Activation of NF-κB is involved in 6-hydroxydopamine—but not MPP⁺-induced dopaminergic neuronal cell death: its potential role as a survival determinant

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Abstract

The nuclear factor-kappaB (NF-κB) family plays an important role in the control of the apoptotic response. Its activation has been demonstrated in both neurons and glial cells in many neurological disorders. In the present study, we specifically examined whether and to what extent NF-κB activation is involved in culture models of Parkinson’s disease following exposure of MN9D dopaminergic neuronal cells to 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-4-phenylpyridinium ion (MPP⁺). Both analysis by immunocytochemistry and of immunoblots revealed that NF-κB–p65 was translocated into the nuclei following 6-OHDA but not MPP⁺-treatment. A time-dependent activation of NF-κB induced by 6-OHDA but not MPP⁺ was also demonstrated by an electrophoretic mobility shift assay. A competition assay indicated that not only NF-κB–p65 but also –p50 is involved in 6-OHDA-induced NF-κB activity. Co-treatment with an antioxidant, N-acetyl-L-cysteine, blocked 6-OHDA-induced activation of NF-κB signaling. In the presence of an NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), 6-OHDA-induced cell death was accelerated while PDTC did not affect MPP⁺-induced cell death. Our data may point to a drug-specific activation of NF-κB as a survival determinant for dopaminergic neurons.

Keywords: NF-κB; 6-Hydroxydopamine; MPP⁺; PDTC; MN9D cells
proteins of the IκB family [5]. Upon stimulation, IκB protein is phosphorylated and proteolytically degraded, culminating in the nuclear translocation of active NF-κB dimers and thereby leading to the transcriptional activation of target genes [5,6]. In the nervous system, activation of NF-κB is demonstrated under various pathophysiological conditions including acute or chronic neurodegenerative disorders [7,8]. While activation of NF-κB is known to play an important role in preventing apoptotic cell death during the immune and inflammatory response, however, its potential role in the nervous systems is still controversial and requires further elucidation.

In this report, therefore, we specifically examined whether and to what extent activation of NF-κB is involved in dopaminergic neuronal death. For this purpose, we utilized the MN9D dopaminergic neuronal cell line established from embryonic mesencephalic dopaminergic neurons by a somatic fusion with N18TG2 neuroblastoma cells [9,10]. Neuronal death was induced by treatment of MN9D cells with two well-known dopaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenylpyridinium (MPP+). Our data suggest that (i) an ROS-mediated nuclear translocation and activation of NF-κB were apparent in cells treated with 6-OHDA but not after MPP+ and (ii) inhibition of NF-κB signaling accelerated 6-OHDA but not MPP+-induced cell death. Our data indicate a drug-specific activation of NF-κB as a survival determinant for dopaminergic neurons.

Materials and methods

Cell culture and drug treatment. MN9D cells were plated on 48-well plates or p-100 dishes (Corning Glass Works, Corning, NY) coated with 25 μg/ml poly-d-lysine. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA) in an incubator with an atmosphere of 10% CO2 at 37 °C for 3 days. Cells were subsequently switched to serum-free N2 medium [11] containing either 100 μM 6-OHDA or 50 μM MPP+ in the presence or the absence of 1 mM N-acetylcysteine or 1.25–10 μM pyrrolidine dithiocarbamate (PDTC; Sigma) for the time periods indicated [13]. Following drug treatment, cells were washed with PBS, incubated in a buffer containing 10 mM Hepes, pH 7.5/1.5 mM MgCl2/10 mM KC1/1 mM DTT/0.1% Tween 20 and insulin-like growth factor (ILG) 1% non-fat milk followed by an incubation with a rabbit polyclonal anti-NF-κB-p65 (1:3000). The blots from the cytosolic fraction were reprobed with mouse monoclonal anti-actin antibody (1:3000; Sigma) to serve as a control for gel loading. Specific bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Electrophoresis mobility shift assay. For electrophoresis mobility shift assay (EMSA), nuclear fractions were prepared by high-salt extraction from MN9D cells treated with either 100 μM 6-OHDA or 50 μM MPP+ in the presence or the absence of 1 mM N-acetylcysteine or 1.25–10 μM pyrrolidine dithiocarbamate (PDTC; Sigma) for the time periods indicated [13]. Following drug treatment, cells were washed with PBS, incubated in a buffer containing 10 mM Hepes, pH 7.5/1.5 mM MgCl2/10 mM KC1/1 mM DTT/0.1% Tween 20 and insulin-like growth factor (ILG) 1% non-fat milk followed by an incubation with a rabbit polyclonal anti-NF-κB-p65 (1:3000). The blots from the cytosolic fraction were reprobed with mouse monoclonal anti-actin antibody (1:3000; Sigma) to serve as a control for gel loading. Specific bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry and immunoblot analysis. For the localization of the p65 protein of NF-κB complexes (NF-κB-p65), MN9D cells were treated with either 100 μM 6-OHDA or 50 μM MPP+ for the time periods indicated. Cells were then fixed in 4% paraformaldehyde followed by three washes with phosphate-buffered saline (PBS). Cells were incubated with a rabbit polyclonal antibody to NF-κB-p65 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1:100 in 5% normal goat serum in PBS for 30 min followed by incubation with Alexa fluor 488 goat anti-rabbit IgG (1:200; Molecular Probes, Eugene, OR) for 50 min. After three washes with PBS, cells were incubated further with 1 μM Hoechst 33258 for 10 min to localize the nuclei of cells. Cells were then examined under an Axiovert 100 Microscope equipped with epifluorescence (Carl Zeiss, Zena, Germany). For immunoblot analysis, MN9D cells were treated with either 100 μM 6-OHDA or 50 μM MPP+ for the time periods indicated. Following drug treatment, both cytosolic and nuclear fractions of cells were collected as described above. Proteins (5–20 μg) were separated on a 12% SDS-PAGE, transferred to pre-wetted polyvinylidene difluoride (PVDF) nitrocellulose membranes, and processed for immunoblot analysis as described previously [12]. Briefly, membranes were blocked in Tris-buffered saline containing 5% non-fat milk followed by an incubation with a rabbit polyclonal anti-NF-κB-p65 (1:3000). The blots from the cytosolic fraction were reprobed with mouse monoclonal anti-actin antibody (1:3000; Sigma) to serve as a control for gel loading. Specific bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Results

A drug-specific localization of NF-κB-p65 into the nuclei

As we previously reported, approximately 60–70% of MN9D cells were dying after treatment with either 100 μM 6-OHDA for 24 h or with 50 μM MPP+ for 48 h. In these paradigms of cell death, we have raised the possibility that 6-hydroxydopamine (6-OHDA) and MPP+ act on distinct cell death pathways not only in dopaminergic neuronal MN9D cells [14–17] but also in primary cultures of dopaminergic neurons [18,19].
More specifically, reactive oxygen species (ROS) and/or an ROS-mediated signaling pathway may play an important role in 6-OHDA- but not in MPP⁺-induced cell death. Therefore, we wished to first examine whether these two neurotoxins distinctly trigger nuclear translocation of NF-κB–p65 in MN9D dopaminergic neuronal cell lines. MN9D cells were treated with either 100 μM 6-OHDA or 50 μM MPP⁺ for the time periods indicated and subjected to immunofluorescent localization of NF-κB–p65. In control MN9D cells, NF-κB–p65 was localized mainly in the cytosol (Figs. 1A–C). In MN9D cells treated with 6-OHDA for 9 h, nuclear translocation of NF-κB–p65 was apparent (Figs. 1D–F). In contrast, treatment of cells with 50 μM MPP⁺ for up to 24 h did not induce nuclear translocation of NF-κB–p65 (Figs. 1G–I). To further examine this phenomenon, we carried out immunoblot analysis of both the cytosolic and the nuclear fractions harvested from MN9D cells after treatment with either 100 μM 6-OHDA or 50 μM MPP⁺ for the time periods indicated. As shown in Fig. 2, a majority of NF-κB–p65 was present in the cytosolic fraction in MN9D cells before drug treatment. Upon exposure of cells to 6-OHDA, nuclear translocation of NF-κB–p65 was apparent between 3 and 9 h, and slightly decreased thereafter (Fig. 2A). In MN9D cells treated with MPP⁺, however, translocation of NF-κB–p65 into the nuclear fraction was not evident over 24 h of observation (Fig. 2B). In three independent experiments, the levels of nuclear NF-κB–p65 were quite similar among the groups treated with or without MPP⁺ (100% for control vs 96.7 ± 3.1–112 ± 4.9% for MPP⁺-treated groups).

**Drug-specific activation of the NF-κB signal**

One of the cellular responses to oxidative stress involves an increased expression of early transcription factors including NF-κB [3]. We previously demonstrated in MN9D cells treated with 100 μM 6-OHDA that a dramatic surge of both dihydroethidium-sensitive reactive oxygen species (ROS) and DAF-FM-sensitive nitric oxide (NO) appeared as early as 1 h after treatment, peaked at 6–12 h, and then decreased thereafter [19]. In contrast, dihydroethidium-sensitive ROS was not detected in MN9D cells treated with 50 μM MPP⁺ for up to 24 h [16]. To measure NF-κB activity, we performed electrophoretic mobility shift assays (EMSA) using end-labeled double-stranded oligonucleotides containing NF-κB consensus sequence. As shown in Fig. 3A, NF-κB activity in MN9D cells treated with 100 μM 6-OHDA increased between 3 and 6 h, peaked at 9 h, and decreased...
thereafter. Only a marginal level of NF-κB activity was detected in MN9D cells treated with 50 μM MPP⁺ for up to 24 h. Co-treatment for 9 h with an antioxidant, 1 mM N-acetylcysteine, significantly blocked the 6-OHDA-induced activation of the NF-κB signal, indicating that ROS comprises at least one trigger for NF-κB signaling in 6-OHDA-treated dopaminergic neuronal cells. To ascertain the specificity of NF-κB DNA binding, competition assays were conducted after preincubation of nuclear extracts with either polyclonal antibody against the p50 or p65 of NF-κB or with excess amounts of unlabeled probe. As demonstrated previously in 6-OHDA-treated PC12 cells [20], a super-shift of p65 or p50 was not detected after preincubation with anti-NF-κB-antibodies or unlabeled probe. Rather, 6-OHDA-induced NF-κB binding to DNA sequences was hindered in all cases (Fig. 3B), indicating that both p65 and p50 are involved in 6-OHDA-induced NF-κB activity.

**Difference in survival for dopaminergic neurons depending on drugs used to attempt activate NF-κB**

Data from postmortem Parkinsonian brains or from in vitro models of PD have indicated a nuclear translocation of NF-κB proteins, although its potential role in dopaminergic neuronal cell death is still largely controversial. For examples of controversy, in PC12 cells following treatment with dopamine [21,22] or 6-hydroxydopamine [20,23], NF-κB signal has been proposed to serve as either an anti-cell death or pro-cell death promoter. Therefore, to investigate whether NF-κB signaling affects 6-OHDA-induced cell death,
MN9D cells were treated with 100 µM 6-OHDA in the presence or the absence of varying doses of pyrrolidine dithiocarbamate (PDTC; a suppressor of NF-κB activity). The nuclear extracts from each group were subjected to EMSA as described in Materials and methods. To assess cell viability, MN9D cells were treated with (B) 100 µM 6-OHDA for 9 h; or (C) 50 µM MPP⁺ for 36 h in the presence or the absence of 10 µM PDTC. Cell viability was measured by the MTT reduction assay as described in Materials and methods. Values from each treatment were expressed as a percentage of the untreated control (100%). Each bar represents the means ± SEM from the three independent experiments done in triplicate. *p < 0.001; ANOVA with post hoc Student’s t test.

Fig. 4. Effect of pyrrolidine dithiocarbamate (PDTC) on NF-κB activity and cell viability in neurotoxin-treated MN9D cells. (A) MN9D cells were treated with 100 µM 6-OHDA for 9 h in the presence or the absence of the doses of PDTC indicated. Nuclear extracts from each group were subjected to EMSA as described in Materials and methods. To assess cell viability, MN9D cells were treated with (B) 100 µM 6-OHDA for 9 h; or (C) 50 µM MPP⁺ for 36 h in the presence or the absence of 10 µM PDTC. Cell viability was measured by the MTT reduction assay as described in Materials and methods. Values from each treatment were expressed as a percentage of the untreated control (100%). Each bar represents the means ± SEM from the three independent experiments done in triplicate. *p < 0.001; ANOVA with post hoc Student’s t test.

Discussion

We demonstrated previously that 6-OHDA triggers reactive oxygen species (ROS)-mediated signaling pathway(s) in a MN9D dopaminergic neuronal cell line, in primary cultures of dopaminergic neurons, and in cortical neurons [14–19,24]. By contrast, metabolic abnormalities and activation of calpain seem to be involved primarily in MPP⁺-induced cell death. The data presented here provide a further example of the presence of distinct cell death signals recruited by these two well-known dopaminergic neurotoxins. More specifically, we have demonstrated here that 6-OHDA induces nuclear translocation of the NF-κB–p65 and the subsequent activation of NF-κB signal. Evidence from EMSA suggests that 6-OHDA-induced activation of NF-κB is an ROS-dependent event. Therefore, co-treatment with the antioxidant, N-acetylcysteine, completely abolished 6-OHDA-induced NF-κB activity. Our present data support a notion that NF-κB activation may play a role for preventing 6-OHDA-induced neuronal cell death as shown by morphological criteria and by the MTT reduction assay. By contrast, we have demonstrated that another dopaminergic neurotoxin, MPP⁺, does not induce either nuclear translocation of the NF-κB–p65 or NF-κB activity. Therefore, the rate of cell death induced by MPP⁺ is not affected by co-treatment with pyr-
rolidine dithiocarbamate over 36 h, indicating a drug-specific activation of NF-κB as a survival determinant for dopaminergic neurons.

In the nervous system, activation of NF-κB is demonstrated in such pathophysiological conditions as acute or chronic neurodegenerative disorders including Parkinson’s disease (PD) [7,8]. For example, studies of post-mortem brain tissue from patients with PD have revealed a nuclear translocation of NF-κB in melanized neurons [25]. Data from in vitro culture models of PD have also shown a nuclear translocation of NF-κB in various cell types including PC12 cells, SH-SY5Y neuroblastoma cells or primary cultures of dopaminergic neurons following treatment with MPP+, 6-OHDA, dopamine, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or ceramide [20-23,26-28]. However, its potential role in promoting or alleviating cell death in dopaminergic neurons is still largely controversial. Indeed, the importance of NF-κB activation is ascribed to either the prevention or acceleration of cell death induced by dopamine [21,22] or by 6-hydroxydopamine [20,23]. Several parameters including culture conditions, severity, and duration of insult may account for the discrepancy among several groups. In our study, we failed to observe any changes in the pattern of NF-κB activation and its protective role in 6-OHDA-induced cell death over a wide concentration of 6-OHDA (10–200 μM) up to 36 h. Therefore, both the severity and the duration of 6-OHDA treatment do not seem to be responsible for the discrepancy. Although it is speculative at present, one rather plausible explanation could be that distinct cellular context may be responsible for determining transcriptional activation of the subsets of genes by activating specific combinations among the NF-κB family [4].

MPP+ is an active metabolite of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) known to be transported into dopaminergic cell types via the high affinity dopamine uptake system; it is sequestered within mito-

References


