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6-hydroxydopamine-induced nuclear factor-kappaB activation in PC12 cells

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Abstract

The involvement of nuclear Factor-kappaB (NF-κB) transcription factor in PC12 cell death triggered by the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) was investigated. Results show that oxidative stress generated by 6-OHDA activates NF-κB. When the NF-κB activation was inhibited by parthenolide, PC12 cell death induced by 6-OHDA was significantly increased, thus suggesting an involvement of this transcription factor in a protective mechanism against 6-OHDA toxicity. To further assess this hypothesis, we studied the involvement of NF-κB in the protective effect of two anti-apoptotic genes, bcl-2 and bfl-1. Although Bcl-2 and Bfl-1 expression normally protects PC12 cells from 6-OHDA, parthenolide strongly decreased the beneficial effects afforded by transgene expression. These results suggest: (1) that the transcription factor NF-κB is likely associated with the protection of catecholaminergic PC12 cells and (2) that the protective effects afforded by bcl-2 and bfl-1 expression may be dependent on NF-κB activation.

Keywords: NF-kappaB; PC12 cells; 6-Hydroxydopamine; Bcl-2; Parkinson's disease

1. Introduction

The transcription factor NF-κB is a dimeric complex of proteins of the Rel-family that can regulate several cellular functions upon activation by a variety of extracellular stimuli [1]. NF-κB, frequently composed of p50 and p65/RelA proteins, is retained in an inactive state in the cytoplasm by interaction with an inhibitory protein of the IκB family. Upon stimulation, the IκB protein is phosphorylated and proteolytically degraded, allowing nuclear translocation of NF-κB, its binding to cognate DNA sequences, and induction of transcription of target genes [2].

Recently, NF-κB has gained increasing attention for its possible role as a critical regulator of cell death [3]. In the nervous system, NF-κB is found in both glia and neurons [4] and is activated in response to neurotoxic stimulations such as glutamate [5] β-amyloid peptide [6], kainate [7], quinolinic acid [8], or after cerebral vessel occlusion in rat [9,10]. Likewise, NF-κB activity is high in post-mortem brains of Alzheimer's disease patients [6]. However, the functional significance of these findings is still unclear, and NF-κB activation leads to various effects on neuronal cell death [11]. The inhibition of NF-κB activity has indeed been shown to protect cortical neurons against glutamate or quinolinic acid-induced neuronal injury both *in vitro* or *in vivo* [5,8] and to participate in the neuroprotective effect of melatonin and normelatonin against hydrogen peroxide insult [12]. Conversely, the anti-apoptotic function of NF-κB is supported by several studies showing that the activation of NF-κB protects primary neuronal cells against β-amyloid [13,14], mediates NGF-promoted survival of PC12 cells [15], and increases the resistance of neuronal cells against hydrogen peroxide [16].

Oxidative stress-induced neuronal cell death appears to be involved in certain neurodegenerative disorders such as Alzheimer's and Parkinson's disease (PD) [17,18]. Among NF-κB-inducing agents, reactive oxygen species (ROS) are widely used [1,19], and recent evidence strongly suggests

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Abbreviations: 6-OHDA, 6-hydroxydopamine; EMSA, electrophoretic mobility shift assay; GSH, glutathione; IAP, inhibitory apoptosis protein; MAP, mitogen-activated protein; NAC, *N*-acetyl-cystein; NF-κB, nuclear factor-κB; and PD, Parkinson's disease.

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that NF-κB may afford neuroprotection against oxidative insults [16,20,21] in particular with respect to Alzheimer's disease [6]. The situation is, however, much less clear for PD, although Hunot *et al*. [22] suggested, on the basis of their observation of nuclear translocation of NF-κB in degenerating substantia nigra in PD post-mortem brains, that oxidative stress might be related to apoptotic death in PD through NF-κB-related signal transduction.

6-OHDA, a hydroxylated derivative of dopamine that, possibly, is formed endogenously in patients with PD [23, 24], induces common features of PD [25]. 6-OHDA lesion triggers nigral cell degeneration in rats with the production of high levels of ROS [26,27]. *In vitro*, this neurotoxin, similarly to other catechol derivatives [28], induces oxidative stress [29,30] and apoptosis of various catecholaminergic cell lines [31–33]. In the present study, we therefore investigated the effect of 6-hydroxydopamine (6-OHDA) upon NF-κB activity in the catecholaminergic PC12 cell line in an attempt to further characterize the role of NF-κB in nigral neuron degeneration.

2. Material and methods

2.1. Cell culture

PC12 cells (ATCC) were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Life Technology) containing 10% (v/v) fetal bovine serum, 5% (v/v) horse serum, 4 mM L-glutamine, 50 units/mL of penicillin, and 50 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°.

2.2. Stable transfections

PC12 cells (ATCC) were transfected by lipofection with 10 μg of either empty pcDNA3-neo (InVitrogen), pcDNA3-Bcl-2 (kindly provided by Dr. Bonner), or pcDNA3-Bfl-1 (kindly provided by Dr. D'Sa Eipper) expression plasmids. Briefly, PC12 cells were plated at 2.10^4 cells/cm^2 in 100-mm diameter dishes. Twenty-four hours later, cultures were transfected with 100 μL 0.9% NaCl solution containing 10 μg plasmids and 33 μL polyethylenimine 22 kD (6 eq of PEI nitrogen per DNA phosphate; Euromedex) prepared 15 min before addition at room temperature. Culture medium was changed 3 hr after transfection. After 24 hr, cells were switched to culture medium containing 400 μg/mL of G418 (Sigma) and selection was performed for 3 weeks. Colonies were cloned by trypsinization in cloning cylinders and expanded at 37° in the presence of G418. Clones were screened for Bcl-2 and Bfl-1 expressions by Western blotting analysis (data not shown) using anti-Bcl-2 monoclonal antibody (Dako) or anti-Bfl-1/A1 polyclonal antibody (Santa Cruz Inc).

2.3. Transient transfections and determination of constitutive NF-κB transcriptional activity

PC12 cells, seeded at 10^5 cells/cm^2 in 24-well poly-D-lysine-coated dishes, were transfected 24 hr after plating with either pLTRX-Luc or mpLTRX-Luc plasmids (kindly provided by Dr. Schwartz and Dr. Israël) using the same transfection protocol as above (2 μg of plasmid complexed with 6 eq polyethylenimine 22 kD). The NF-κB-driven luciferase (pLTRX-Luc) reporter plasmid contained 2 copies of the consensus κB sites (GGGACTTTCC). Luciferase transcription independent of NF-κB activity was determined by transfection of the mpLTRX-Luc plasmid that contained two mutations in the consensus κB sites. The luciferase activity resulting from the transfection of this latter was used to normalize assays for the transcriptional activity in the different cellular clones. Twenty-four hours after transfection, cells were incubated for 15 min at room temperature with lysis buffer (25 mM Tris pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X100). Luciferase activity was then determined using the Luciferase assay system (Promega) and a Turner TD-20 luminometer (Promega). Twelve replicates from three independent experiments were analysed for each condition (clone type and plasmid used) and values were expressed as means ± SD.

2.4. Drug treatments and cell viability assay

Cells were plated at the density of 10^5 cells/cm^2 on 24-well poly-D-lysine coated dishes. Twenty-four hours later, cells were treated for 24 hr with 100 μM 6-OHDA (hydrobromide; 10 mM stock solution in ascorbic acid 10 mM; Sigma) with or without the anti-oxidants glutathione (GSH) or *N*-acetyl-cysteine (NAC) (Sigma). When indicated, PC12 cells were pretreated with the NF-κB inhibitors parthenolide (20 or 40 μM; Biomol Research Laboratories Inc) 1 hr before 6-OHDA addition. The number of viable cells following drug exposure was determined using the Alamar Blue assay (Biosource). The significance of findings was assessed using the unpaired Student's *t*-test performed with Statview 512^+ (Brain-Power).

2.5. Electrophoretic mobility shift assay (EMSA)

Cytoplasmic and nuclear extracts were prepared as described previously [34] and protein concentrations were determined using the BCA protein assay (Pierce). The NF-κB double-stranded oligonucleotide corresponding to the NF-κB consensus sequence in the κ light chain enhancer of B cells (5′-AGT TGA G**GG GAC TTT CC**C AGG C-3′; NF-κB motifs in boldface) was purchased from Promega. The double-stranded oligonucleotide-bearing mutation in the NF-κB consensus sequence (5′-AGT TGA GTT CAC TTT CCC AGG C-3′; mutations underlined) was synthesized by Eurogenetec. The NF-κB oligonucleotide

was end-labelled with [γ-^{32}P]ATP (4500 Ci/mmol) and T4 polynucleotide kinase (Promega), then purified on a G-25 column (Pharmacia). Reactions were performed in a final volume of 20 μL buffer C (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl, 10% v/v glycerol) containing 10 μg bovine serum albumin, 5 μg polydeoxyinosinic-deoxycytidylic acid (Pharmacia), and radiolabelled probe (50,000 cpm per sample) for 30 min at room temperature. Competition studies were performed after a 20-min preincubation with a 50-fold excess of either unlabelled, double-stranded normal, or mutated NF-κB oligonucleotides or 2 μL specific antibodies against either p50 or p65 subunits of NF-κB prior to probe addition (Transcruz polyclonal anti-rabbit p50 sc-144X or polyclonal anti-rabbit p65 sc-109X, Santa Cruz Inc). Reaction mixtures were loaded on 5% non-denaturing polyacrylamide gels which were then run at 10 V/cm for 2 hr in 0.25 $\times$ TBE. The gels were dried and exposed to Agfa Curix ray films at $-80°$ for 14–96 hr.

2.6. Immunoblotting

Protein gel electrophoresis was performed as described elsewhere [32]. Equal amounts of proteins (10 μg) were heated to 100° for 5 min in Laemmli buffer, run on a 12% SDS–polyacrylamide gel and blotted onto a PVDF membrane (Millipore). Membranes were then successively incubated with either anti-IκBα antibody (1:1000; sc-371, Santa Cruz Inc) or anti-p65 antibody (1:1000; sc-109, Santa Cruz Inc), biotin-conjugated anti-rabbit IgG F(ab′)$_2$ (1:2000; Boehringer Mannheim) and extravidin peroxidase (1:5000; Sigma). Immunoreactive bands were visualized by chemiluminescent Covalab reagent (Dako).

3. Results

3.1. Reactive oxygen species-dependent NF-κB activation following exposure of PC12 cells to 6-OHDA

EMSA analysis demonstrated the presence of three specific NF-κB DNA-binding complexes (I, II, III) in untreated control PC12-neo cells as well as parental PC12 cells, thus indicating a constitutive activation of the transcription factor (Fig. 1A). To ascertain the specificity of NF-κB DNA binding, reactions were performed in the presence of a 50-fold molar excess of unlabeled NF-κB oligonucleotide. This resulted in the disappearance of the complexes I to III (Fig. 1B). No competition for the binding of complexes I to III was observed when a mutated NF-κB oligonucleotide was used (Fig. 1B). To determine the subunit composition of the three specific complexes, supershift analysis was carried out by incubation of nuclear extracts with antibodies raised against p65 or p50 NF-κB subunits prior to EMSA (Fig. 1B). Under our experimental conditions, both antibodies failed to produce a clear supershift but rather caused a decrease in the intensity of the specific bands (Fig. 1B). This lack of clear supershift appears to be a common observation in PC12 cells [15,16]. The addition of anti-p65 antibody strongly reduced complexes I and III, whereas anti-p50 antibody decreased the intensity of all the specific bands. This indicates that the upper and lower specific complexes (I and III) are composed of both p50 and p65 proteins and that complex II is formed by p50 homodimers.

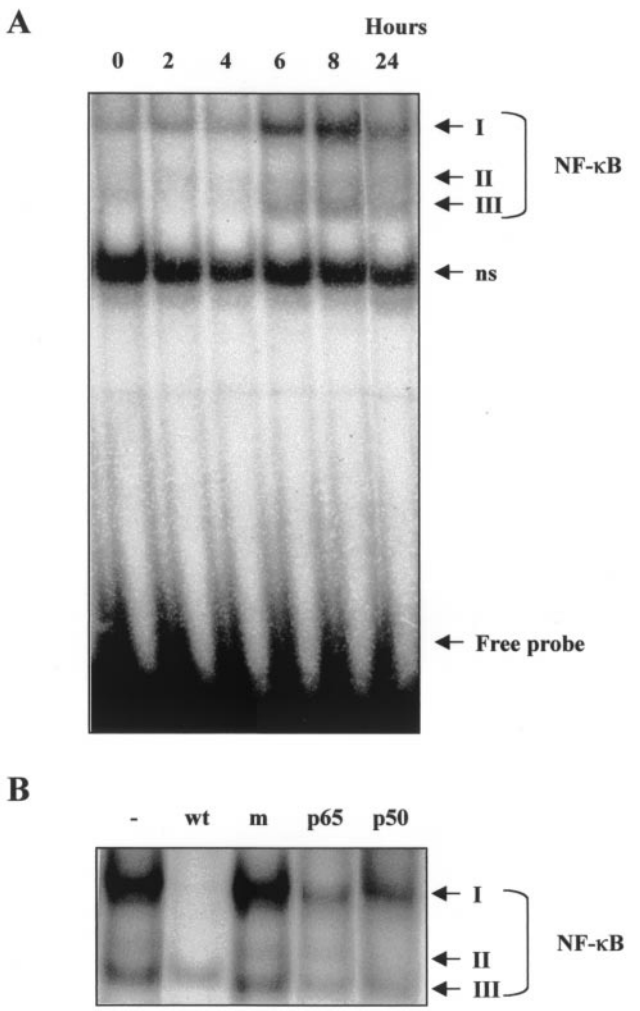

Fig. 1. 6-OHDA induces NF-κB DNA-binding activity in PC12 cells. (A) EMSA analysis of DNA-binding activity of NF-κB in nuclear proteic fractions of PC12 cells exposed to 6-OHDA. Three specific shifts (I–III) are induced by treatment. Complex III can be clearly detected from 6 to 24 hr after 6-OHDA exposure. ns represents the non-specific DNA-binding activity. Free non-bound DNA probe is also indicated. (B) Specificity and characterization of binding complexes. For competition experiments, nuclear extracts of PC12 cells treated 8 hr with 100 μM 6-OHDA were incubated with either a 50-fold excess of cold NF-κB oligonucleotide (wt), a 50-fold excess of cold mutated oligonucleotide (m), or 2 μL of anti-p65 antibody or 2 μL of anti-p50 antibody.

When PC12-neo cells were exposed to 100 μM 6-OHDA (the highest apoptosis-inducing concentration previously described in dose–response experiments [31,32]) for various durations (0 to 24 hr), NF-κB DNA-binding activity, especially complexes I and III, increased after 6 hr, peaked

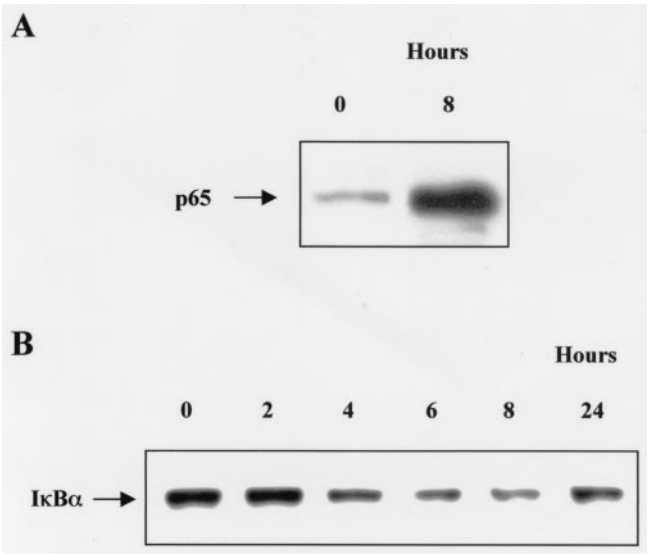

Fig. 2. Nuclear p65 expression and time-course modulation of cytoplasmic IκBα levels following 6-OHDA exposure. (A) The immunoblot analysis of p65 protein (65 kD) was performed from nuclear fractions using equal amounts of proteins of either non-treated PC12-neo cells or 100 μM 6-OHDA treated-cells for 8 hr. (B) The immunoblot analysis of IκBα protein (37 kD) was performed from cytoplasmic fractions using equal amounts of proteins of either non-treated PC12-neo cells or 100 μM 6-OHDA-treated cells for various periods of time.

at 8 hr, and then decreased (Fig. 1A). In agreement with these results, Western blot analysis showed that 6-OHDA treatment leads to an increase in p65 nuclear content (Fig. 2A) and to a reduction, within 4 hr, in the inhibitory subunit IκBα cytoplasmic content (Fig. 2B). The late increase in IκBα cytoplasmic levels observed between 8 and 24 hr of drug treatment is consistent with the presence of NF-κB binding sites in the repressor gene promoter and with the regulatory control that NF-κB exerts on its own activation [35]. Therefore, our results provide evidence that 6-OHDA is able to induce NF-κB translocation in PC12/PC12-neo cells at a concentration known to induce a regulated cell death process [32].

6-OHDA toxicity is thought to be mediated through ROS generation, mainly hydrogen peroxide [29,30], a relevant NF-κB-inducing agent [1]. To determine whether NF-κB activation triggered by 6-OHDA was dependent upon ROS generation, PC12-neo cells were incubated with 100 μM 6-OHDA and 10 mM of either GSH or NAC. Although a 24-hr incubation with the neurotoxin alone led to a 65% decrease in cell viability, the presence of anti-oxidants strongly protected PC12 cells from 6-OHDA and clearly decreased the 6-OHDA-induced DNA-binding activity of NF-κB observed 8 hr after drug addition (Fig. 3). These experiments thus demonstrate that ROS are required for NF-κB activation and cell death following 6-OHDA exposure.

3.2. Effects of parthenolide on 6-OHDA toxicity and Bcl-2/Bfl-1 protective effects

The above findings suggested that NF-κB may participate in cell death transduction pathways initiated by

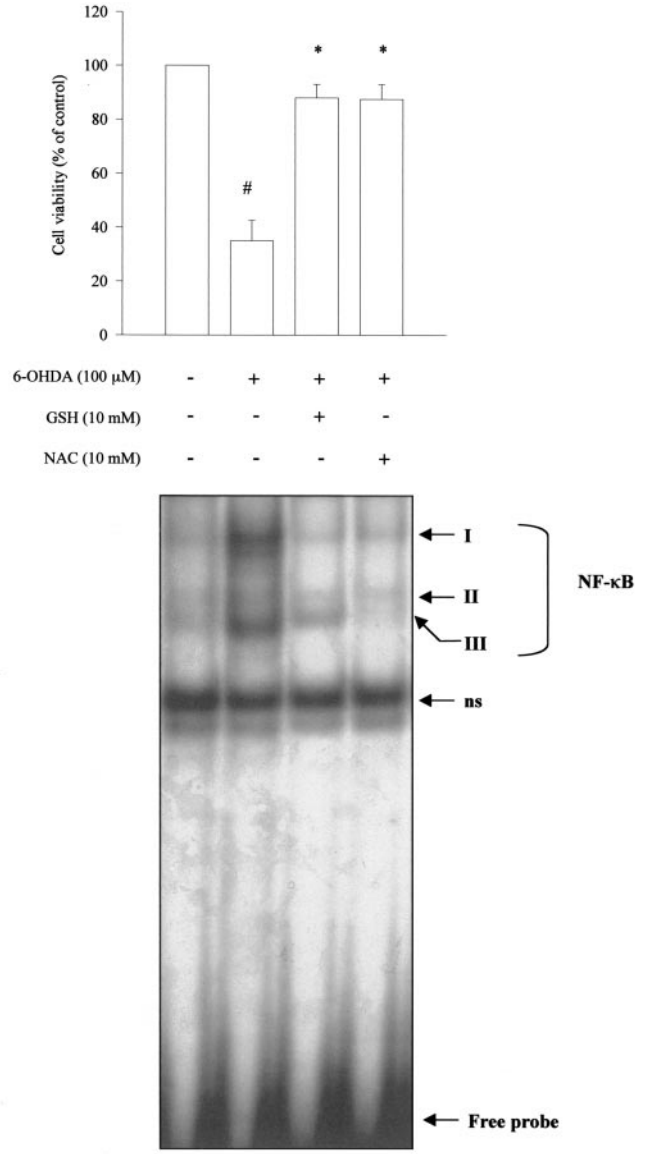

Fig. 3. Effect of GSH and NAC on 6-OHDA-induced cell death and NF-κB activation in PC12-neo cells. Twenty-four hours after seeding, cells were incubated with both 100 μM 6-OHDA and either GSH or NAC. Cell viability was determined by the Alamar Blue assay 24 hr after 6-OHDA addition. Results are given as a percentage of the untreated control and represent the means ± SD of triplicate determinations from a representative experiment. Similar results were obtained in 3 independent experiments. $^{\#}P < 0.05$ vs untreated cells, $^{*}P < 0.05$ vs 6-OHDA-treated cells using Student's *t*-test. EMSA experiment was performed using nuclear protein fraction of PC12-neo cells treated for 8 hr with or without both 100 μM 6-OHDA and either GSH or NAC. The three specific complexes are indicated as well as the non-specific DNA-binding activity (ns) and the free non-bound DNA probe.

6-OHDA since its inhibition with anti-oxidants protected PC12 cells from 6-OHDA toxicity. In order to determine the putative role of NF-κB in 6-OHDA-induced PC12 cell death, we investigated the impact of its inhibition on cell viability following 6-OHDA exposure. We first attempted to create stable transfectants expressing the negative-dominant super-repressor IκB to irreversibly block NF-κB ac-

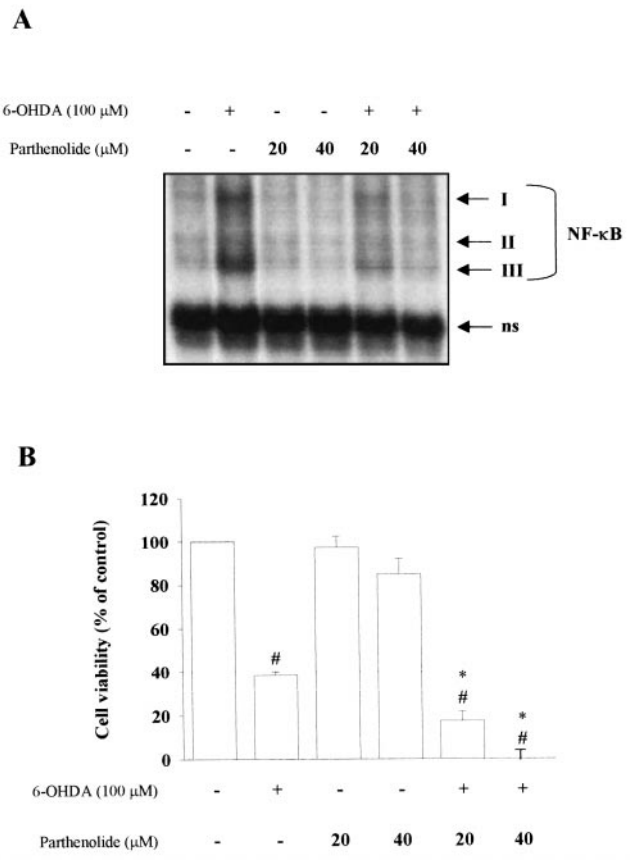

Fig. 4. Effect of parthenolide on NF-κB activation and PC12 cell death induced by 6-OHDA. PC12-neo cells were pretreated for 1 hr with 20 or 40 μM parthenolide before addition of 6-OHDA and then incubated for 8 hr (A) or 24 hr (B). (A) EMSA analysis of DNA-binding activity of NF-κB. The three NF-κB complexes are indicated as well as the non-specific DNA-binding activity (ns). (B) Cell viability was determined by the Alamar Blue assay. Results are given in percentage of non-treated control and represent the means ± SD of quadruplicate determinations from a representative experiment. Similar results were obtained in 3 independent experiments. $^{\#}P < 0.05$ vs untreated control and $^{*}P < 0.05$ vs 6-OHDA treated cells using Student's *t*-test.

tivation. Unfortunately, we were unable to obtain IκB-expressing cells due to the rapid degenerescence of the clones. We thus investigated the effects of parthenolide, an inhibitor of NF-κB activation devoid of anti-oxidant effects [36,37], on cell viability following 6-OHDA exposure. Parthenolide (20 and 40 μM) alone slightly decreased NF-κB constitutive DNA-binding activity (Fig. 4A) without affecting PC12 cell viability (Fig. 4B). Treatment with parthenolide decreased NF-κB activation induced by 6-OHDA in a dose-dependent manner (Fig. 4A); indeed, NF-κB activation was reduced by 50% in the presence of 20 μM parthenolide and completely inhibited when parthenolide concentration was raised to 40 μM. Similarly, the effect of parthenolide on 6-OHDA-induced PC12 cell death was also dose-dependent; while 40% of cells survived a 24-hr exposure to 6-OHDA, cell viability was decreased to 16% with the addition of 20 μM parthenolide, and cell killing was almost complete in the presence of 40 μM of the inhibitor (Fig. 4B). Similar results were obtained in both PC12-neo and parental cells.

To further explore a potential protective role of NF-κB activation and because Bcl-2 and Bfl-1 proteins are known to interact with this transcription factor [38,39], we examined the effects of their ectopic expression on 6-OHDA-induced cell death. While a 24-hr exposure to 100 μM 6-OHDA decreased cell viability down to 34 ± 4% in PC12-neo cells when compared to untreated control cultures, 61 ± 5% and 67 ± 7% of cells were still healthy in Bcl-2 or Bfl-1-expressing PC12 cell cultures, respectively (Fig. 5A). Therefore, Bcl-2 and Bfl-1 proteins are able to efficiently protect PC12 cells from 6-OHDA cell killing. We then determined whether parthenolide would impair the ability of these anti-apoptotic proteins to rescue PC12 cells from 6-OHDA-mediated cell death. Parthenolide alone did not affect PC12-Bcl-2 and PC12-Bfl-1 cell survival. By contrast, the presence of parthenolide together with 6-OHDA decreased PC12-Bcl-2 and PC12-Bfl-1 cell survival by approximatively 50% when compared to cultures treated by 6-OHDA alone (cell viability following a 24-hr exposure: 80% in the presence of 6-OHDA alone; 42 ± 12 and 35 ± 10% for PC12-Bcl-2 and PC12-Bfl-1 cell cultures, respectively, treated with both 6-OHDA and parthenolide [Fig. 5B]). Interestingly, results from luciferase reporter gene assays indicated that basal NF-κB-dependent transcriptional activity was significantly increased in PC12-Bcl-2 and PC12-Bfl-1 cells (4.3- and 4.5-fold increase, respectively) when compared to that of control PC12-neo cells (Fig. 5C).

4. Discussion

Several studies suggest a role for the transcription factor NF-κB in neuronal cell death. However, very little is known about its exact function in neurodegenerative diseases, especially in PD. The present study provides evidence that 6-OHDA, a neurotoxin widely used for animal models of PD, is a potent activator of NF-κB in the catecholaminergic PC12 cell line. Moreover, pharmacological inhibition of NF-κB activity potentiates the lethal effect of the neurotoxin, and impaired the protection afforded by ectopic expression of either Bcl-2 or Bfl-1/A1 genes, suggesting a protective role for NF-κB activation in this model of neuronal cell death.

Treatment of PC12 cells with 100 μM 6-OHDA, a concentration previously shown to induce a regulated cell death pathway [32], activates nuclear translocation of NF-κB as demonstrated by the increase in the DNA-binding activities of NF-κB (especially p50-p65-containing complexes I and III), the increase in the nuclear content of p65, and the cytoplasmic degradation of IκBα repressor protein. To our knowledge, this is the first demonstration that 6-OHDA neurotoxin induces NF-κB transcription factor activation in catecholaminergic cells. These results are in line with recent studies demonstrating that auto-oxidized dopamine and

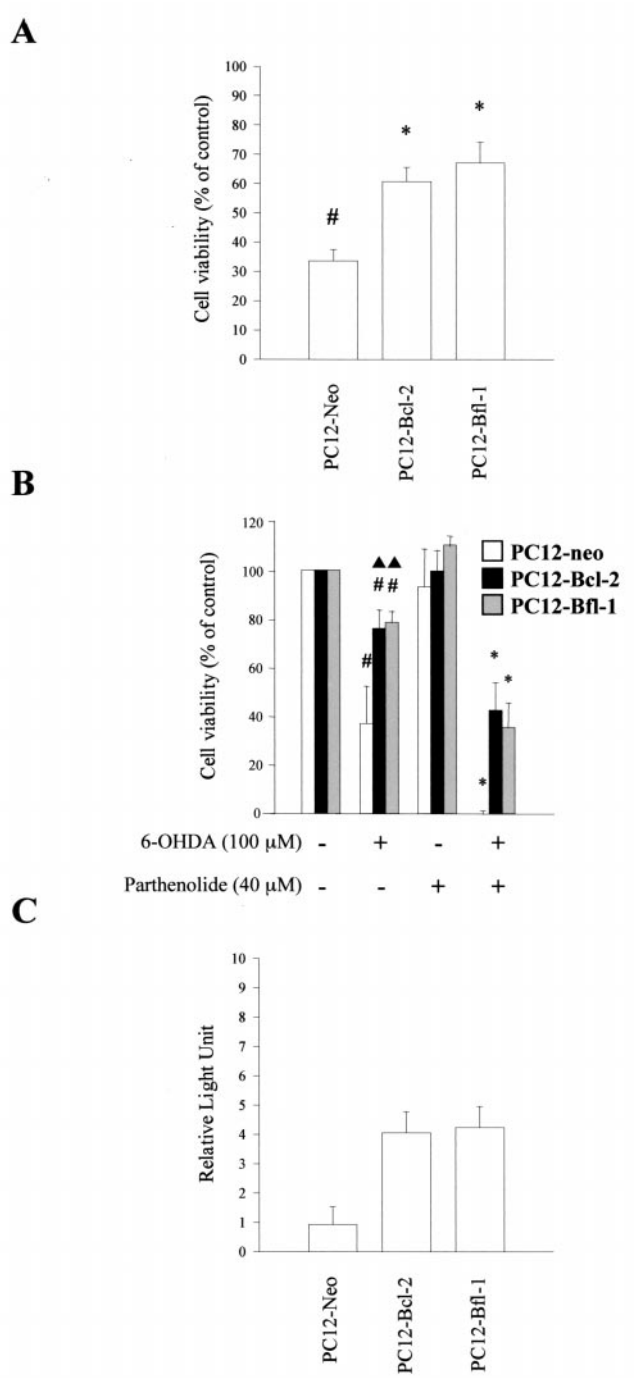

Fig. 5. Relationship between ectopic expression of Bcl-2 and Bfl-1 and NF-κB transcription factor. (A) Protective effects of Bcl-2 and Bfl-1 expression against 6-OHDA. Cell viability in cultures of PC12-neo, PC12-Bcl-2, and PC12-Bfl-1 cells exposed for 24 hr to 100 μM 6-OHDA was determined by the Alamar Blue assay. Results are given as a percentage of the untreated control and represent the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in 3 independent experiments. $^{\#}P < 0.05$ vs untreated cells, $^{*}P < 0.05$ vs 6-OHDA-treated cells using Student's *t*-test. (B) Effect of parthenolide on the protection afforded by Bcl-2 and Bfl-1 expression against 6-OHDA toxicity. Cell viability in cultures of PC12-neo (white bars), PC12-Bcl-2 (black bars), and PC12-Bfl-1 (grey bars) cells pretreated for 1 hr with parthenolide before addition of 6-OHDA for 24 hr was determined by the Alamar Blue assay. Results are given in percentage of non-treated control and represent the means ± SD of quadruplicate determinations from a representative experiment. Similar results were obtained in 3 independent experiments. $^{\#}P < 0.05$ vs untreated control, $^{\blacktriangle}P < 0.05$ vs 6-OHDA-treated PC12-neo cells, and $^{*}P < 0.05$ vs 6-OHDA-treated cells using Student's *t*-test. (C) Constitutive NF-κB activity in cells expressing anti-apoptotic genes. PC12-neo, PC12-Bcl-2, and PC12-Bfl-1 cells were transiently transfected with luciferase reporter plasmids containing, in their promoter, either NF-κB binding elements (pLTRX-Luc) or mutated NF-κB binding sites (mpLTRX-Luc). Twenty-four hours after transfection, cells were lysed and assessed for luciferase activity. Data were compared with their respective control groups and are presented as the means ± SD of fold increase from control. Experiments were repeated 3 times with four replicates for each condition.

MPP^{+}, another classical dopaminergic neurotoxin, triggers NF-κB activation in catecholaminergic cell lines [40,41].

Competition studies revealed that complexes I and II represent the classical NF-κB dimers p50/p65 and p50/p50 found to be inducible in PC12 cells [15,16]. Another complex (III) that was not previously observed in this cell type is also found to be strongly increased upon 6-OHDA exposure. This fast-migrating complex may be a truncated form of the native p50/p65 dimer resulting from a specific proteolysis in the C-terminal portion of p65 occurring during proteic extraction [42]. Nevertheless, the exact function of this complex remains to be further elucidated.

NF-κB activation is usually considered as a regulator of cell stress response, in particular following oxidative stress. However, the precise activation pathway(s) induced by oxidants and leading to NF-κB activation are still ill-defined [1,19], although low levels of ROS, especially H_2O_2, are thought to act as messengers [43,44]. In keeping with these results, the anti-oxidants GSH and NAC inhibit 6-OHDA-induced NF-κB activation and prevent PC12 cell death. A similar protective function of anti-oxidants against 6-OHDA was found by Choi *et al*. [45] using the mesencephalon-derived dopaminergic neuronal cell line MN9D. Taking into account the oxidative properties of 6-OHDA [29,30], one can thus assume that the activation of NF-κB by 6-OHDA requires reactive oxygen intermediates as messengers. Thus, the decrease in NF-κB activation induced by GSH and NAC may be causally related to their protective effects, and it follows that activation of NF-κB factor may be a component of a cell death pathway induced by 6-OHDA. This conclusion is consistent with several studies which suggested that NF-κB promotes apoptosis in a variety of *in vitro* and *in vivo* neuronal cell injury models [5,9,10, 12,22,46]. However, the action of anti-oxidants upon NF-κB may be indirect, either scavenging ROS [30] or altering the function of proteins that can interfere with NF-κB activity [47]. Thus, anti-oxidants might suppress the toxic stimulus at the onset of the cell death pathway, upstream of NF-κB. On the sole basis of experiments using anti-oxidants, it is therefore difficult to clearly ascertain whether NF-κB exerts either a death-promotor or death-repressor function in catecholaminergic PC12 cells following 6-OHDA stimulation.

To clarify this point, experiments were performed with parthenolide, a sesquiterpene lactone compound devoid of direct or indirect anti-oxidant properties and previously shown to prevent the activation of NF-κB in response to various stimuli, including hydrogen peroxide [36,37]. The treatment of PC12 cell cultures with parthenolide together with 6-OHDA completely blocks NF-κB DNA-binding activity and enhances cell killing. The negative correlation found between the 6-OHDA-induced NF-κB activation and cell killing suggests that the neurotoxic stress activates NF-κB, which in turn turns on/off genes that repress/induce cell death. This response is not, however, by itself sufficient to repress cell death in this paradigm, possibly due to the strength of the toxic insult. Nevertheless, the above data suggest that NF-κB activation may be involved in a protective mechanism against deleterious effects of 6-OHDA-induced oxidative stress. Considering the possibility that parthenolide may exert effects on other cellular signalling pathways such as MAP kinases [48], this point remains to be further explored. However, the idea of NF-κB as a cell-death repressor, especially against oxidative stress, agrees with previous findings [16] and is in accordance with two recent studies demonstrating the potential neuroprotective role of this transcription factor in other cellular models of PD induced by MPP^+ and dopamine [40,41]. This conclusion is reinforced by the data we gathered using Bcl-2 and Bfl-1 expressing cells. Bcl-2 anti-apoptotic gene expression has previously been shown to protect neuronal cells against 6-OHDA toxicity *in vitro* [49,50] as well *in vivo* [51]. We confirm these findings and extend them to another member of the Bcl-2 family, A1/Bfl-1. Interestingly, the protection afforded by Bcl-2 or Bfl-1 may require NF-κB activation, since parthenolide impaired their ability to rescue cells from 6-OHDA-mediated apoptosis. Our results thus suggest that a relationship between NF-κB and Bcl-2/Bfl-1 exists which could be involved in the protective effects of these anti-apoptotic genes.

A role for NF-κB-dependent transcription in the regulation of cell death genes has recently been demonstrated [3]. Although mostly unknown, a few target genes of NF-κB involved in the regulation of cell death have been identified. Several reports thus suggest that some bcl-2 family genes, i.e. bcl-x_L and bfl-1/A1, contain NF-κB sites in their promoter [39,52,53] and are up-regulated through NF-κB activation [54–56]. Hence, it is possible that NF-κB activation following 6-OHDA exposure induces a positive regulation of anti-apoptotic proteins leading to increased cell survival. Although this possibility has not been examined in detail yet, we have some evidence against this hypothesis. Indeed, Bcl-2 and Bfl-1 proteins are not constitutively expressed by PC12 and PC12-neo cells and Bcl-2 expression is not induced by 6-OHDA.[1] Moreover, we previously reported that Bcl-x_L anti-apoptotic protein, strongly and constitutively expressed by PC12 cells, is not increased following 6-OHDA treatment [32], although it is a known target gene for NF-κB transcription factor [53]. Thus, although other anti-apoptotic proteins could be involved, the protective effect of NF-κB activation induced by 6-OHDA seems unlikely to be related to a positive regulation of these three anti-apoptotic proteins. Given that the death-repressing ability of Bcl-2 and Bfl-1 was potently impaired by the inhibition of NF-κB activation, other cytoprotective proteins may be induced by NF-κB in 6-OHDA-treated PC12 cells. From this point of view, several anti-apoptotic factors besides Bcl-2 family members are regulated by Rel/NF-κB, including IAP proteins [57–59], and further studies will help to clarify their possible involvement in the protection brought by Bcl-2 and Bfl-1/A1 against 6-OHDA toxicity.

The present study suggests that NF-κB activation participates in a cellular neuroprotective response against an oxidative neurotoxic insult in an *in vitro* model of nigral degeneration [31,32]. However, addressing the exact function of NF-κB activation in dopaminergic neurons from PD brains needs further studies using *in vivo* models more closely reproducing the pathological situation.

[1] Blum D *et al.*, unpublished data.

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