with respect to control. The iron is likely to accelerate the oxidation of dopamine to produce quinones which covalently bind to proteins and induce high-molecular protein aggregates. We evidenced that dopamine/iron combination induced cell death in undifferentiated PC12 cells via an active cellular process evaluated in terms of morphological and biochemical changes indicative of apoptosis. We also demonstrated induction of lipid peroxidation when dopamine and ferrocene were present in high concentrations. Moreover, ascorbic acid diminished apoptosis but not the lipid peroxidation process. It might indicate that ferrocene and dopamine could produce oxidative stress of a different nature. These results show that the actions of dopamine and iron are essential in the induction of apoptosis and lipid peroxidation. However, there is no necessary causual link between lipid peroxidation and apoptosis. Our data also suggest that iron is capable of increasing the cytotoxicity of dopamine merely by increasing its rate of oxidation and without intervention of the monoamine oxidase B enzyme and, hence, both phenomenons may occur independently from each other in rat pheochromocytoma PC12. These observations may have relevance to the understanding of the mechanism by which dopaminergic neurones are destroyed in some neurodegenerative disorders.

The molecular mechanisms underlying neuronal cell death in degenerative disorders such as idiopathic Parkinson's disease are still poorly understood but dopamine has often been considered to be a contributing factor. Both the autoxidation of dopamine and its enzymatic catabolism by monoamine oxidase (MAO) is well known to produce hydrogen peroxide and oxyradicals. These reduced forms of oxygen are highly reactive and the "oxidative stress" which they produce has been held responsible for the loss of monoamine-containing neurones (Fahn and Cohen 1992). In addition, the autoxidation of dopamine produces semiquinones and quinones which are capable to bind covalently to proteins and to glutathione, one of the major cellular defence mechanisms against oxidative stress (Graham *et al.* 1978; Ito *et al.* 1988; Meister, 1988). The occurrence of 5-S-cysteinyl adducts of catechols in mammalian brains is thought to reflect the *in vivo* autoxidation of the catechols and the coupling of the resulting quinones to glutathione (Fornstedt *et al.* 1986). In this respect, 5-S-cysteinyl adducts of catechols have been suggested to constitute markers in the diagnosis of Parkinson's disease (Carlsson and Fornstedt 1991).

It seems that, besides dopamine, iron could also play an important role in the pathogenesis of Idiopathic parkinson's disease (Fahn and Cohen 1992; Youdim and Riederer 1993; Gerlach *et al.* 1994). Indeed, the substantia nigra from parkinsonian patients contains high levels of total iron and a reduced ferritin buffering (Dexter *et al.* 1989; Riederer *et al.* 1989). Iron is known to promote monoamine oxidation and to catalyse the transformation of hydrogen peroxide into the more reactive hydroxyl radicals. Moreover, iron has also been reported to decrease the intracellular concentration of glutathione and, thereby, to induce oxidative stress.

In vitro studies have provided some insight into the molecular mechanisms by which iron and dopamine may confer cytotoxicity. At the subcellular level, Fe^{2+} has been shown to promote oxidative damage of lipid membranes (Braghler *et al.* 1986), DNA damage (Spencer *et al.* 1994), oxidation of sulphydryl groups of proteins (Stadtman 1990; Korge & Campbel 1993) and the co-valent binding of monoamines such as dopamine and serotonin to proteins (Jimenez Del Rio *et al.* 1993b). This binding was investigated in detail for proteins in brain extracts (initially denominated as "Serotonin Binding Proteins") and for actin (Velez-Pardo *et al.* 1995a). As a result, a mechanism was proposed in where a) Fe^{2+} reacts with dissolved molecular oxygen to produce superoxide radicals, b) the radicals oxidise monoamines into quinone derivatives and c) the oxidation products bind covalently to sulphydryl groups of proteins. Recently, it was also found that protein-associated dopamine is able to cata-

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lyse redox cycling processes (Velez-Pardo et al.1996) and that Fe^{2+} also promotes the binding of dopamine and serotonin-related neurotoxins to Serotonin Binding Proteins (Jimenez Del Rio *et al.* 1994). These findings support the suggestion that the iron-mediated co-valent binding of monoamines to proteins represent an *in vitro* model for cytotoxicity (Vauquelin *et al.* 1994, Jimenez Del Rio *et al.* 1995; Velez-Pardo *et al.* 1995a,b & 1996).

Several animal models as well as neuronal and non-neuronal cells lines have also been used to investigate the toxic effect of iron and catecholamines (Michel & Hefti 1990; Ben-Shachar & Youdim 1991; Sengstock *et al.* 1992; Mytilineou *et al.* 1993). PC12 cells, a clonal catecholaminergic cell line derived from rat pheochromocytoma which responds to nerve growth factor by undergoing differentiation into a sympathetic-like neuronal phenotype (Greene & Tischer 1976), have been particularly fruitful for the study of catecholamine toxicity. When serum-deprived, these cells go into 'apoptosis', a type of cell death exhibiting distinct morphological and biochemical features such as cell shrinkage, chromatin condensation, break-up of the nucleus, plasma membrane blebbing followed by fragmentation of the cell into discrete apoptotic bodies (Kerr *et al.* 1972; Batistatou and Greene 1991; Martin *et al.* 1994). A particularly striking observation was that dopamine-related compounds as L-DOPA and the neurotoxin 6-hydroxydopamine are also able to induce apoptosis in these cells (Walkinshaw & Waters 1994 & 1995). The effect of 6-hydroxydopamine encompassed the activation of an endonuclease in the death pathway (Walkinshaw & Waters 1994) and the effect of L-DOPA was inhibited by antioxidants, suggesting the involvement of oxygen radicals (Walkinshaw & Waters 1995).

The combined action of catechols and iron was investigated by Tanaka *et al.* (1991) on a dorsal root ganglion neuronal culture. These authors showed that both dopa and dopamine cause cell death in the presence of iron, and this was attributed to lipid peroxidation-mediated membrane cleavage. Since no evidence was found for the involvement of hydroxyl- and superoxide radicals, they concluded that a catechol-iron complex with oxidising reactivity is responsible for the damage to the lipids and, consequently, for the cell death. These conclusions clearly deviate from the usually alluded role of oxyradicals and (semi)quinone- derivatives in dopamine/iron-related cell toxicity.

To obtain a better insight into the neurotoxic effects of dopamine and iron in terms of morphological and biochemical changes, we used PC12 cells in the present study. Iron was shown to increase the covalent binding of [^{3}H]dopamine to intracellular proteins. Iron and dopamine were also able to produce lipid peroxidation and cell death via an apoptotic process.

Materials and Methods

Materials. [7,8-^{H}]dopamine (48 Ci/mmol) was obtained from Amersham (UK); dopamine hydrochloride, ferrocene, acridine orange, agarose, RNAse A, Proteinase K, Triton X-100, pargyline and ethidium bromide were from Sigma Chemical Co. (U.S.A.); iron sulphate ($FeSO_4$) was from Aldrich Chemie (Belgium); sodium metabisulfite, N-lauroylsarcosine from Fluka (Switzerland); RPMI 1640 medium, L-glutamine and Penicillin-Streptomycin from Gibco (UK); fetal calf serum from PAA (Austria); L(+) ascorbic acid from Merck (Germany); DL-buthionine-S,R-sulfoximine (BSO) from ICN (Belgium); LPO-586 kit from Bioxytech S.A. (France). PC12 cells were purchased from European Collection of Animal Cell Culture (ECACC number 88022401).

Experiments with calf brain extract "Serotonin binding proteins".

Protein preparation and binding assay. Calf brains were obtained from a local slaughter house and kept in ice during transportation. Protein preparation was performed according to Jimenez Del Rio *et al.* 1993b. Samples of protein (0.1 mg/ml) were incubated at 20° in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.5) with 0.2 μM of [^{3}H]dopamine and freshly prepared $FeSO_4$ or ferrocene (50 μM). $FeSO_4$ or ferrocene was added immediately after the radioligand. After 15 min. incubation, 0.3 ml of the mixture was applied to a small Sephadex G-50 column (0.7×15 cm) equilibrated with phosphate buffer, and eluted with the same buffer. The void volume (1.5 ml) was discarded and the fraction containing labelled protein (1.8 ml) was collected and counted by liquid scintillation counting.

Experiments with PC12 cells.

Binding of [^{3}H]dopamine to soluble proteins. Undifferentiated PC12 cell lines (passage 2-12) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% foetal calf serum (Greene & Tishler 1976), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells in suspension were plated in 24-wells tissue culture plates (1 ml/well), preincubated for 30 min. with 1 mM pargyline and incubated with 0.25 mM [^{3}H]dopamine and 50 mM iron ($FeSO_4$ or ferrocene) for 2 hr. Cells were collected by centrifugation (1000 rpm for 2 min. at room temperature, washed two times with phosphate-buffered saline (PBS) (pH 7.5) and suspended in 0.5 ml of ice-cold lysis buffer A consisting of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% w/v N-lauroylsarcosine and 1% Triton X-100. Protein concentration was determined according to Lowry *et al.* (1951). Total protein was diluted to 0.1 mg/ml and, for the determination of protein-associated radioactivity, 0.3 ml of the mixture was applied to a sephadex G-50 column as described above.

High molecular weight aggregates. PC12 cells were incubated for 24 hr at 37° in culture medium containing 1 mM pargyline and the indicated combinations of dopamine, homovalinic acid and ferrocene. Cells were collected by centrifugation, washed two times with PBS and suspended for 30 min. in 0.5 ml of ice-cold lysis buffer A. Protein concentration was determined as described above. SDS-Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and the gel (20 mg protein/slot) was stained with Coomassie Brilliant Blue R 250.

Assessment of apoptotic index. PC12 cells were incubated for 24 hr at 37° in culture medium containing 1 mM pargyline and 0.3 or 1 mM dopamine in the absence or presence of 50 μM ferrocene and other products of interest. Cells were then used for parallel microscopic examinations and biochemical assays. For viability studies, treated cells were mixed with 1 ml acridine orange/ethidium bromide (0.1 mg/ml) and 10 ml of the suspension was placed onto a slide and examined under fluorescence on a confocal microscope (Leitz Wetzlar, Germany). Based on the differential uptake of the fluorescent DNA binding dyes acridine orange and ethidium bromide, normal cells (NC, bright green chromatin) can be discriminated from early apoptotic cells (EA, bright green highly condensed or fragmented chromatin), late apoptotic cells (LA, bright orange highly condensed or fragmented chromatin) and necrotic cells (N, bright orange chromatin) (McGahon *et al.* 1995). Quantification of apoptosis was done by counting a minimun of 200 total cells as follows: % apoptotic cells=100× (total number of early and late

apoptotic cells/total number of cells counted). Necrotic cells were not detected under the present experimental conditions. For additional morphological evaluation, treated cells (100 ml suspension) were placed on to a microscope slide by centrifugation at 200 r.p.m. for 2 min. After haematoxylin/eosin staining based on Romanowsky Staining (RS) (Rapi-diff II, DiaChem Diagnostic development, Southport, U.K.), the number of apoptotic cells were evaluated under a light microscope. A minimun of 200 cells were screened and cells were regarded to be apoptotic when there was chromatin condensation or nuclear fragmentation.

Assessment of DNA fragmentation. Cells were incubated as described above; centrifuged at 1200 rpm for 10 min. at 20° and resuspended in lysis buffer B consisting of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) sodium lauryl sarcosine, 1% Triton X-100, 0.25 mg/ml RNAse A and 100 mg/ml proteinase K. After incubation at 50° for 2 hr, samples were extracted twice with phenol and chloroform/isoamyl alcohol (24:1 v/v) and then DNA was precipitated overnight at −70° by adding 1/10 volume of 3M sodium acetate (pH 5.2) and two volumes of ethanol. DNA was pelleted by centrifugation at 13,000×g for 10 min., washed with 1 volume of 70% ethanol, and dried. DNA was resuspended in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA and supplemented with loading buffer (50% glycerol-0.05% bromophenol blue). Electrophoresis was performed for 3 hr at 20 mA in 1% agarose slab gels containing ethidium bromide at a final concentration of 0.1 mg/ml in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA at pH 8.0). A DNA 123 ladder (Gibco-BRL) was applied to each gel to provide molecular size markers. DNA banding was evidenced with a UV transilluminator.

Lipid peroxidation assay. Lipid peroxidation assay was performed according to a colorimetric assay using the LPO-586 method (Bioxytech S.A., France). Briefly, supernatant (0.2 ml) from treated cell cultures were collected by centrifugation at 1200 r.p.m. for 10 min. at room temperature. Freshly prepared (0.65 ml) of a chromogenic reagent was added to each sample. Reaction was started by addition of 0.15 ml of 10 M methanesulfonic acid. Samples were incubated at 45° for 40 min. A stable chromophore product was measured spectrophotometrically at 586 nm. The concentration of malondialdehyde (MDA) and 4-hydroxyalkenals was determined in the samples using standard curves.

Spectrophotometric measurement of quinone formation. Dopamine (1mM) was incubated in presence or absence of iron and/or (1mM) asorbic acid for up to 24 hr in 1 ml (final volume) of serum-supplemented culture medium (RPMI-1640). Quinone formation was evaluated by spectrophotometry at 490 nm (Heikkila & Cohen 1973).

Results

[^{3}H]Dopamine binding to proteins in PC12 cells.

Ferrous iron ($FeSO_4$) has previously been demonstrated to promote the binding of dopamine and serotonin to proteins by an oxidative mechanism (Jimenez Del Rio *et al.* 1993b). To assess whether such phenomenon also takes place in an *in vitro* cell culture, we compared the ability of 50 mM iron sulphate ($FeSO_4$) to increase the binding of 0.2 μM [^{3}H]dopamine to proteins extracted form calf frontal cortex (15 min. incubation time) and to undifferentiated PC12 cells (2 hr incubation time). As shown in fig. 1, $FeSO_4$ increased the binding in both instances but the increase was more important for the soluble proteins (up to 5.6 fold the control binding in the absence of iron) than for PC12 proteins (2.1 fold).

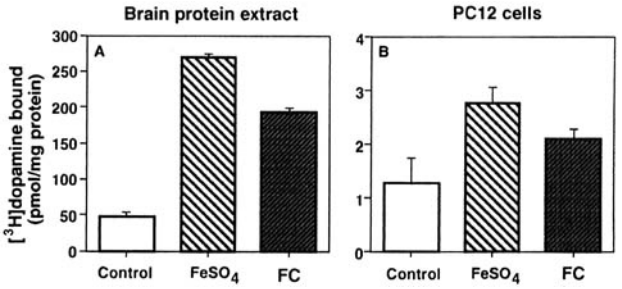

Fig. 1. (A). Samples of protein extract from calf brain "serotonin binding proteins" were incubated for 15 min. with 50 μM of either sulphate ($FeSO_4$) or ferrocene iron and 0.2 μM [^{3}H]dopamine. (B) In parallel, PC12 cells were also incubated in the same conditions, except that incubation time was 2 hr. The protein-associated radioactivity was measured as described in Materials and Methods. The binding is expressed as pmol/mg protein. Values (means±S.D. of three experiments). FC=ferrocence.

A major drawback for the use of $FeSO_4$ in PC12 cell suspensions is that it precipitates and, by doing so, interferes with microscopic evaluations and staining. This can be avoided by replacement of $FeSO_4$ by ferrocene. Ferrocene has already been used for iron loading in living systems (Ward *et al.* 1991). Under same experimental conditions, ferrocene increases the binding of [^{3}H]dopamine to the soluble proteins form calf frontal cortex (4 fold) and to the PC12 cells (1.6 fold) to about the same level as $FeSO_4$ (fig. 1).

Formation of high molecular weight aggregates.

High molecular weight protein aggregates are readily formed when PC12 cells are treated for 24 hr with a combination of 50 μM ferrocene and dopamine at concentrations

Table 1.

Dopamine/ ferrocene induced lipid peroxidation in PC12 cells. PC12 cells were incubated for 24 hr with 0.3 or 1 mM of dopamine in the presence or absence of 0.05 mM iron (ferrocene) and the listed additives. The evaluation of LPO was performed as described in Materials and Methods using colorimetric assay LPO-586 method. The amount of lipid peroxidation is expressed in μM of production of MDA and 4-hydroxyalkanes. Values are means±S.D. of three independent experiments.

Treatments	Malonaldehyde+4-hydroxyalkanes production (μM)
Untreated cells (Control)	0
Serum Free	0
Ferrocene iron (50 mM)	0.98±0.09
Dopamine (1 mM)	1.98±0.03
Dopamine+Ferrocene	4.64±0.50
BSO (1 mM)	0.27±0.05
BSO+Ferrocene	1.09±0.38
BSO+Dopamine	3.30±0.29
BSO+Ferrocene+Dopamine	3.85±0.16
Ascorbic Acid (AA, 1 mM)	0
AA+Ferrocene	1.90±0.04
AA+Dopamine	0.73±0.02
AA+Dopamine+Ferrocene	2.05±0.05

BSO=L-buthionine (S, R)-sulfoximine.

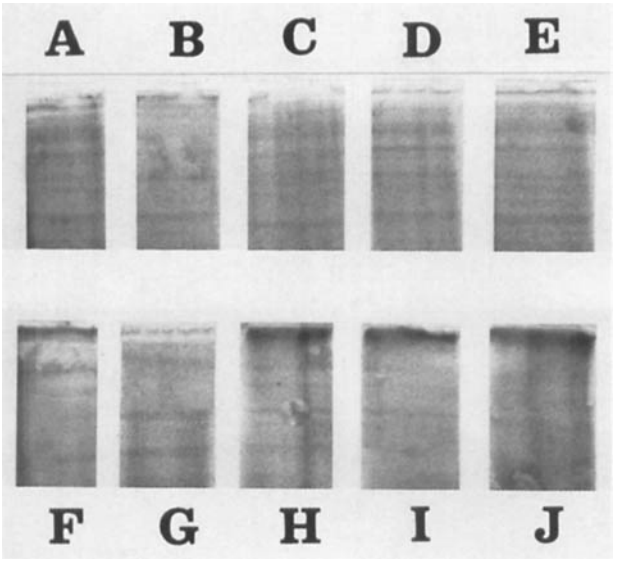

Fig. 2. Ferrocene iron-induced formation of high molecular weight aggregates (HMW-a) in PC12 cells detected by SDS-PAGE. PC12 cells were incubated for 24 hr at 37° with 100 mM, 300 mM and 1 mM dopamine (lanes D, E, F). As controls, no additives (lane A); 50 mM ferrocene (lane B), 1 mM homovanillic acid (HVA) (lane C). Combinations of ferrocene and increasing concentrations of dopamine (100 μM, 300 μM, 1 mM) are in lanes (H, I, J) and ferrocene/HVA is in lane (G). Samples containing 20 μg protein, prepared as described in Materials and Methods, were subjected to SDS-PAGE electrophoresis. Proteins were stained with Coomassie Brilliant Blue R 250. The figure represents one of three independent experiments.

as low as 0.1 mM. As shown in fig. 2, these aggregates are present on the top of the separating gel when protein preparations from such treated cells are subjected to SDS-PAGE (fig. 2, lanes H to J). In the absence of iron, the dopamine concentration has to be raised to 1 mM for the aggregates to become equally detectable (Fig. 2, lanes D to F). It has previously been shown that, unlike dopamine, homovanillic acid does not bind to proteins in the presence of iron (Jimenez Del Rio *et al.* 1992 & 1993a). In the same vein, no high molecular aggregates can be detected when the cells are treated with 1 mM homovanillic acid alone (lane C), 50 μM ferrocene alone (Fig. 2, lane B), or a combination of both (lane G).

Lipid peroxidation.

Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids in cell membranes as a consequence of free radical production (Halliwell & Gutterridge 1989). As shown in Table 1, no detectable lipid peroxidation (i.e. production of malondialdehyde and 4-hydroxyalkanes) was observed in control, untreated PC12 cells, in cells grown in serum-free medium or in cells treated with 1 mM ascorbic acid for 24 hr. An appreciable increase in the extent of lipid peroxidation occured in the presence of 50 μM ferrocene alone as well as in presence of 1 mM dopamine alone (table 1). There was also a marked increment when ferrocene and dopamine were given in combination. Ascorbic acid was unable to change the effect of ferrocene alone but it attenuated the ability of 1 mM dopamine to trigger lipid peroxidation and the effect of ferrocene thereon.

Apoptosis of PC12 cells.

Recently, it has been shown that L-DOPA and 6-hydroxydopamine induce PC12 cell death via a mechanism which has the morphological and biochemical hallmarks of apoptosis (Walkinshaw & Waters, 1994 & 1995). To evaluate the effects of iron and dopamine, undifferentiated PC12 cells were challenged for 24 hr against increasing concentrations of ferrocene (1 to 50 mM) and dopamine (0.3 mM and 1 mM) either alone or in combination. The treated cells were analysed according to cell viability and apoptotic index by acridine orange/ ethidium bromide (AO/EB) uptake and stained cytospin preparation of cells (fig. 3A-D). Apoptosis was evaluated in terms of chromatin condensation and fragmentation as described in Materials and Methods (fig. 4). Since undifferentiated PC12 cells die within 24-48 h in serum-free medium (Greene & Tischler 1976), this condition was tested in parallel as a positive control. As shown in fig. 4, serum deprivation induced a more than 10-fold increase in the amount of cells undergoing apoptosis after 24 hr i.e. 15±1.4% (n=3, AO/EB index) and 12±1.5% (n=3, Romanovsky Staining) as compared to 1±0.6% (n=6, AO/EB index and RS) for control cells. Ferrocene alone did not increase the apoptotic index at concentrations ranging between 1 and 50 mM (fig. 4) but, at 10 μM and above, it provoked vacuolisation of the cytoplasm (fig. 3B). This phenomenon was clearly observable upon RS staining and it became more severe when increasing the ferrocene concentration. Dopamine alone did not increase the apoptotic index at 0.3 mM and it produced a small increase at 1 mM i.e. 3.5±0.7% (n=3, AO/EB index) and 2±0.3 % (n=3, RS). However, a combination of 50 mM ferrocene and dopamine induced a marked increase in the number of apoptotic cells: 10±4.2% (n=3, AO/EB) and 7.5±1% (n=3, RS) for 0.3 mM dopamine and 14±5% (n=3, AO/EB) and 15±2% (n=3, RS) for 1 mM dopamine. This increase was quite similar

Table 2.

Relation between quinone formation and apoptotic index. Apoptotic index of PC12 cells (in %) was obtained from acridine orange/ ethidium bromide uptake experiments (Fig. 4). Quinone formation referred to the extent of absorbance at 490 nm after 24 hr incubation at room temperature of dopamine (1mM) with ferrocene (50 μM) either in the absence or presence of ascorbic acid (1 mM). Incubation was performed in supplemented culture medium (1 ml final volume). Values are means±S.D. of 3 experiments.

Treatments	Apoptotic index (%)	Quinone formation (λ=490 nm)
Untreated cells (control)	1.0±0.6	-
Dopamine+ferrocene	14±5	1.49±0.1
Ascorbic Acid (1 mM)	1.2±0.1	-
Ascorbic acid+dopamine+ferrocene	3.5±0.5	0.30±0.0

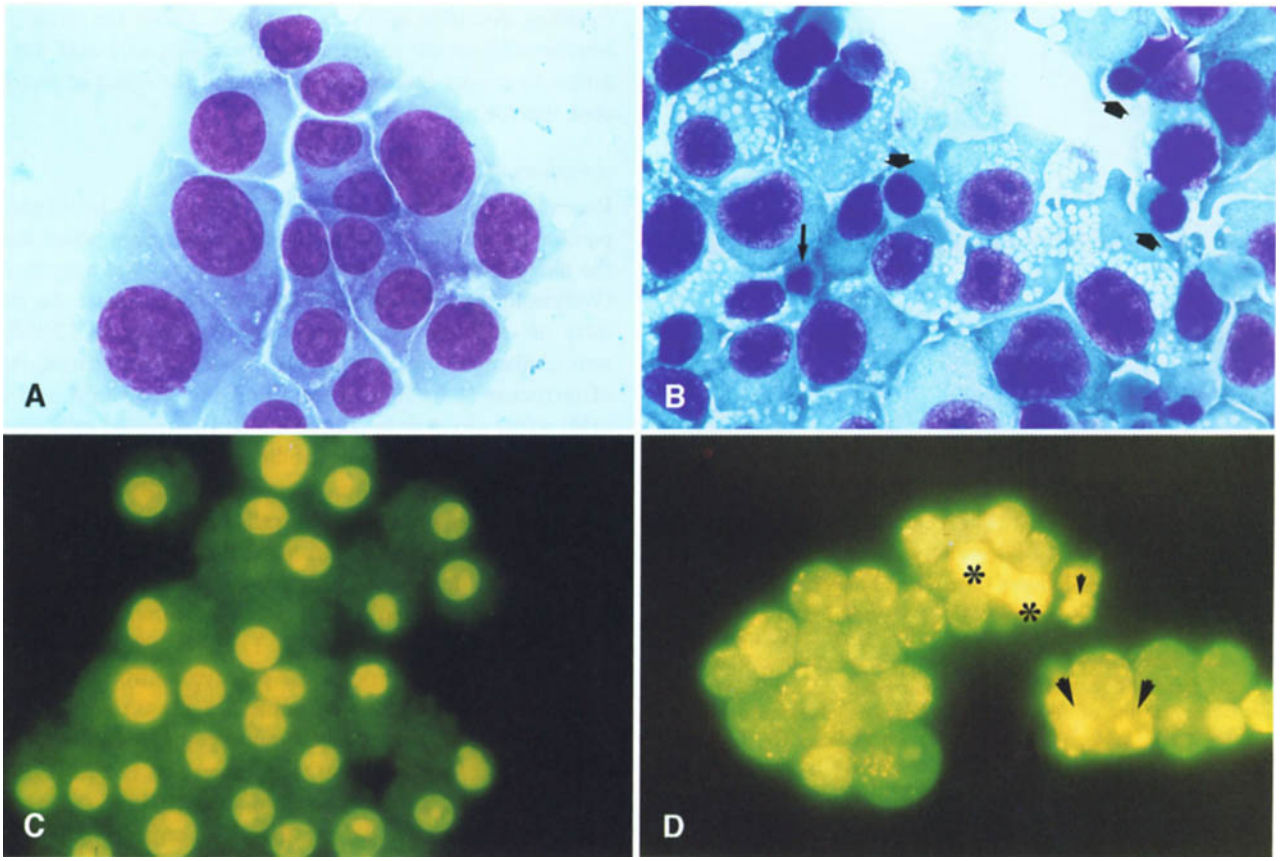

Fig. 3. Morphological characteristics of dopamine/ iron-induced apoptosis. (A and B) Photomicrograph of PC12 cells stained with Romanowsky (RS) and observed under light microscopy. (A) Untreated control cells. (B) Cells from cultures treated with 50 μM ferrocene and 1mM dopamine for 24 hr. These cells present characteristic features of apoptosis including highly condensated and fragmented chromatin (arrows) and extensive vacuolization. (C) Photomicrograph of untreated PC12 cells and (D) treated PC12 cells with 50 μM ferrocene and 1 mM dopamine for 24 hr. Cells were stained with 1 μl (AO/EB) acridine orange/ethidium bromide (0.1 mg/ml). Normal cells (bright green chromatin) can be discriminated from early apoptotic cells (bright green highly condensed or fragmented chromatin, asterisk), late apoptotic cells (bright orange highly condensed or fragmented chromatin, arrowheads). Magnifications: A, B C and D ×1000.

to the one observed after treatment of the cells with serum-free medium.

Depletion of the cell's glutathione content by irreversibly blocking the γ-glutamyl- cysteine synthetase enzyme with compounds such as L-buthionine (S,R)-sulfoximine (BSO) (Meister 1983) is typically used to evaluate the biological role of glutathione in the central nervous system (Pileblad & Magnusson 1990) and to accentuate the cellular effects of an oxidative insult. Addition of 1 mM BSO to the PC12 cells yielded a substantial increase in the extent of apoptosis in the presence of dopamine alone as well as in the presence of dopamine/ferrocene combinations (fig. 4). On the other hand, ascorbic acid (1 mM), produced a four-fold reduction in the dopamine/ferrocene- induced apoptosis (fig. 4). This protective effect of ascorbic acid on dopamine/iron toxicity correlated with a reduced formation of quinones (table 2).

Internucleosomal cleaveage.

The characteristic cleavage of DNA at internucleosomal sites to produce a DNA ladder constitutes a biochemical hallmark of apoptosis in many cells, but not all (Wyllie 1980; Oberhammer *et al.* 1993). The effects of the various treatments on the integrity of the PC12 cell's DNA was tested by agarose gel electrophoresis. However, internucleosomal DNA laddering was not detectable when PC12 cells were deprived from serum for 24 hr or after dopamine and ferrocene treatments (data not shown).

Discussion

In this report, it is shown that iron and dopamine act synergistically to induce apoptosis in undifferentiated PC12 cells. This event is associated with a variety of structural changes such as the co-valent linking of dopamine to proteins, the formation of protein aggregates and lipid peroxidation. These changes are indicative of the formation of quinone oxidation products and free oxygen radicals, two processes which are well known to be associated with catecholamine autoxidation. The cytotoxic actions of dopamine and iron could also result from an alternative pathway involving the turnover of dopamine by monoamine oxidase B enzyme. This gives rise to an increased production of hydrogen per-

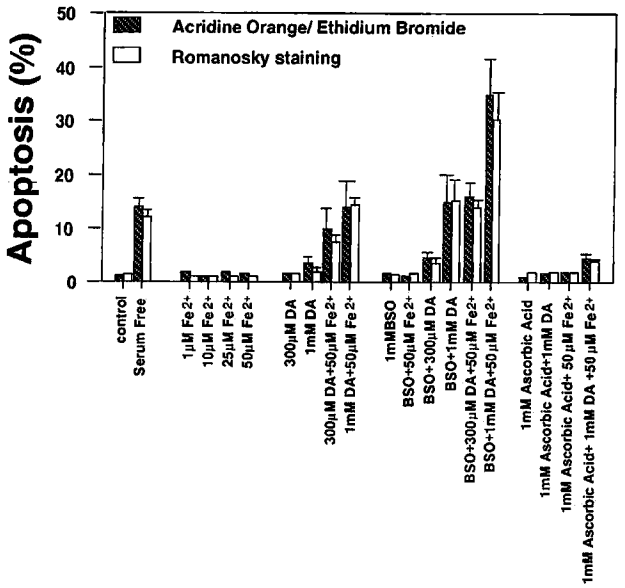

Fig. 4. Dopamine/ ferrocene induced apoptosis in PC12 cells. PC12 cells were incubated for 24 hr with 300 μM or 1 mM of dopamine in the presence or absence of 50 μM ferrocene iron and the listed additives. The evaluation of apoptosis was performed as described in Materials and Methods using parallel techniques of acridine orange/ethidium bromide (AO/EB) and Romanowsky staining (RS). The amount of apoptosis is expressed as a mean of percentage±S.D. from three independent experiments.

oxide which can be transformed by ferrous ions into the highly reactive hydroxyl radical. However, this latter pathway has been prevented by including monoamine oxidase B inhibitor pargyline in the incubation media.

Iron under the form of $FeSO_4$ has been used in our previous studies (Jimenez Del Rio *et al.* 1993b; Velez-Pardo *et al.* 1995a) but since it interferes with microscopic evaluations and staining of PC12 cells, it was replaced by ferrocene. This form of iron has already been used for iron loading in living systems and it has the advantage to be taken up by cells in a relatively short time (Ward *et al.* 1991). $FeSO_4$ and ferrocene increased the binding of [^{3}H]dopamine to "Serotonin binding proteins" in calf brain extracts to nearly the same extent (fig. 1). This effect has previously been shown to involve an oxidative mechanism in where Fe^{2+} reacts with dissolved molecular oxygen to produce superoxide radicals. These radicals then oxidise dopamine to produce semi-quinones and quinones which are able to undergo covalent associations to sulphydryl groups of proteins (Jimenez Del Rio *et al.* 1993b). When added to intact PC12 cells, ferrocene and $FeSO_4$ also produced a comparable increase in the covalent binding of [^{3}H]dopamine to the cell's proteins (fig. 1). These findings comply with the proposal of Maguire *et al.* (1974) that the binding of [^{3}H]dopamine in intact cells involves its oxidation and that this oxidation process is mediated by iron.

Covalent binding of quinone derivatives of catecholamines to nucleophilic groups of proteins is also known to induce their crosslinking (Streeter *et al. 1986; Ito et al.* 1988). This gives rise to the formation of high molecular weight protein aggregates which can be detected on the top of the separating gel after SDS-PAGE. Such aggregates have already been observed when intact cells are treated with catechol derivatives such as menadione (Mirabelli *et al.* 1988) and 6-hydroxydopamine (Rotman *et al.* 1976). The present findings reveal that, if its concentration is sufficiently elevated, dopamine alone is also capable to induce the appearance of high molecular weight protein aggregates in PC12 cells (fig. 2). Low concentrations of dopamine have little effect on their own but the concurrent presence of ferrocene greatly increases the formation of protein aggregates (fig. 2). This complies with earlier in vitro studies on the synergetic effect of iron on a) the ability of dopamine and related neurotoxins to produce protein aggregates in calf brain extracts (Velez-Pardo *et al.* 1996) and b) the ability of catechol oxidation products to covalently crosslink neurofilaments, leading to Lewy body formation (Montine *et al.* 1995).

Cell death commonly occurs by one of two distinct processes, necrosis or apoptosis. Apoptosis, derived from ancient Greek for 'falling off of tree leaves', was introduced by Kerr *et al.* (1972) to describe a type of cell death exhibiting a distinct set of morphological and biochemical features such as cell shrinkage, chromatin condensation, break up of the nucleus, cytoplasmic vacuolisation, plasma membrane blebbing followed by fragmentation of the chromatin into discrete apoptotic bodies (Kerr *et al.* 1972; Martin *et al.* 1994). These morphological features were clearly observed in PC12 cells by light and fluorescence microscopic examination after serum deprivation as well as after 24 hr treatment of the cells with a combination of dopamine and ferrocene but, at the same concentration, dopamine and ferrocene provoked little apoptosis by themselves (fig. 4).

Internucleosomal cleavage of the chromatin with the formation of DNA fragments that are multiples of 180-200 base pairs has often been regarded to constitute one of the main biochemical hallmarks of apoptosis. However, recent studies reveal that this phenomenon is not universal and it has now been put forward that, during apoptosis, DNA fragmentation proceeds through an ordered series of steps commencing with the production of 300- and 50 kb fragments. These large fragments are then susceptible to further degradation into small oligonucleosome fragments which appear as a distinctive "ladder" on electrophoretic DNA gels (Walker *et al.* 1993). Although it has been reported that serum-deprived PC12 cells leads to internucleosomal cleavage of their cellular DNA (Batistaou & Greene 1991), this result has not been reproduced neither by Mesner *et al.* (1992), nor in the present study. Indeed, no DNA "ladders" were detected when the PC12 cells underwent apoptosis upon 24 hr serum deprivation as well as after their treatment with a combination of dopamine and ferrocene. These findings comply with the view that chromatin condensation may be independent of the various types of DNA degradation (Walker *et al.* 1993) and that the cleavage of DNA may be restricted to large fragments (Oberhammer *et al.* 1993; Wyllie 1993).

It has been reported by Tanaka *et al.* (1991) that a combination of iron and dopa or dopamine causes lipid peroxidation in the membranes of cultured dorsal root ganglia and that this complex was responsible for the observed cell death. In the present study, ferrocene and dopamine alone are both capable to increase the extent of lipid peroxidation in PC12 cells and this effect is clearly accentuated when they are given in combination (table 1). Lipid peroxidation is generally regarded to reflect an increased formation of free oxygen radicals and, in this context, it is noteworthy that the effects of ferrocene and dopamine can be differentiated by ascorbic acid (vitamin C).

The most striking chemical property of ascorbic acid is its ability to act as a reducing agent (electron donor). This property allows ascorbic acid to reduce quinones and semiquinones back to catechols (MacDonald & Sirvio 1993; Pardo *et al.* 1993) and this provides a satisfactory explanation for its preventive action on the formation of quinones out of dopamine (table 2 and Pardo *et al.* 1993) and on the formation of dopamine-protein conjugates (Jimenez Del Rio *et al.* 1993b). The inhibitory action of ascorbic acid on dopamine- mediated lipid peroxidation as well as on the ability of dopamine to accentuate the effect of ferrocene (table 1) could also be explained by a decreased formation of quinones, at least if one assumes that the oxidation of dopamine is somehow able to produce/contribute to lipid peroxidation.

In agreement with the findings of Mohanakumar *et al.* (1994), there is no protective effect of ascorbic acid with respect to the ferocene- mediated lipid peroxidation (table 1). These observations are also in accordance with the reported use of ascorbic acid/iron- combinations to generate free radicals (Ramassamy *et al.* 1994) and with the ability of ascorbic acid to undergo autoxidation in presence of iron salt (Wayner *et al.* 1986). The data with ferrocene therefore suggest that ascorbic acid does not prevent oxygen free radical-mediated lipid peroxidation in PC12 cells.

Taken together, the opposite effect of ascorbic acid on the ferrocene and dopamine-mediated lipid peroxidation suggests that the effect of dopamine is mediated by its oxidation products rather than by free oxygen radicals that could be produced during the oxidation process. A quite similar conclusion was reached by Tanaka *et al.* (1991), who found that superoxide and hydroxyl radicals are not essential for the catecholamine/iron-mediated lipid peroxidation in cultured dorsal root ganglia. Their proposal for the involvement of catecholamine-iron complexes with oxidising reactivity therefore merits special attention.

The ability of ascorbic acid to reduce the extent of dopamine/ferrocene-mediated apoptosis as well as dopamine oxidation (table 2) suggests that this process constitutes a potential trigger for apoptosis as well. This conclusion is further supported by the observation that L-buthionine (S,R)-sulfoximine (BSO), an irreversible inhibitor of the g-glutamylcysteine synthetase enzyme (Griffith & Meister 1979), increases the percentage of apoptotic PC12 cells when given in the presence of dopamine either alone or in combination with ferrocene. In contrast, ferrocene does still not produce significant apoptosis in the presence of BSO. These latter data even suggest that dopamine oxidation plays a more important role than the formation reactive oxygen species in the apoptotic process.

Since lipid peroxidation experiments clearly reveal that ferrocene is able to promote an oxidative stress in PC12 cells, it can be concluded that there is no necessary causual link between lipid peroxidation and apoptosis and, hence, that both phenomena may occur independently from each other. This notion is further supported by the ability of serum deprivation to induce apoptosis in the absence of detectable lipid peroxidation.

The observed link between apoptosis and the other cellular processes which were found affected by a combination of dopamine and iron (i.e. protein cross-linking and their covalent modification by reactive quinones) could also be merely accidental rather than causal. In this context, it is known that transglutaminases, which cross-link proteins through ε-(η-glutamyl)lysine bonds (Folk 1980), maintain cellular integrity during cell damage process (Byrd & Lichti 1987) and make part of the biochemical pathway of apoptosis (Fesus *et al.* 1987). It can therefore not be excluded that the dopamine/ferrocene-mediated cross-linking of proteins may produce the same result, but since this is not as well organised as enzyme-mediated process, we feel that it is more likely to disrupt the physiological organisation of cytoskeletal structures.The present findings on PC12 cells are in agreement with current views about iron and catecholamine-mediated neurotoxicity, i.e. a) iron greatly accelerates the autoxidation of monoamines such as dopamine (Langston *et al.* 1987; Jimenez Del Rio *et al.* 1993b & 1994) and b) the autoxidation of dopamine produces reactive quinones and oxyradicals (Graham 1978; Brunmark & Cadenas 1989) whose accumulation induces oxidative stress in tissues resulting in a number of events such as damage of the mitochondrial electron transport, increase in the cytosolic free calcium concentration, activation of proteases, lipid peroxidation and DNA fragmentation (Halliwell & Aruoma 1991; Halliwell B, 1992; Orrenius *et al.* 1992; Ben-Shachar *et al.* 1995). The cytotoxic actions of dopamine and iron could also result from an alternative pathway involving the turnover of dopamine by the monoamine oxidase B enzyme but, since the present experiments were carried out in the presence of pargyline, our data suggest that iron is also capable of increasing the cytotoxicity of dopamine by increasing its rate of oxidation.

The cause (or causes) for the degeneration of dopaminergic neurons in Parkinson's disease is still unknown but several suggestions have been made over the years. Among them, the endogenous toxins hypothesis, or more commonly the oxidant stress hypothesis, is regarded by some (Jenner 1992; Fahn & Cohen 1992) to be the best currently available. This hypothesis refers to an unbalanced condition in which the protecting system is overcome by oxidising species resulting from the abnormal metabolism of e.g. dopamine. Some indications for oxidant stress have

indeed been found in the substantia nigra of parkinsonian patients; i.e. depletion of reducing substances such as glutathione (Sofic et al.1992), a high concentration of iron (Riederer *et al.* 1989; Dexter *et al.* 1991; Sofic *et al.* 1991; Sengstock *et al.* 1992), oxidation of proteins and membrane lipids (Dexter *et al.* 1989). The present data indicate that cultured PC12 cells represent a convenient model system to investigate such different cellular aspects of iron and dopamine-related oxidative stress and, hence, that such cells could be used to investigate potential factors which contribute to the degeneration of dopaminergic neuroens in Parkinson's disease.

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