Dopamine Induces Phenotypic Differentiation or Apoptosis in a Dose-Dependent Fashion: Involvement of the Dopamine Transporter and p53

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Neurodegeneration · Parkinson's disease · Programmed cell death · DNA damage · Aging · Catecholamine

Abstract
The effect of dopamine on the growth, phenotypes (morphological and biochemical) and programmed cell death (apoptosis) of the human neuronal NMB cell line was examined. Exposure to 20–50 μM of dopamine decreased cell growth, induced an apparent differentiated cell morphology and increased 3H-dopamine uptake. At higher concentrations (100–300 μM) dopamine was neurotoxic and induced apoptosis, as reported previously. The observed effects of both low and high doses of dopamine were blocked by cocaine, which suggested involvement of dopamine transporters. Indeed, several experiments demonstrated the relationship between dopamine uptake of cells and their vulnerability to the toxic effect of dopamine. High concentrations of dopamine, which induced apoptosis, also increased p53 levels, detected by RT-PCR analysis and immunoblotting, whereas lower dopamine concentrations, which induced a differentiated phenotype, did not increase p53 immunoblotting. Dibutyryl-cAMP and dimethyl sulfoxide, which induced differentiation but not apoptosis of the NMB cells, did not increase p53 expression. These findings provide an insight into the role of dopamine, dopamine transporters and p53 in the differentiation and apoptosis of dopaminergic neurons, which will further our understanding of neuronal development and neurodegenerative diseases.

Introduction
Dopamine, a major catecholamine neurotransmitter in the central nervous system, controls motor activity, cognition, hormone secretion and several normal and pathological behaviors [Cooper et al., 1996]. A developmental role for dopamine has also become evident during the last decade. For example, the development and innervation of certain brain structures are altered by lesioning of defined neuronal pathways or by treatment with dopamine uptake inhibitors...
inhibitors (such as cocaine) or drugs that induce dopamine depletion [Graybiel, 1990; Gerfen et al., 1991; Levitt et al., 1997; Stanwood et al., 2001]. Dopaminergic innervation also controls the patch/matrix organization of the striatum in organotypic cocultures [Snyder-Keller et al., 2001], and dopamine receptor knockout mice display abnormal axonal arborization, behavior and cortical activity [Rubinstein et al., 2001; Parish et al., 2001]. The suggested involvement of dopamine in neurogenesis is in line with the role of other neurotransmitters, such as acetylcholine, serotonin and γ-aminobutyric acid, in development [for reviews, see Levitt et al., 1997; Pendleton et al., 1998].

Whether a neuron in the developing brain multiplies, differentiates or dies depends on the expression of various genes [Freeman et al., 1994; Thompson, 1995; Ross, 1996]. In fact, the normal brain development is accompanied by the death of many neurons [Cowan et al., 1984; Oppenheim, 1991], and programmed cell death (apoptosis) has been established as a major mechanism underlying cell loss in the developing nervous system [Naruse and Keino, 1995; Jackson-Lewis et al., 2000]. Apoptosis also plays an important role in neurodegenerative processes in the adult brain and has been implicated as the major cause for several neurodegenerative diseases [Martin, 2001]. Although degeneration of dopaminergic neurons in the substantia nigra underlies the pathophysiology of Parkinson’s disease [Hornykiewicz, 1973], the extent to which apoptosis is involved is uncertain [Offen et al., 2000; Burke and Kholodilov, 1998; Blum et al., 2001]. The underlying cause of Parkinson’s disease is still not understood, despite the extensive study of the etiology of dopaminergic cell loss, the contribution of free radicals, environmental neurotoxins interacting with the mitochondria and genetic factors [Kopin, 1993; Blum et al., 2001; Jenner, 2001; Vaughan et al., 2001].

Dopamine as well as several of its metabolites are now known to be neurotoxic. Dopamine can induce cell cycle arrest, intranucleosomal DNA fragmentation and apoptosis in cultures of chicken sympathetic neurons [Ziv et al., 1994; Barzilai et al., 2000] and human neuronal cell lines [Simantov et al., 1996; Junn and Mouradian, 2001]. Similar events occur when primary mesencephalic dopaminergic neurons and rat pheochromocytoma PC12 cells are treated with 6-hydroxydopamine [Walkinshaw and Waters, 1995; Lotharius et al., 1999]. In these models of apoptotic cell death, several genes are involved, including Bcl2, BclX, cyclin B and p53 [Cassarino et al., 1997; Blum et al., 1997; Shirvan et al., 1997; Daily et al., 1999; Lotharius et al., 1999; Barzilai et al., 2000; Rabinovic et al., 2000]. Involvement of p53 in dopamine-induced cell death is in agreement with the involvement of p53 in regulating cell growth, apoptosis and cancer [Oren, 1999].

In this study, the human neuronal cell line NMB [Brodeur et al., 1977; Simantov et al., 1996] was used as a model system for dopamine-induced apoptosis. In these cells, dopamine-induced apoptosis has previously been shown to be attenuated by the dopamine transporter (DAT) inhibitor cocaine and after knockdown of (DATs) with an antisense phosphothioate oligonucleotide [Simantov et al., 1996]. In addition, glutathione and N-acetyl cysteine, but not vitamin E or ascorbic acid, decreased the neurotoxicity of dopamine, which suggests differential protective effects for endogenous antioxidants [Gabbay et al., 1996]. Herein, we observed that low concentrations of dopamine alter cell proliferation, without being toxic, which prompted us to further analyze the dose-dependent effect of dopamine on the differentiation and apoptosis. The involvement of the DAT and p53 gene in this dose-dependent effect of dopamine, and the effects of dibutyryl-cAMP and dimethyl sulfoxide (DMSO), which also induce differentiation of NMB cells, were studied. Understanding the underlying mechanisms of differentiation and apoptosis induced by dopamine will advance the understanding of neuronal development and neurodegenerative diseases such as Parkinson’s disease.

Materials and Methods

Materials and Cell Cultures

Dopamine, dibutyryl-cAMP, RNase A, ethidium bromide, ethelenediamine-tetracetic acid (EDTA) and Tris-Cl were purchased from Sigma Co., DMSO from Merck and Tri-reagent from MRC Inc. 3H-Dopamine (specific activity 43–49 Ci/mmol) and 125I-protein A (0.5 mCi/ml) were from Radiochemical Centre, Amersham, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell proliferation kit from Roche Diagnostics and iron-supplemented calf serum from Hyclone Laboratories. All other compounds used were of analytical grade.

NMB cells [Brodeur et al., 1977] were kindly provided by Drs. M.N. Goldstein and Y. Wollman, and adapted to Dulbecco modified Eagle medium with 10% Hyclone calf serum as reported previously [Simantov et al., 1996]. Cells were maintained in a 5% CO2 incubator at 37 °C and subcultured every 4 or 5 days. Dopamine was added at the indicated concentrations from a freshly prepared stock, and cultures were incubated with dopamine for 1–3 days. Viability was determined by (a) counting viable cells and (b) the MTT cell proliferation assay [Heeg et al., 1985]. Briefly, 10⁶ cells were seeded into a 96-well plate and 1 day later treated with the indicated concentration of dopamine. After the incubation period (1–3 days), the medium was replaced with fresh medium containing 10% MTT, cells were incubated further for 4 h and 100 μl solubilization solution was added to each well and incubated overnight. Absorbance was measured

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at 550 nm in an ELISA plate reader. Statistical significance was determined with Student’s t test.

Uptake of $^3$H-dopamine in 35-mm plates was performed as follows. Cells were preincubated for 20 min at 37°C in 1.5 ml fresh medium without or with 50 μM cocaine. $^3$H-dopamine was added (final concentration 20 nM) and incubation continued for 15 min. medium was removed and plates were washed 3 times with 2 ml ice-cold phosphate-buffered saline (PBS). Cells were then dissolved with 0.5 M NaOH (2 h at 37°C), neutralized with HCl, and radioactivity was determined with scintillation fluid.

For morphological analysis, cultures were treated 48 h after seeding with 50 μM dopamine, 50 μM cocaine or both, washed with ice-cold PBS, fixed with 4% paraformaldehyde +0.2% glutaraldehyde, stained (May-Grünwald/Giemsa) and photographed. The cocaine concentration used was assumed to be necessary to inhibit the long-term exposure to dopamine. At this concentration cocaine was not toxic to NMB cells and did not change the cells’ growth or morphology.

**DNA Fragmentation**

Cells were collected, washed with PBS and lysed in 10 mM Tris-Cl buffer, pH 7.4, containing 0.6% sodium dodecyl sulfate (SDS) and 10 mM EDTA, and sodium chloride was added (660 mM final concentration). Samples were incubated overnight at 4°C, centrifuged for 30 min at 10,000 g, and DNA was separated after phenol-chloroform extraction, precipitated with ethanol, and the pellet was suspended in distilled water and treated with RNase A (0.01 μg/ml, 30 min at 37°C). NaCl and polyethylene glycol 8000 were added to the DNA to final concentrations of 500 mM and 6.5%, respectively. After incubation for 60 min in an ice-cold water bath, the samples were centrifuged at 10,000 g for 15 min, pellets were washed with 70% ethanol, dried and suspended in SDS + EDTA. Samples of 28 μg DNA were analyzed on 1.5% agarose gel containing 0.15 μg/ml ethidium bromide.

**Analysis of p53 Expression by RT-PCR and Immunoblotting**

RT-PCR analysis of p53 and glyceraldehyde dehydrogenase (GAPDH, used to confirm equal loading on the gels) was performed as follows. Total RNA (about 2.5 μg) extracted with Tri-reagent according to the manufacturer’s protocol was incubated at 42°C for 50 min in 20 μl of 1 × first-strand buffer containing 0.5 μM of the random primer 5’GGACAGCTTC-3’, 200 units Super Script II RNase H reverse transcriptase, 1 μl 10 mM deoxynucleotide triphosphates (dNTPs), 2 μl 10 mM dithiothreitol and 2 μl 0.25 mM MgCl₂. The reaction was terminated at 72°C for 15 min. PCR was carried out in 30 μl containing 2 μl of cDNA sample, 1 × PCR buffer, 200 μM dNTPs, 0.2 μM of each gene-specific primer and 1.5 units of Taq DNA polymerase (Takara, Japan). PCR was performed as follows: denaturation for 3 min at 94°C followed by 1 min at 94°C, 90 s at 58°C and 50 s at 72°C for 27 cycles and a final 5-min incubation at 72°C. Primer pairs for GAPDH and p53, respectively, were 5’-GGACATCATCCCTGCCTCTAGTG-3’ and 5’-AATTTGCGGCCCCAGGCTAAGGT-3’ as well as 5’-TGACTCAGACTGACATTCTCC-3’ and 5’-CTCCACACAAACAAACC-3’. PCR products were separated on 1.5% agarose gel.

For p53 immunoblotting, cells were homogenized in 2% SDS, and 5 × sample buffer (83 mM Tris-Cl buffer, pH 6.8, containing 5% SDS, 0.28 mM EDTA, 1 M β-mercaptoethanol, 1/6 glycerol v/v and 0.06 mg/ml bromophenol blue) was added. Samples of 100 μg protein (determined by Lowry et al. [1951]) were analyzed on 7% SDS-polyacrylamide gels. After blotting to nitrocellulose membranes, membranes were stained with 0.2% Ponceau-S in 3% trichloroacetic acid, and gels were stained with Coomassie blue to verify the efficiency of protein transfer. Nitrocellulose membranes were incubated for 3 h at room temperature with PBS containing 10% low-fat milk, washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) and incubated for 90 min with 1:200 of p53-specific antibodies PAb421 and PAb240 (kindly provided by M. Oren). Membranes were then washed with PBS-T, incubated for 120 min with 125I-protein A and washed with PBS-T. Radioactivity was analyzed by a phosphoimager (Fugix-BSA2000), using MacBAS 2.0 software.

**Results**

NMB neuronal cells exposed to low doses (20–50 μM) of dopamine acquired a spindle-shaped cell body, displayed 1–4 processes of 20–100 μm and had cell bodies 40–70% larger than those of untreated cells. Figure 1 depicts the effect of 50 μM dopamine. In parallel to these morphological changes, cell proliferation was reduced in a time- and dose-dependent fashion, as determined by counting viable cells and the MTT proliferation assay (fig. 2). For example, upon treatment with 20 and 50 μM dopamine for 3 days, the number of viable cells decreased by 32 and 56%, and the MTT values by 11 and 32%, respectively. These relatively low concentrations of dopamine, particularly that of 20 μM, were not cytotoxic, since the number of dead cells (0.2–0.4% of the total cell number) did not increase. Treatment with higher concentrations of dopamine (100–300 μM) caused retraction of cell processes, shrinkage of the cell body and loss of cell brightness, followed by detachment from surfaces and cell death, as previously reported [Simantov et al., 1996]. Such cell death exhibits a characteristic apoptotic profile, including cell cycle arrest, intranucleosomal DNA fragmentation and ladder formation [Simantov et al., 1996]. When NMB cells were incubated with dopamine for several days, a dose-dependent increase in the uptake of $^3$H-dopamine was observed (fig. 3a). Overall, the dose-dependent effects of dopamine suggested that this neurotransmitter has a dual effect in NMB cells, with low concentrations inducing a differentiated phenotype, and higher concentrations exhibiting neurotoxicity and inducing programmed cell death.

Attempting to further verify this issue, two other compounds inducing differentiation, dibutyryl-cAMP and DMSO, were analyzed. Both dibutyryl-cAMP and DMSO induced morphological changes similar to those induced by 50 μM dopamine (data not shown), decreased cell growth and increased $^3$H-dopamine uptake in a dose-dependent way (fig. 3b, c). Analysis of DNA fragmenta-
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Fig. 1. Effect of 50 μM dopamine on the morphology of NMB cells. Three days after treatment with dopamine, with or without cocaine, cells were fixed and stained as described in Materials and Methods. Cocaine (50 μM) was added, where indicated, 1 h prior to dopamine. The bar in the control panel indicates 5 μm.

A...
Fig. 2. Time course of the dopamine effect on the proliferation of NMB cells. Viable cell number (b) or MTT cell proliferation assay (a) were determined 1, 2 and 3 days after treatment with 0, 20, 50 or 100 µM dopamine. Data are means ± SD from triplicates. *p < 0.005, **p < 0.001: significantly different from control cells (0 dopamine).

Table 1. Specific and nonspecific ³H-dopamine uptake after pre-treatment with dopamine

<table>
<thead>
<tr>
<th>Time of treatment, h</th>
<th>Specific uptake</th>
<th>Nonspecific uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.5 ± 4.3</td>
<td>18.2 ± 5.4</td>
</tr>
<tr>
<td>1</td>
<td>51.5 ± 2.8</td>
<td>16.3 ± 2.8</td>
</tr>
<tr>
<td>3</td>
<td>60.5 ± 1.6</td>
<td>16.6 ± 1.3</td>
</tr>
<tr>
<td>6</td>
<td>62.5 ± 1.5</td>
<td>16.1 ± 0.7</td>
</tr>
<tr>
<td>24</td>
<td>15.2 ± 2.1*</td>
<td>10.4 ± 0.6*</td>
</tr>
<tr>
<td>48</td>
<td>8.3 ± 3.2*</td>
<td>10.6 ± 2.6*</td>
</tr>
</tbody>
</table>

Cells were treated with 100 µM dopamine, washed at the indicated time points, and specific and nonspecific ³H-dopamine uptakes were determined without or with cocaine (50 µM). Data are means ± SEM of femtomoles ³H-dopamine/well from 4 experiments. *p < 0.001: significantly different from control (0 time).

Fig. 3. Dose-dependent effect of dopamine (a), dibutyryl (DB)cAMP (b) or DMSO (c) on cell viability and ³H-dopamine uptake. Viable cells (■) and ³H-dopamine uptake (□) were determined 3 days after treatment. Data are means ± SEM of 2–5 experiments.
Fig. 4. DNA fragmentation in cells treated with dopamine, dibutyryl (DB)-cAMP or DMSO. Cells were treated for 1 day with 50 μM dopamine, 0.5 mM dibutyryl-cAMP or 1.5% DMSO, and DNA was extracted and analyzed on agarose gel. Figures on the right indicate DNA markers.

Fig. 5. RT-PCR and Western immunoblotting of p53 in dopamine-treated cells. 

a RT-PCR of p53 in NMB cells treated with 100 μM dopamine for 0–48 h. Total RNA samples, in 2 or 3 replications, were analyzed as described in Materials and Methods. Data are means ± SEM of the intensity of the 204-bp p53 product. 

b Cells were treated for 1 day with 0–300 μM dopamine, proteins were extracted and Western immunoblotting with anti-p53 antibodies was performed. Molecular-weight markers are indicated on the left. 

c Phosphoimage analysis of p53 immunoblotting analysis of cells treated with 300 μM dopamine for 0–48 h. PSL = photostimulated luminescence. Data are means ± SEM from 3 experiments.
munoblotting and phosphoimage analysis. In cells treated with 10, 25 or 50 \( \mu M \) dopamine, p53 levels were 95, 113 and 126\% of those of untreated cells. These changes are much less than those observed with the toxic doses (100–200 \( \mu M \)) of dopamine and are statistically insignificant (d.f. = 9, \( F = 1.67, p = 0.289 \)). Moreover, neither dibutyryl-cAMP nor DMSO, which both induce a differentiated phenotype of NMB cells similar to that induced by dopamine, altered the p53 levels of the cells (fig. 6). It is worth indicating that the p53-immunoreactive band of NMB cells corresponds to that of the human p53 protein detected in rat fibroblasts transfected with a vector expressing human p53 (performed by M. Oren and associates). Also, p53 immunoreactivity in NMB cells was increased by 145 ± 15\% upon UV irradiation (data not shown), which indicates that expression of p53 in NMB cells is regulated upon exposure to toxic agents.

**Discussion**

Besides being able to induce programmed cell death at high doses (100–300 \( \mu M \)) [Ziv et al., 1994; Simantov et al., 1996; Barzilai et al., 2000; Junn and Mouradian, 2001], herein we demonstrate that dopamine, at low, non-neurotoxic doses (20–50 \( \mu M \)) can decrease cell proliferation and induce characteristics compatible with differentiated neurons. These characteristics of differentiation include altered cell morphology, development of processes and increased \(^3\)H-dopamine uptake. Using differential display PCR, we have recently observed that the low dopamine concentrations also increased the expression of several genes, some of them involved in neuronal differentiation [Premkumar and Simantov, in preparation]. These findings with low and high doses of dopamine suggest that dopamine has a dual effect on NMB, and possibly other neuronal cells, depending on the concentration of the neurotransmitter. The observed effect of low concentrations of dopamine on the proliferation and differentiation of NMB cells is similar to the developmental role of other neurotransmitters, such as acetylcholine, serotonin, \( \gamma \)-aminobutyric acid and other catecholamines [Lauder, 1993; Levitt et al., 1997; Pendleton et al., 1998].

Although the mechanism(s) responsible for the effect of dopamine on the proliferation and differentiation of NMB cells are not yet known, blockade of the effect by cocaine strongly implicates a transporter-mediated pathway. Yet, involvement of dopamine-receptor-mediated effect cannot be ruled out, since the expression of dopamine receptors by NMB cells was not determined. However, the potent dopamine receptor inhibitor haloperidol does not block dopamine-induced apoptosis and the dopamine agonist 2-bromoergocriptine is not toxic to NMB cells [Simantov, 1997], which does not support a role for dopamine receptors in the proliferation and differentiation of NMB cells. Similar transporter-mediated dopamine toxicity has recently been reported with another
neuroblastoma cell line [Junn and Mouradian, 2001]. In other cell types, particularly those that lack dopamine transporters, different pathways, including those that involve dopamine receptors, may mediate the effects of dopamine on cell growth, differentiation and apoptosis.

The involvement of several genes, including cyclin A and B2, in dopamine-induced apoptosis was demonstrated in sympathetic neurons of the chicken [Shirvan et al., 1997], which also show p53 activation. Herein, when NMB cells were induced to differentiate by dibutyryl-cAMP, DMSO or low concentrations of dopamine, the expression (determined by immunoblotting and RT-PCR) of p53 did not change basically. In contrast, at high concentrations of dopamine that induce apoptosis, the expression of p53 increased up to 3-fold, with the peak increase within 3–6 h, which was prior to cell death. This early induction of p53 by dopamine is in agreement with the involvement of p53 in programmed cell death of various cell types [Oren, 1999], including cells in the central nervous system [Eizenberg, et al., 1995, 1996].

Metabolism of dopamine results in production of free radicals, and ionizing radiation stimulates cell differentiation via activation of specific genes [for examples, see Chae et al., 1999]. The possibility that dopamine-derived low or high levels of free radicals, depending on their concentration, may activate different genes, associated with differentiation or apoptosis, respectively, is worth close analysis. Dibutyryl-cAMP and DMSO have not been reported to induce excess production of free radicals, therefore cell differentiation induced by these compounds may involve other regulatory pathways, such as induction of genes regulated by protein kinase A or alterations in membrane phospholipids, respectively. Elucidating the genes involved in dopamine neuron differentiation would have obvious developmental implications. Taken together the findings reported herein contribute to the understanding of the underlying mechanisms of differentiation and apoptosis induced by dopamine in neurons expressing dopamine transporters. The in vivo implications of these results to neurogenesis and neurodegenerative diseases such as Parkinson’s disease need further analysis.

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### References


