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Activation of nuclear transcription factor kappa B (NF-κB) is essential for dopamine-induced apoptosis in PC12 cells

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Abstract

The etiology of Parkinson's disease is still unknown, though current investigations support the notion of the pivotal involvement of oxidative stress in the process of neurodegeneration in the substantia nigra (SN). In the present study, we investigated the molecular mechanisms underlying cellular response to a challenge by dopamine, one of the local oxidative stressors in the SN. Based on studies showing that nuclear factor kappa B (NF-κB) is activated by oxidative stress, we studied the involvement of NF-κB in the toxicity of PC12 cells following dopamine exposure. We found that dopamine (0.1–0.5 mM) treatment increased the phosphorylation of the IκB protein, the inhibitory subunit of NF-κB in the cytoplasm. Immunoblot analysis demonstrated the presence of NF-κB-p65 protein in the nuclear fraction and its disappearance from the cytoplasmic fraction after 2 h of dopamine exposure. Dopamine-induced NF-κB activation was also evidenced by electromobility shift assay using radioactive labeled NF-κB consensus DNA sequence. Cell-permeable NF-κB inhibitor SN-50 rescued the cells from dopamine-induced apoptosis and showed the importance of NF-κB activation to the induction of apoptosis. Furthermore, flow cytometry assay demonstrated a higher level of translocated NF-κB-p65 in the apoptotic nuclei than in the unaffected nuclei. In conclusion, our findings suggest that NF-κB activation is essential to dopamine-induced apoptosis in PC12 cells and it may be involved in nigral neurodegeneration in patients with Parkinson's disease.

Keywords: apoptosis, dopamine, nuclear transcription factor-kappa B, Parkinson's disease.

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Parkinson's disease (PD), one of the most common neurological disorders in the elderly, is characterized by progressive and massive loss of midbrain dopaminergic neurons. The mechanism by which these neurons degenerate is still unknown. However, several environmental and genetic factors have been suggested to contribute to this selective vulnerability. Of these, reactive oxygen species (ROS), mitochondrial dysfunction, excitotoxicity and inflammatory processes have been intensively investigated (Hirsch 1992; Marsden and Ollanow 1998). Post-mortem studies of the substantia nigra (SN) have yielded evidence of massive lipid peroxidation, protein and DNA oxidation processes (Jenner and Olanow 1998). The precise trigger for the local oxidative stress in PD is not yet clear. However, it has been suggested that high concentrations of dopamine, neuromelanin and iron, make the nigra particularly vulnerable to degenerative processes (Youdim *et al*. 1989; Ziv *et al*. 1996; Offen *et al*. 1997). Dopamine metabolism by monoamine oxidase or auto-oxidation leads to the formation of hydrogen peroxide (H_2O_2). Normally, H_2O_2 is inactivated by catalase or by reduced glutathione (GSH) peroxidase. Nonetheless, in the presence of iron it can react to form cytotoxic hydroxyl radicals. Auto-oxidation of dopamine can also lead to the formation of superoxide radicals and of reactive quinones and semiquinones. Similarly, neuromelanin in the dopaminergic neurons binds ferric iron (Fe^{3+}), can reduce it to its ferrous (Fe^{2+}) form, and can be highly toxic (Youdim *et al*. 1989; Offen *et al*. 1997).

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; NF-κB, nuclear transcription factor-kappa B; FCS, fetal calf serum; NR, neutral red; PBS, phosphate-buffered saline; PD, Parkinson's disease; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SN, substantia nigra; TBS, Tris-buffered saline.

One of the transcription factors known to be activated by oxidative stress is the nuclear transcription factor kappa B (NF-κB) (Lezovalch *et al*. 1998; Mattson *et al*. 1997, 2000). NF-κB, a member of the Rel transcription factor family, participates in the regulation of a broad array of genes involved primarily in immune and defense mechanisms (Baeverle 1991, Baeverle and Henkel 1994, Baichwal and Baeverle 1997, Lipton 1997). In nonstimulated cells, NF-κB is sequestered in the cytoplasm by an inhibitory subunit, the IκB protein. Stimulation of cells by diverse agents causes phosphorylation of IκBα and, subsequently, its degradation by the proteosome (Brown *et al*. 1995). Liberated NF-κB is transported into the nucleus where it induces the transcription of target genes, including IκBα as an autoregulatory loop (Baeverle and Henkel 1994).

In recent years, major advances have been made towards understanding the mechanisms of cell death. Apoptosis is a genetically controlled cell 'suicide' program, inherent to every eukaryotic cell. Normally, apoptosis plays a major role in tissue development and homeostasis. However, 'inappropriate' activation of apoptosis is fundamental to the pathogenesis of tissue damage, both in acute and chronic disease states (Mochizuki *et al*. 1997). Indeed, apoptosis is now recognized to be a major factor in the pathogenesis of numerous neurological diseases, ranging from acute cerebral stroke to Parkinson's and Alzheimer's diseases (Marks and Berg 1999). We have shown that the endogenous neurotransmitter dopamine and its oxidative-derived metabolites can trigger apoptosis in various cultured neurons, lending support to the view that apoptosis is important to the degenerative processes occuring in the SN of patients with PD. (Ziv *et al*. 1994, 1997; Offen *et al*. 1997, 1998). Indeed, several postmortem studies of PD patients demonstrated morphological signs of apoptosis in dopaminergic neurons (Mochizuki *et al*. 1996, 1997; Tompkins *et al*. 1997; Tatton *et al*. 1998), although others failed to detect such a process (Kingsbury *et al*. 1998; Banati *et al*. 1998; Wullner *et al*. 1999).

The involvement of NF-κB in the pathogenesis of PD was first suggested by Hunot *et al*. (1997) who found an increase in NF-κB immunoreactivity of more than 70-fold in the nuclei of dopaminergic cells of patients with PD compared to control subjects. However, its precise role in the degenerative process remains controversial. The aim of the present study is to investigate the role of NF-κB activation in the toxicity of PC12 cells, following dopamine exposure.

Materials and methods

Cell culture

PC-12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% heat inactivated fetal calf serum (FCS), 8% horse serum, penicillin (25 μg/mL), streptomycin (25 μg/mL) and 2 mM L-glutamine (Biological Industries, Beit Haemek, Israel). Confluent cultures were washed with phosphate-buffered saline (PBS) pH 7.0, detached with 0.5 mM EDTA, centrifuged and subcultured to poly L-lysine coated 96-well microtiter plates (Nalge Nunc, Naperville, IL, USA), 0.1 mL of 5×10^5 cells/mL in each well.

Pharmacological treatments

Cells were treated for the indicated incubation periods with various concentrations of dopamine and untreated cells served as controls. The inhibitory agents were dissolved directly into the culture medium at the indicated concentration 1 h before dopamine treatment.

Neutral red (NR) assay

NR is a selective staining for lysosomes in living cells. Culture medium was washed out and 100 μL NR (0.39%, Sigma, St Louis, MO, USA) was added to the cells in 96-well microtiter plates in DCCM-1 medium (dissolved 1 : 100) and incubated for 2 h at 37°C. Wells were then washed with cold PBS containing 1 mM $MgCl_2$ solution and incubated for 15 min in 50% Sorenson buffer (70 mM sodium citrate, 30 mM citric acid, 0.1 M HCl) and 50% ethanol. The developed color was measured by ELISA reader (590 nm).

Western blot

After the appropriate treatments, PC12 cells (10^6 each sample) were washed with PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4), scraped and pelleted by centrifugation at 1500 *g* for 5 min. The pellet was resuspended in 400 μL of cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then allowed to swell on ice for 15 min, followed by the addition of 0.025 mL NP-40 (10%) and vortexed for 10 s at a high speed. The mixture was centrifuged for 30 s and the supernatant was designated and used as a cytoplasm fraction. The pellet was resuspended with 0.05 mL of ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 25% glycerol. The extract was then homogenized, rocked vigorously for 15 min, and centrifuged for 5 min. The supernatant was used as a nuclear fraction (Hallahan *et al*. 1995). Protein content was determined with BCA kit (Pierce, Rockford, IL, USA). Nuclear and cytoplasmic fractions were resolved by electrophoresis on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel and electrotransferred to PVDF membranes as recommended by the manufacturer (Amersham, Buckinghamshire, UK). Membranes were probed with rabbit polyclonal IGg against NF-κB (p65) antibody (SC-109, Santa-Cruz, CA, USA) for 1 h at room temperature and washed three times (5 min each) with TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20). The membranes were then incubated with horseradish peroxidase conjugated goat anti-rabbit antibodies for 1 h at room temperature, then washed and incubated with the enhanced chemiluminescence system (ECL, Amersham).

Gel shift assay

Electrophoretic mobility shift assays of nuclear protein extracts from dopamine-treated PC12 cells were preformed as previously described. (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with [^{32}P]ATP (Amersham) using polynucleotide kinase (Promega, Madison, WI, USA). Nuclear extracts from PC12 cells

(10 μg) were incubated for 30 min at room temperature with the end labeled DNA (1μg) in binding buffer containing 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 20% glycerol, 50 mM Tris-HCl pH 7.5 and 2 μg/sample of poly (dI-dC) in a final volume of 25 μL. Competition with unlabeled oligonucleotide of NF-κB binding sequence at a 100-fold molar excess was used to analyze specific bands. The reaction products were analyzed by 6% nondenaturating polyacrylamide gel electrophoresis. The specific bands were visualized by X-ray autoradiography and phosphoimaging.

Immunocytochemistry

PC12 cells treated with dopamine were fixed in 4% paraformaldehyde (in PBS pH 7.4) for 15 min at room temperature. Cells were then washed three times with Tris-buffered saline (TBS): 50 mM Tris-HCl, 0.9% NaCl (pH 7.6) and quenched in 3% H_2O_2 for 5 min at room temperature. After three more washes in TBS, the cells were blocked in 10% normal goat serum (0.1% Triton X-100) in TBS for 1 h at 4°C, washed twice with TBS, followed by incubation with anti-NF-κB p65 antibody or anti-phosphorylated IkB antibody (New England Bio-Labs, Beverly, MA, USA, diluted in 2% normal goat serum and 0.1% Triton X-100) for 24 h at 4°C. The cells were then washed three times with TBS and incubated with biotinylated secondary antibody (diluted 1 : 500 in 3% bovine serum albumin) for 1 h at room temperature. After further washes with TBS, the cells were incubated with ABC reagent (Vector, Burlingame, CA, USA) at room temperature and washed three times with TBS followed by incubation for 10 min with 3,3′-diaminobenzidine (DAB) reagent. The cells were washed with water and examined under bright field microscopy (Olympus IMT-2).

Reactive oxygen species production assay

ROS production was measured using a membrane penetrative diacetate derivative of 2′,7′-dichlorofluorescein (DCFH-DA). Upon entering the cells, the diacetate group is cleaved enzymatically. Both DCFH-DA and DCFH are nonfluorescent fluorescein analogs. However, during oxidative burst they are oxidized to highly fluorescent compounds (DCF, Rosenkranz *et al*. 1992).

PC-12 cells were exposed to an oxidative burst by incubation with dopamine (0.5 mM, 24 h, 37°C) with or without SN-50 (10 μM) cell permeable inhibitory peptide. Cells were then scraped, washed in fresh medium and counted, 4×10^5 distributed to a 96-well microtiter plate with 10 μM DCF. After 2 h incubation at 37°C, fluorescence was measured at 544 nm exitation and 590 nm emission wavelength (Fluo star BMG Labtechnologies, Offenburg, Germany).

Flow cytometry analysis

Dopamine-treated PC12 cells (10^6) were centrifuged (200 *g*, 5 min) and incubated with reagents from the Intrastain kit, as recommended by the manufacturer (Dako, Glustrup, Denmark). The treatment enabled intracellular and intranuclear probing with antibodies. Cells were then incubated with anti-NF-κB-p65 antibodies for 1 h at room temperature. The nuclei of the treated cells were isolated and stained with propidium iodide as described previously (Ziv *et al*. 1996). Cells were stored in the dark at 4°C before flow cytometric analysis. For evaluation, the nuclei were divided into two groups by the degree of granulation and condensation and level of anti-NF-κB-p65, as indicated by fluorescein-conjugated goat anti-rabbit antibodies, using FACSORT (Becton-Dickinson, Franklin Lakes, NJ, USA).

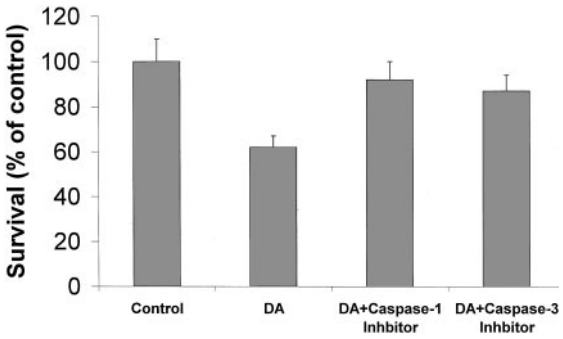

Fig. 1 Caspases inhibitors inhibit dopamine toxicity. PC12 cells treated with dopamine (0.5 mM for 24 h) were incubated as indicated with caspase 1 and 3 inhibitor peptides (0.01 mM). Cell survival was measured by neutral red assay (mean ± SD $n = 3$).

Statistical analysis

Results are presented as mean ± SEM. The significance of intergroup differences were evaluated by the two-tailed Student's *t*-test. In all tests, significance was assigned at $p < 0.05$.

Results

Dopamine toxicity is inhibited by caspase-3 and -1 inhibitors

To test whether dopamine-induced apoptosis is dependent on caspase activity, PC12 cells were exposed to dopamine in the presence or absence of caspase 3 and 1. Neutral red assay showed that exposure to dopamine (0.5 mM) for 24 h reduced cell survival to 59 ± 5% (Fig. 1). Addition of 10 μL of specific caspase-3 (CPP32) inhibitor (DEVD-CHO, Bio-Mol, Plymouth Meeting, PA, USA) 30 min before dopamine application, rescued the cells and resulted in 94 ± 5% cell survived ($p < 0.01$). Similarly, addition of 10 μL of specific caspase-1 (ICE) inhibitor (YVAD-CHO, Bio-Mol) 30 min before dopamine application rescued the cells (90 ± 7% survived, $p < 0.01$, Fig. 1). Cell exposure to caspases inhibitors alone was not toxic at concentrations of up to 50 μM (data not shown).

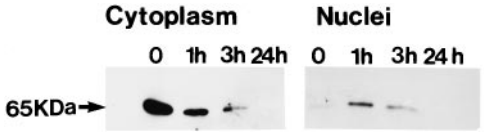

Fig. 2 NF-κB p65 immunoreactivty in dopamine-treated PC12 cell fractions. Cytoplasmic and nuclear extracts from PC12 cells were treated with dopamine for the indicated times and subjected to immunoblotting assay using anti-NF-κB-p65 antibodies. Within 1 h the presence of NF-κB p65 was reduced in the cytoplasmic fraction while in the nuclear fraction it increased, peaking at 3 h and disappearing after 24 h.

Dopamine induces activation of NF-κB

To verify whether NF-κB is associated with dopamine-induced apoptosis, we incubated PC12 cells with 1 mM dopamine for 1, 3 and 24 h. Extracts of dopamine-treated cells were separated into cytoplasmic and nuclear fractions and analyzed by immunoblot assay. Using specific antibodies we found that the presence of the NF-κB-p65 protein was reduced in the cytoplasmic fraction within 1 h, was further reduced to a very small amount after 3 h and had totally disappeared after 24 h (Fig. 2). In contrast, the NF-κB-p65 protein accumulated after 1 h, in the nuclear fraction, declined after 3 h of treatment and disappeared at 24 h (Fig. 2).

The translocation of the NF-κB-p65 protein to the nuclei was evident also by immunocytochemistry. Incubation with anti-NF-κB-p65 showed cytoplasmic staining within untreated cells, whereas cells treated with dopamine (1 mM for 3 h) showed marked nuclear staining (Fig. 3).

Dopamine-treated cells demonstrated cytoplasmic staining, indicative of IκBα phosphorylation, while those untreated exhibited only background staining (Fig. 4). The activation of NF-κB was also evidenced by gel-shift assay wherein nuclear extracts from PC12 cells that had been treated with dopamine for 15, 30, and 60 min were incubated with radiolabeled NF-κB consensus DNA sequence. Analysis on nondenaturated 6% acrylamide gel showed several bands,

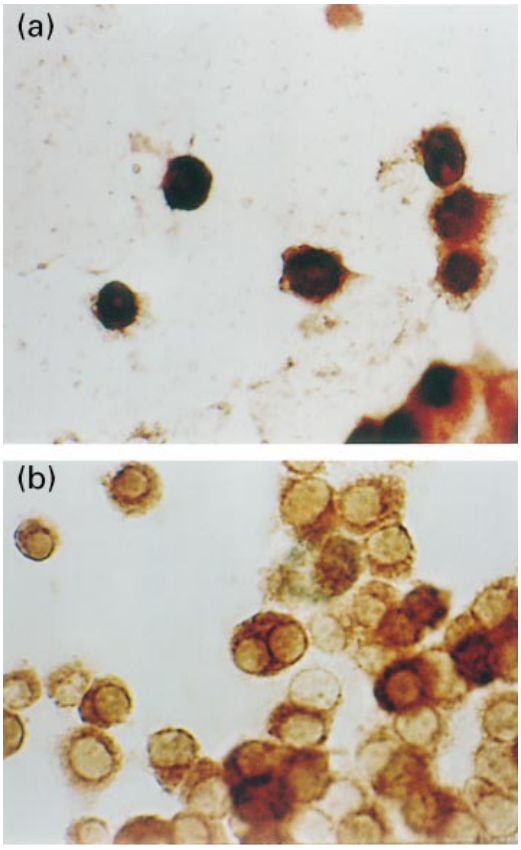

Fig. 3 NF-κB-p65 translocation following dopamine treatment. PC12 cells that were treated with dopamine (1 mM for 3 h) hybridized with anti-NF-κB-p65 showed nuclear staining (a), whereas untreated cells that were hybridized with anti-NF-κB-p65 showed mainly cytoplasmatic staining (b).

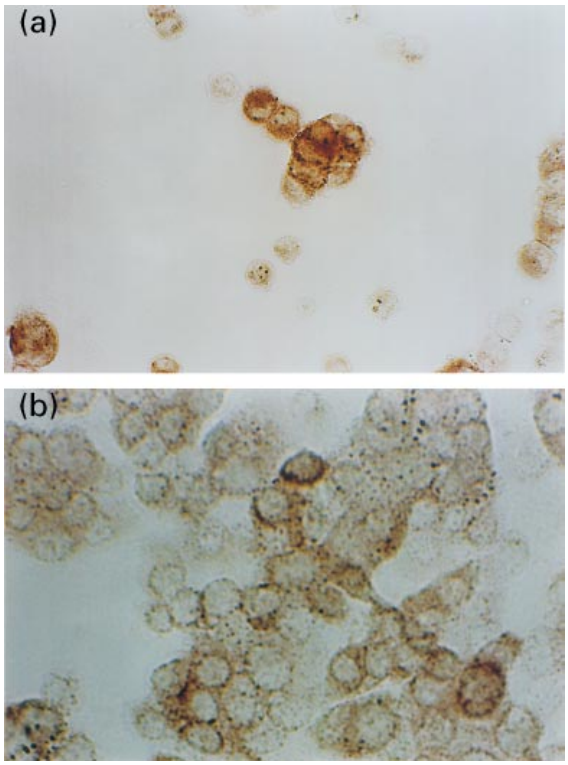

Fig. 4 Cytoplasmic IκBα is phosphorylated following dopamine treatment. PC12 cells that were treated with dopamine (1 mM for 3 h) hybridized with anti-phosphorylated IκBα showed cytoplasmatic staining (a) whilst untreated cells showed only background staining (b).

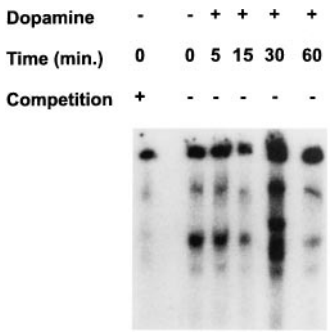

Fig. 5 Exposure to dopamine activates protein binding to NF-κB consensus DNA sequence. Nuclear fractions from PC12 cells treated with dopamine (1 mM for the indicated time) were incubated with ^{32}P-labeled NF-κB consensus DNA sequence. The extracts were then separated on polyacrylamide gel and visualized by using a phosphoimager.

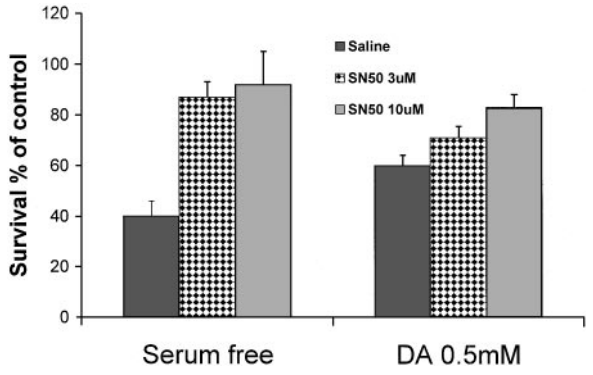

Fig. 6 NF-κB inhibitor rescues cells from dopamine toxicity. (a) NF-κB inhibitor SN-5O (3 μM and 10 μM) was added to dopamine-treated PC12 cells (0.4 mM for 24 h) which were incubated for 24 h in serum-free medium. Cell survival was determined by neutral red assay (mean ± SD).

indicating the binding of these proteins to the radiolabeled NF-κB DNA. The addition of unlabeled DNA as a specific competitor to the reaction blocked the binding of these proteins (Fig. 5, lane 1). The major bands which represent the NF-κB proteins were markedly increased after 30 min of dopamine treatment (Fig. 5, lane 5).

NF-κB inhibitor rescues cells from dopamine toxicity

PC12 cells were treated for 24 h with SN-50 (Bio-Mol), a specific inhibitor of NF-κB, 30 min prior to dopamine exposure. As shown in Fig. 6, dopamine (0.5 mM) alone caused 55% cell death and the addition of 3 μM and 10 μM of SN-50 increased cell survival to 75% and 85%, respectively ($p < 0.01$, Fig. 6). A similar effect was demonstrated in cells grown for 24 h in a serum-free medium, a treatment that promotes apoptosis. SN-50 administration (3 μM and 10 μM) rescued the cells and 87% and 92% survived, respectively, whilst in untreated cells there was only 39% survival (Fig. 6, $p < 0.01$).

To rule out the possibility that the neuroprotective effect is due to the antioxidant effect of SN-50, in addition to its NF-κB inhibitory effect, we measured dopamine-induced radical formation in the presence and absence of SN-50.

Using ROS production assay, by penetrative diacetate derivative DCFH-DA, we found that dopamine incubation for 24 h increased ROS production to 4830 ± 278 relative fluorescence units as compared to 3160 ± 194 in untreated cells ($p \leq 0.01$). However ROS production did not change significantly following the addition of 10 μM SN-50 to dopamine-treated and -untreated PC12 cells (5610 ± 110 and 3110 ± 194, respectively).

Nuclear NF-κB-p65 in dopamine-treated cells

Flow cytometric analysis of extracted nuclei of PC12 cells following dopamine exposure (0.5 mM for 3 h), demonstrated a high percentage of granulation [high side scatter (SSC) values]. We therefore separated the cell population into two subgroups (R1 and R2) according to their granulation values (lower and higher than channel 425

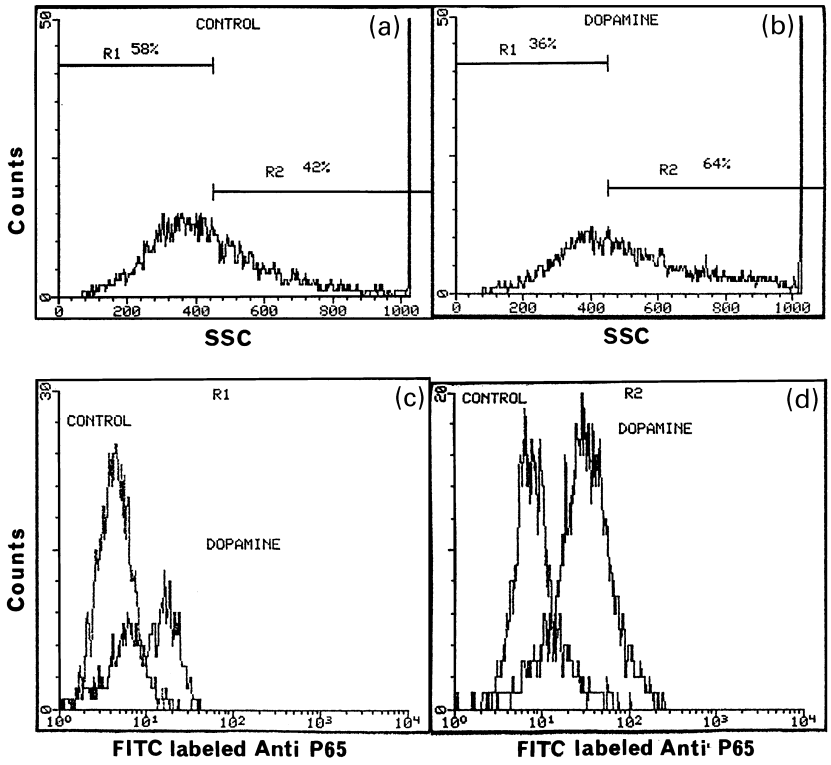

Fig. 7 Dopamine-treated cells show high levels of NF-κB-p65 in their nuclei. Nuclei from PC12 cells were treated with dopamine (0.4 mM for 18 h), divided into two groups by their membrane granulation (a and b). These groups were further analyzed by staining with anti-NF-κB-p65 and swine antibodies conjugated with fluoroscein isothiocyanate (FITC) (c and d).

SSC). We found that the percentage of the highly granulated nuclei (SSC > 425) was higher in the dopamine-treated cells (63%) compared with controls (42%) (Figs 7a and b). The two nuclear type subgroups were further analyzed for the presence of NF-κB-p65. Nuclei from the control cells showed only background staining (mean = 7.5) in the less-granulated groups (R1, Fig. 7c) while dopamine-treated cells nuclei showed a slightly higher level of positive staining to NF-κB-p65 antibodies (mean = 22). In contrast, in the highly granulated nuclei subgroup (R2, Fig. 7d), we observed two distinct peaks. Most of the nuclei from the dopamine-treated cells were positive for NF-κB-p65 antibodies (mean = 50), while the control nuclei showed only background staining (mean = 8). The fact that cells with high NF-κB activation, as evident by increased level of nuclear p65, also demonstrated apoptotic morphology, indicates that activation of NF-κB is associated with apoptotic induction, rather than inhibition.

Discussion

Our study attempted to determine the potential role of NF-κB in dopamine toxicity. First, we showed that caspase inhibitors (1 and 3) attenuated dopamine-induced death in PC12 cells. These results support previous studies showing that dopamine causes apoptosis in PC12 cells and other neuronal cultures (Ziv *et al*. 1994; Offen *et al*. 1997; Offen *et al*. 1998; Simantov *et al*. 1996). Several other studies also demonstrated that caspases play a role in 6-hydroxydopamine- and amyloid-induced apoptosis and that specific caspase inhibitors attenuated cell death (Ochu *et al*. 1998; Ivins *et al*. 1999). Our data also showed that dopamine toxicity in PC12 cells is associated with activation of NF-κB. According to earlier studies, IkB-protein, the inhibitory subunit of the NF-κB complex, upon activation, undergoes phosphorylation and degradation processes that enable the translocation of the NF-κB-p65 and NF-κB-p50 proteins (Beg *et al*. 1993; Brown *et al*. 1995). Using antibodies against the phosphorylated form of IκBα protein, we observed cytoplasmic staining in PC12 cells treated with dopamine for 3 h, indicating the initiation of NF-κB activation. Western blot analysis of cytoplasmic and nuclear extracts demonstrated that NF-κB-p65 protein translocates into the nucleus after 1 h of dopamine treatment and degraded 2 h later. In contrast, the untreated cytoplasmic fraction showed high levels of NF-κB-p65 which decreased after 3 h of dopamine treatment and disappeared after 24 h. Gel-shift analysis, routinely used to demonstrate transcription factor activation, indicated that NF-κB-p65 proteins bound after 30 min to the consensus [^{32}P]DNA sequence in dopamine-treated nuclei. Thus, taken together, our data demonstrate that dopamine exposure stimulates NF-κB during the first hours (0.5–3 h) while cell death can be seen only after 18–24 h.

To further investigate the role of NF-κB in dopamine toxicity, we used NF-κB cell-permeable inhibitor peptide, which contains the nuclear localization sequence of NF-κB-p50 linked to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor. The addition of this NF-κB inhibitor to PC12 cells significantly protected the cells against dopamine toxicity in a dose-dependent manner.

The positive contribution of NF-κB activation to the cell death process, and not to the defence mechanism, was also demonstrated by flow cytometry. We analyzed the nuclei of dopamine-treated cells and found that they showed apoptotic morphological features and had a higher content of NF-κB-p65, compared to the less granulated nuclei that showed low content of NF-κB-p65. These data indicate that NF-κB activation is essential for the initiation and execution of dopamine-induced apoptosis in PC12 cells.

The transcription factor NF-κB has been shown to be activated by different types of stimuli in various cells and tissues (Baeverle and Henkel 1994; Lipton *et al*. 1997; May *et al*. 1999). There is a line of evidence indicating that NF-κB may be involved in promoting apoptosis. First, many potent NF-κB stimulators, such as tumor necrosis factor (Barger *et al*. 1995), ceramide (Hunot *et al*. 1997), H_2O_2 and serum deprivation (Tong and Perez-Polo 1995; Grimm *et al*. 1996) ultimately induce apoptosis. Second, both apoptosis and activation of NF-κB are suppressed by antioxidants such as *N*-acetyl-L-cysteine (Schulze-Osthoff *et al*. 1995; Offen *et al*. 1996; Sen and Packer 1996; Lezovalch *et al*. 1998). Third, several of the apoptosis-induced genes such as Fas/Apo-1 ligand, c-myc, p53, and caspases are also induced by NF-κB (Baichwal *et al*. 1997). Fourth, the upstream promoter regions of several 'death genes' contain potential NF-κB binding motifs (Grimm *et al*. 1996; Sen *et al*. 1996). These experimental data accumulated strongly suggest that exogenous signals (toxins, ROS, etc.) activate NF-κB. The resulting transcription genes later execute the apoptosis program (Tong *et al*. 1995; Grilli *et al*. 1996, 1999a, 1999b; Kuner 1998; Qin *et al*. 1998).

There are, however, contradictory findings demonstrating that the activation of NF-κB rescues cells from apoptosis and that the process is a protective one. For instance, Maggirwar *et al*. (1998) showed that nerve growth factor-dependent activation of NF-κB contributes to the survival of sympathetic neurons. Moreover, it was reported that high constitutive NF-κB activity mediates resistance to oxidative stress in neuronal cells (Lezovalch *et al*. 1998). In addition, agents that inhibited NF-κB activation-induced apoptosis in response to several neurotoxins (Barger *et al*. 1995; Taglialatela *et al*. 1998; Kaltschmidt *et al*. 1999; Mattson *et al*. 1997, 2000).

Thus, the dual activity of NF-κB may reflect differences in tissue specificity, stimulus dose or duration of stimulation (Lipton *et al*. 1997). The same type of phenomena has also

been observed in neuroblastoma and cerebral granule cells, where low doses of amyloid peptide activated NF-κB and induced neurotrophic activity while high doses were toxic (Kaltschmidt *et al*. 1999). Thus, NF-κB may exert multiple functions with respect to neuronal cell survival, depending on the experimental and cellular paradigm and the mode and kinetics of the activation.

The toxicity of free radicals produced during normal cell metabolism is prevented by the naturally occuring protective antioxidant systems. These systems include enzymes such as glutathione peroxidase and superoxide dismutase, and small molecules such as reduced glutathione (GSH), and vitamins C and E. NF-κB as a sensor of the increased levels of ROS in neurons may direct and activate the defence system. However, under chronic stimulation and/or stimulation with a high amount of dopamine, it might initiate transcription of pro-apoptotic genes. One candidate for such activated genes is the inducible nitric oxide synthetase (iNOS) which is up-regulated by a rise in intracellular calcium due to ROS accumulation (Minc-Golomb and Schwartz 1994; Gerlach 1999; Liberatore *et al*. 1999).

In the context of Parkinson's disease, it was suggested that apoptosis associated with the disease is a result of oxidant-mediated transduction of sphingomyelino-dependent signaling pathway that activates NF-κB (Kaltschmidt *et al*. 1993; Hunot *et al*. 1997; Taglialatela *et al*. 1999). However, it could not determine conclusively if the latter promoted protection or destruction. Our study links toxic dopamine metabolites and the production of ROS signal, activating in turn NF-κB and inducing apoptosis. We speculate that NF-κB activation may initiate a pro-apoptotic gene expression program, which might be involved in the development of neurodegenerative diseases. A better understanding of the transcription factors and gene products generated in response to oxidative stress will enable us to define specific drug targets for retarding or arresting the progression of PD.

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