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Lack of correlation between NF-κB activation and induction of programmed cell death in PC12 pheochromocytoma cells treated with 6-hydroxydopamine or the cannabinoid receptor 1-agonist CP55,940

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Abstract

NF-κB is a transcriptional regulator that plays a key role in immunity, inflammation and programmed cell death. We generated a PC12 cell line termed PC12κBluc that contains an integrated NF-κB-responsive reporter gene to directly measure NF-κB activity. The ''classical'' activators of NF-κB, phorbol 12-*O*-tetradecanoate-13-acetate and tumor necrosis factor α, strongly induced NF-κB activity in PC12κBluc cells. Activation of NF-κB could be attenuated by preincubating the cells with the cAMP analogue dbcAMP or *via* expression of the superrepressor IκBαS32A/S36A. PC12κBluc cells were subjected to several apoptotic paradigms, including treatment with 6-hydroxydopamine, H_2O_2, $K_2Cr_2O_7$, $MnCl_2$, C_2-ceramide or the cannabinoid receptor-1 agonist CP55,940. A simultaneous measurement of the NF-κB activity revealed that only administration of 6-hydroxydopamine or CP55,940 increased NF-κB activity. Using pharmacological and genetic strategies to attenuate NF-κB transcriptional activity, we demonstrate that the elevation of NF-κB activity by 6-hydroxydopamine and CP55,940 is not an integral part of the apoptotic signaling cascade in PC12 cells.
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Keywords: Cannabinoid receptor; 6-Hydroxydopamine; IκB; PC12 cells; Neuronal cell death; NF-κB

1. Introduction

NF-κB is an inducible transcriptional regulator that plays a key role in immunity, inflammation and programmed cell death [1,2]. NF-κB is a dimer composed of five mammalian family members (Rel/p65, RelB, c-Rel, p105/p50 and p100/p52) in almost any combination. The prototypical NF-κB consists of a heterodimer of p50/p65 that resides in resting cells in the cytoplasm as a complex with one of the three inhibitory proteins (IκBα, IκBβ or IκBε). Upon stimulation with a great variety of extracellular signaling molecules including cytokines, phorbol esters, viral infection or ultraviolet irradiation, the IκB proteins become *de novo* phosphorylated on critical serine residues by the IκB kinase complex. This process targets them for ubiquitination and degradation by the proteasome. The degradation of IκB results in the rapid translocation of NF-κB to the nucleus where NF-κB can stimulate transcription of NF-κB-responsive target genes. NF-κB represents a focal point for stimulus-transcription coupling, connecting extracellular signals with changes in gene transcription.

Originally, NF-κB was identified as a transcription factor specific for mature B-cell lines regulating immunoglobulin κ light chain gene expression [3]. Subsequently, NF-κB was detected in many cell types including neurons which show either constitutive or signal-induced activation of NF-κB.

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Abbreviations: dbcAMP, *N*,2′-*O*-dibutyryladenosine-3:5-cyclic monophosphate; IBMX, isobutyl-1-methylxanthine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 6-OHDA, 6-hydroxydopamine; TNFα, tumor necrosis factor α; TPA, phorbol 12-*O*-tetradecanoate-13-acetate; TUNEL, terminal deoxynucleotidyl transferase fluorescein-dUTP 3′-end labeling.

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NF-κB has been described to promote cell survival, due to the upregulation of antiapoptotic and antioxidant genes. Thus, NF-κB functions as an anti-apoptotic transcription factor [4–6]. Conversely, an elevation of NF-κB activity was proposed to trigger cell death [5–8]. In particular, NF-κB is activated in a rodent model of stroke. Interestingly, ischemic damage was significantly reduced in p50 knockout mice, suggesting that NF-κB plays a cell death-promoting role in focal ischemia [7]. This paradoxical situation locating NF-κB "at the crossroads of life and death" [4] makes it very interesting to identify the signals leading to an enhanced transcription *via* NF-κB and to elucidate the biological outcome of an activated NF-κB transcription factor. To directly monitor signal-induced activation of NF-κB, we generated a PC12 cell line with an integrated NF-κB responsive reporter gene. This cell line was used to study activation of NF-κB by cytotoxic compounds. Here, we show that from many neurotoxic compounds tested, only 6-hydroxydopamine (6-OHDA) and the cannabinoid receptor 1 agonist CP55,940 simultaneously stimulated cell death and activated NF-κB. We further provide evidence that the activation of NF-κB is not necessary for 6-OHDA or CP55,940 induced cell death.

2. Materials and methods

2.1. Material

1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), H_2O_2, 6-hydroxydopamine (6-OHDA), isobutyl-1-methylxanthine (IBMX), $K_2Cr_2O_7$, $MnCl_2$, phorbol 12-*O*-tetradecanoate-13-acetate (TPA), and puromycin were purchased from Sigma-Aldrich Chemie GmbH. Tumor necrosis factor α (TNFα) was purchased from Biotrend. CP55,940 was purchased from Calbiochem-Novagen, G418, Neurobasal medium and N2-supplement were from Invitrogen GmbH.

2.2. Cell culture

PC12 cells were maintained in RPMI medium supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine at 37° in 5% CO_2. PC12 cells (1×10^6 cells in PBS) were electroporated in 0.4 cm cuvettes (450 V, 250 mFa) with 20 mg of plasmid 3xκBluc and 1 mg of plasmid pSVpuro. Plasmid 3xκBluc contains a κB-dependent luciferase reporter transcription unit consisting of three copies of the Igκ-κB-motif immediately upstream of the β-globin TATA box [9]. PC12 cells were selected with 2.0 mg/mL puromycin and a single clone termed PC12κBluc was selected for further analysis. To measure signal-induced NF-κB activity in PC12κBluc cells, cells were seeded at the density of 2×10^5 cells/well into 12-well plates in RPMI medium supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine, and incubated overnight. The next day, the medium was changed to Neurobasal medium containing 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM glutamine and 1% N2-supplement. The cells were incubated for a further 24 hr period, the medium was renewed and the stimuli were applied to the cells as indicated. Cells were harvested in cold PBS, washed twice and lyzed using reporter lysis buffer (# 397A) from Promega GmbH.

2.3. Reporter gene assays

Luciferase activities were measured as described [10]. Protein concentrations were determined using the BCA assay (Pierce). Luciferase activities were expressed as luciferase light units per μg of protein. The experiments were done at least twice with similar results obtained each time.

2.4. Retroviral gene transfer

The retroviral vector pLNCX [11] was a kind gift of A. Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, USA. The expression vector CMV-MAD-3 encoding IκBαS32A/S36A was a kind gift of P. Baeuerle [12]. The IκBαS32A/S36A coding region was excised and cloned into the Hpa I site of pLNCX, generating plasmid pLNCX-IκBαS32A/S36A. The Phoenix-Eco packaging cell line was obtained from Gary Nolan, Stanford University, USA. Cells were transfected with retroviral vectors according to the protocol developed by G. Nolan (www.stanford.edu/group/nolan/NL-helper.html) using the calcium coprecipitation procedure. Viral supernatants were harvested 72 hr after transfection, filtered through a 45 μm filter, and used to infect PC12κB cells in the presence of 8 μg/mL polybrene at 37°. Six hours later the medium was removed, the cells were supplied with fresh complete medium, and cultured for 72 hr before adding selection medium containing 0.6 μg/mL G418. Mass pools of stable transfectants were selected and used for all experiments in order to eliminate the possibility of specific clonal effects.

2.5. Survival assays and detection of apoptotic markers

Cells were seeded in quadruplicate at the density of 1×10^4 cells/well in 96-well plates in serum-containing medium. Twenty four hours later, the medium was removed, replaced by Neurobasal medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine and 1% N2-supplement. The cells were incubated for 24 hr and the medium was renewed before the application of the cytotoxic compounds. The mitochondrial reduction capacities were determined by quantification of the levels of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-

lium bromide (MTT) reduction to formazan dye crystals. MTT solution (0.5 mg/mL final concentration/well, dissolved in PBS) was added to cultures and incubated for 4 hr at 37° in 5% CO_2. Cells were solubilized in 10 mM HCl containing 10% SDS, and the plates were incubated overnight at 37°. Absorbance was quantified on a BioRad Model 550 microplate reader, using a test wavelength of 595 nm. Alternatively, the medium was removed following the 4 hr incubation with the MTT solution and 100 μL of DMSO was added to each well. The formazan dye crystals were solubilized for 15 min and quantified spectrophotometrically as described. MTT-reduction was expressed as a percentage of controls. Apoptotic DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated 3′-tailing with TMRred-labeled dUTP (TUNEL) using the ''in situ cell death detection kit'' (Roche Diagnostics GmbH) according to the manufacturers protocol.

3. Results

3.1. Stimulation of NF-κB activity in PC12κBluc cells by TPA and TNFα

There are several methods to detect activation of NF-κB. Most frequently, an enhanced DNA-binding activity is used as an indicator for an increase in the transcriptional activity of NF-κB. Likewise, detection of IκB phosphorylation or translocation of NF-κB into the nucleus are indicators for a possible activation of NF-κB. Phosphorylation of IκB, translocation of NF-κB into the nucleus and binding of NF-κB to its cognate DNA binding site are prerequisites for an activation of NF-κB, but these events only indirectly indicate an activation of NF-κB. We have therefore generated a PC12 cell line termed PC12κBluc expressing a reporter transgene under control of three κB-sites. Hence, enhanced reporter gene transcription is solely the result of an activation of NF-κB within the cell. Thus, we were able to directly measure NF-κB activity following incubation of the cells with extracellular signaling molecules. The cytokine tumor necrosis factor α (TNFα) and the tumor promoter phorbol 12-*O*-tetradecanoate-13-acetate (TPA) are ''classical'' activators of NF-κB. We tested whether TNFα and TPA function as inducers of NF-κB in PC12κBluc cells. Cells were seeded in serum-containing medium, then cultured in a defined medium (Neurobasal plus N2-supplement) for 24 hr, and stimulated with TNFα or TPA for 24 hr. Cell extracts were prepared and the protein concentrations and luciferase activities determined. Luciferase activities were normalized to the protein concentration of the extracts and expressed as light units/mg protein. Fig. 1 shows that PC12κBluc cells do not contain constitutively active NF-κB. In fact, TNFα (right panel) and TPA (left panel) strongly activated NF-κB activity in PC12κBluc cells. The stimulation was on the order of 50-fold.

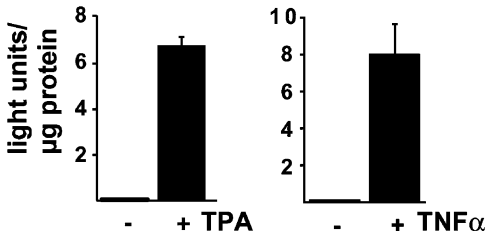

Fig. 1. Tumor necrosis factor α and the tumor promoter TPA strongly activate NF-κB in PC12κBluc cells. PC12κBluc cells were seeded in serum-containing medium and incubated overnight. The medium was replaced by Neurobasal medium containing the N2-supplement and the cells were incubated for 24 hr. The Neurobasal medium was renewed and the cells were stimulated with 50 ng/mL TPA or 10 nM TNFα for 24 hr. Cell extracts were prepared and the protein concentrations and luciferase activities determined. Luciferase activities were normalized to the protein concentration of the extracts and expressed as light units/mg protein.

3.2. Induction of neuronal cell death by cytotoxic compounds

Curiously, activation of NF-κB has been correlated with pro-apoptotic as well as anti-apoptotic signaling [4–8]. To investigate the relationship between NF-κB activity and cell death, PC12κBluc cells were subjected to several apoptotic paradigms, including treatment with 6-hydroxydopamine (6-OHDA), H_2O_2, $K_2Cr_2O_7$, $MnCl_2$, C_2-ceramide or the CB_1 receptor agonist CP55,940. Cells were incubated for 24 hr with the indicated concentrations of these compounds and the mitochondrial reduction capacities were measured using the reduction of MTT to formazan dye crystals. In this assay, colorless tetrazolium salts are reduced by mitochondrial NADP(H)-dependent dehydrogenases to form a colored product termed formazan that was subsequently detected spectrophotometrically. The data are presented in comparison to the amount of formazan formed in the absence of the cytotoxic compound. Fig. 2 shows that a concentration of 50 μM H_2O_2, 3–5 μM $K_2Cr_2O_7$, 1 mM of $MnCl_2$, 20 μM of C_2-ceramide, 2 μM of CP55,940, or 100 μM of 6-OHDA were neurotoxic for PC12κBluc cells. TPA or TNFα did not exhibit cytotoxic effects in PC12κBluc cells (data not shown).

3.3. Simultaneous activation of cell death and NF-κB in 6-OHDA or CP55,940 treated PC12κBluc cells

The integrated NF-κB-responsive reporter gene in PC12κBluc cells allowed us to directly measure the NF-κB activity in cells treated with these neurotoxic compounds. Cells were incubated for 8 hr with these cytotoxins, cell extracts were prepared and the relative luciferase activities determined. Treatment of PC12κBluc cells with either 6-OHDA or CP55,940 activated NF-κB. The activation was in the order of 45- and 10-fold, respectively (Fig. 3). No activation of NF-κB was detected following treatment of PC12κBluc cells with H_2O_2, $K_2Cr_2O_7$, $MnCl_2$ or C_2-ceramide (data not shown).

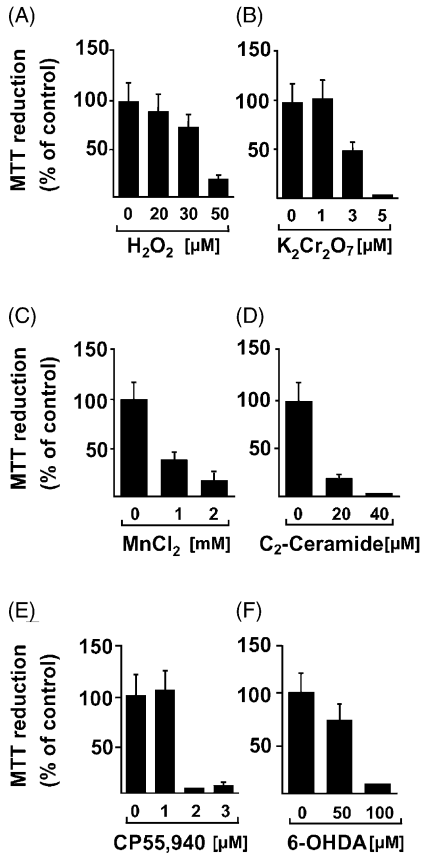

Fig. 2. Induction of cell death in PC12κBluc cells. PC12κBluc cells were cultured in Neurobasal medium containing N2-supplement. The cells were exposed to H_2O_2 (A), $K_2Cr_2O_7$ (B), $MnCl_2$ (C), C_2-ceramide (D), CB_1 receptor agonist CP55,940 (E), or 6-OHDA (F) for 24 hr. The cytotoxic effects of the applied compounds was determined spectrophotometrically by the MTT method.

3.4. Attenuation of NF-κB activity by increased intracellular cAMP concentrations

For human pancreatic MIA PaCa-2 cells, forskolin and cAMP have been reported to inhibit interleukin-1α induced NF-κB induction [13]. Likewise, vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide, both increasing the intracellular cAMP concentration, were shown to inhibit NF-κB-dependent gene activation in human monocytic and endothelial cells [14]. We tested whether an increase in the intracellular cAMP concentration impairs NF-κB activity in PC12κBluc cells. Cells were preincubated with the cAMP analogue dibutyryl cAMP and the phosphodiesterase inhibitor IBMX for 48 hr and then stimulated with either TPA, TNFα, 6-OHDA or CP55,940 for 8 hr. Cells were harvested, cell extracts prepared and the relative luciferase activities determined. Fig. 4A reveals that the combination of dbcAMP and IBMX strongly attenuated TPA or TNFα-induced NF-κB activity. Likewise, induction of NF-κB by 6-OHDA or CP55,940 was efficiently inhibited by an increase in the intracellular cAMP concentration (Fig. 4B).

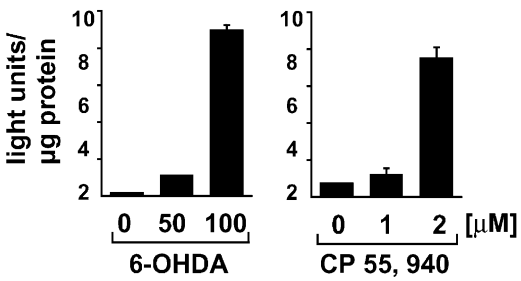

Fig. 3. Activation of NF-κB in PC12κBluc cells by the cytotoxic compounds 6-OHDA and CP55,940. PC12κBluc cells were seeded in serum-containing medium and incubated overnight. The medium was replaced by Neurobasal medium containing N2-supplement and the cells were incubated for 24 hr. The Neurobasal medium was renewed and the cells were treated for 8 hr with either 50 or 100 μM of 6-OHDA (left panel) or 1 or 2 μM of CP55,940 (right panel). Cell extracts were prepared and the protein concentrations and luciferase activities determined. Luciferase activities were normalized to the protein concentration of the extracts and expressed as light units/μg protein.

3.5. Attenuation of NF-κB activity by expression of the superrepressor IκBαS32A/S36A

The activation process of NF-κB requires phosphorylation and subsequent degradation of IκB. Thus, expression of a nondegradable form of IκB impairs NF-κB activity by retaining NF-κB in the cytoplasm. To generate PC12κBluc cells expressing the nondegradable IκBαS32A/S36A protein that contains alanine residues instead of the critical serine residues, we infected PC12κBluc cells with a recombinant retrovirus encoding IκBαS32A/S36A, thus

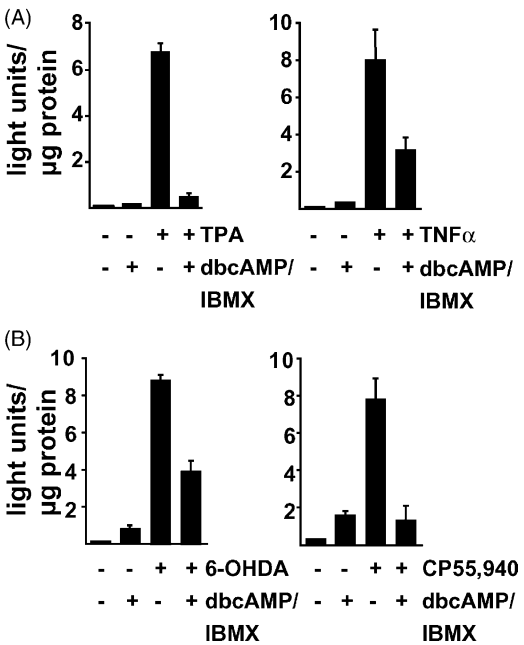

Fig. 4. Elevated intracellular cAMP concentrations inhibit NF-κB activity in PC12κBluc cells. PC12κBluc cells were cultured in Neurobasal medium containing N2-supplement. Cells were preincubated for 48 hr with 0.2 mM dbcAMP and 0.1 mM IBMX. The stimulation was performed with 50 ng/mL TPA, 10 nM TNFα (A), 100 μM 6-OHDA, or 2 μM CP55,940 (B) for 8 hr. Cell extracts were prepared, the protein concentrations and the luciferase activities determined, and the relative luciferase activities calculated.

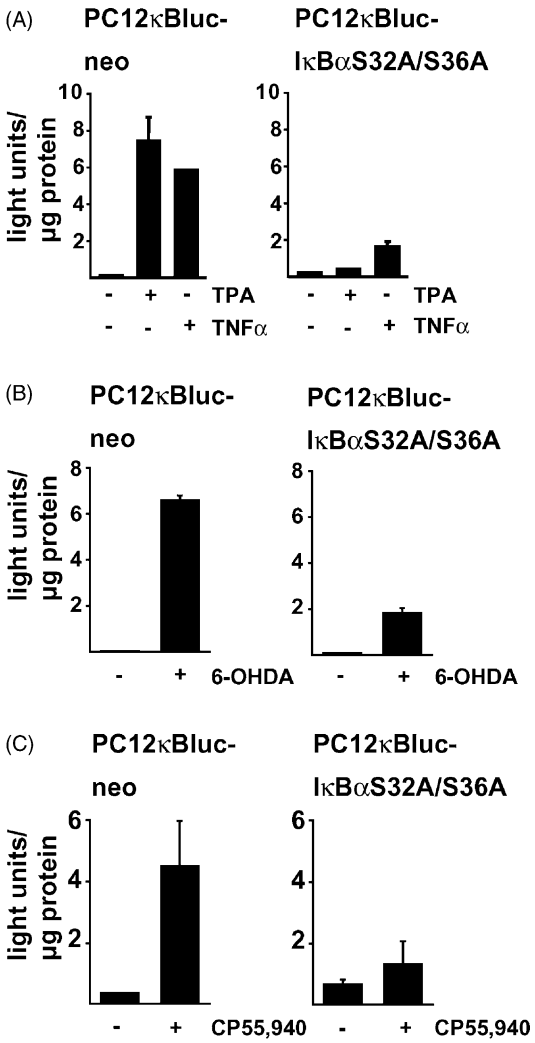

Fig. 5. Expression of the superrepressor IκBα32A/S36A in PC12κBluc cells impaires activation of NF-κB in PC12κBluc cells. PC12κBluc-neo (left panel) and PC12κBluc-IκBαS32A/S36A cells (right panel) were cultured in Neurobasal medium containing N2-supplement for 24 hr and stimulated with either 50 ng/mL TPA, 10 nM TNFα (A), 100 µM of 6-OHDA (B), or 2 µM of CP55,940 (C) for 8 hr. Cell extracts were prepared, the protein concentrations and the luciferase activities determined, and the relative luciferase activities calculated.

generating PC12κBluc-IκBαS32A/S36A cells. As a control, PC12κBluc cells were infected with recombinant retroviruses encoding the neomycin resistance gene (PC12κBluc-neo cells). The biological effect of IκBαS32A/S36A was tested in cells stimulated with TPA or TNFα. Fig. 5A (right panel) shows that expression of the superrepressor efficiently blocked the TPA-mediated elevation of NF-κB activity. Likewise, TNFα-induced activation of NF-κB was severely attenuated. In contrast, PC12κBluc cells expressing the neomycin resistance gene (PC12κBluc-neo) showed a strong induction of NF-κB following stimulation of the cells with either TPA or TNFα (Fig. 5A, left panel). Moreover, induction of NF-κB by 6-OHDA or CP55,940 was efficiently inhibited by the mutated IκBα protein IκBαS32A/S36A (Fig. 5B and C).

3.6. Role of NF-κB activation for 6-OHDA or CP55,940 induced cell death of PC12κBluc cells

Using pharmacological and genetical tools, we tested whether activation of NF-κB is an integral part of 6-OHDA or CP55,940 induced cell death. We tested the neurotoxic activity of 6-OHDA and CP55,940 under conditions of suppressed NF-κB. PC12κBluc cells were incubated with dbcAMP and IBMX for 48 hr and then challenged with 6-OHDA or CP55,940 for 24 hr. The mitochondrial reduction capacities were measured using the MTT assay. Fig. 6A reveals that an impairment of NF-κB had no

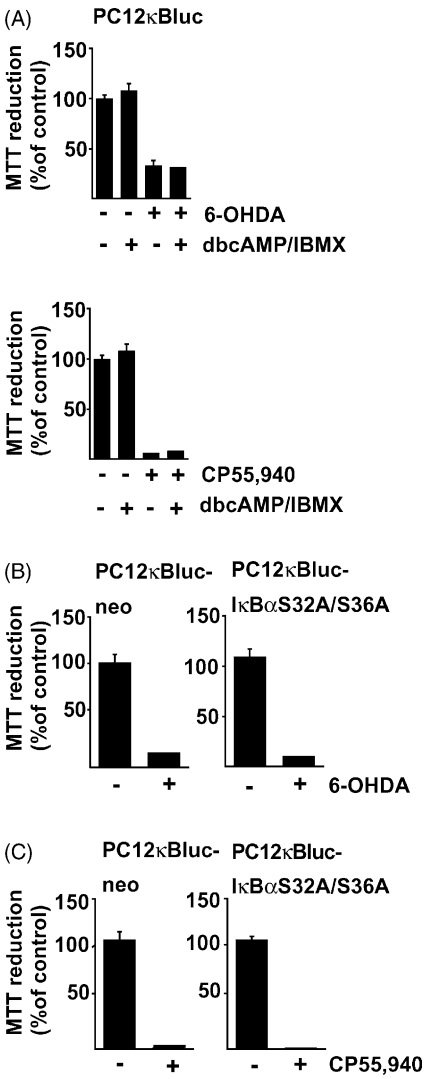

Fig. 6. Attenuation of NF-κB activity in 6-OHDA or CP55,940 treated cells does not increase the mitochondrial reduction capacities. (A) PC12κBluc cells were cultured in Neurobasal medium containing N2-supplement. Cells were preincubated for 48 hr with 0.2 mM dbcAMP and 0.1 mM IBMX and then exposed to 100 µM of 6-OHDA or 2 µM of CP55,940 for 24 hr. Cytotoxicity was determined spectrophotometrically by the MTT method. (B, C) PC12κBluc-neo (left panels) and PC12κBluc-IκBαS32A/S36A cells (right panels) were cultured in Neurobasal medium containing N2-supplement for 24 hr and exposed to 100 µM of 6-OHDA (B) or 2 µM of CP55,940 (C) for 24 hr. Cytotoxicity was determined spectophotometrically by the MTT method.

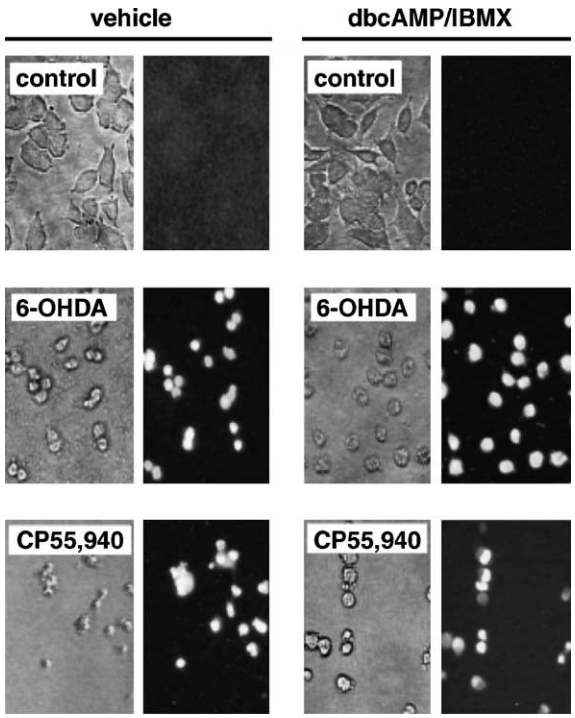

Fig. 7. Attenuation of NF-κB activity in 6-OHDA or CP55,940 treated PC12κBluc cells by elevated cAMP concentration does not block programmed cell death. PC12κBluc cells were cultured in Neurobasal medium containing N2-supplement. Cells were preincubated for 48 hr with vehicle (left panels) or 0.2 mM dbcAMP and 0.1 mM IBMX (right panels) and then exposed to 100 μM of 6-OHDA or 2 μM of CP55,940 for 15 hr as indicated. Control cells are shown on top. Cells were analyzed using the TUNEL technique with TMR-labeled UTP. Phase contrast and fluorescence micrographs of the same locations are depicted.

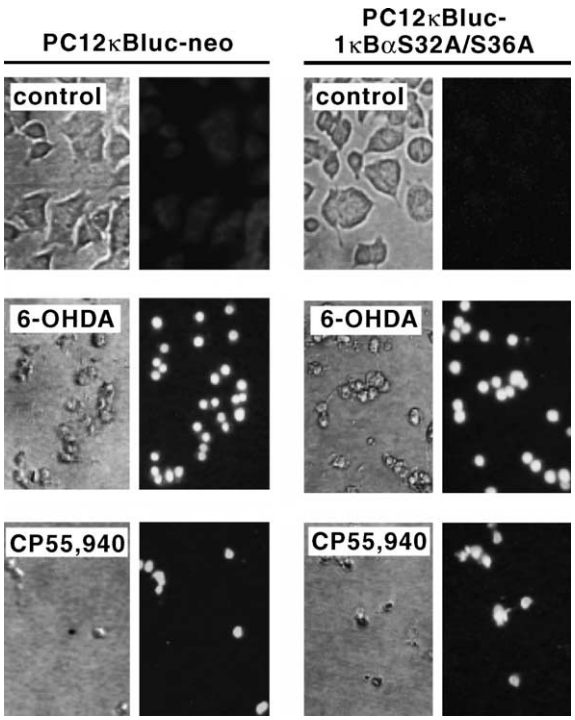

Fig. 8. Attenuation of NF-κB activity in 6-OHDA or CP55,940 treated cells *via* expression of the superrepressor IκBαS32A/S36A does not block programmed cell death. PC12κBluc-neo (left panels) and PC12κBluc-IκBαS32A/S36A cells (right panels) were cultured in Neurobasal medium containing N2-supplement for 24 hr and exposed to vehicle (top), 100 μM of 6-OHDA or 2 μM of CP55,940 for 15 hr as indicated. Cells were analyzed using the TUNEL technique with TMR-labeled UTP. Phase contrast and fluorescence micrographs of the same locations are depicted.

impact in cell survival. Likewise, the mitochondrial reduction capacities were reduced in IκBαS32A/S36A expressing cells as a result of 6-OHDA or CP55,940 treatment (Fig. 6B and C). We did not observe significant differences in the neurotoxic activities of 6-OHDA or CP55,940 in PC12 cells expressing either neomycin acetyltransferase or the IκB superrepressor IκBαS32A/S36A (compare Fig. 6B and C left and right panels). These results indicate that NF-κB is not involved in the signaling pathway initiated by 6-OHDA or CP55,940 that leads to neuronal cell death.

To confirm these data, we analyzed the fragmentation of chromatin as a result of the neurotoxic challenge by 6-OHDA or CP55,940 using the terminal deoxynucleotidyl transferase-mediated dUTP 3′-end-labeling (TUNEL) technique. Cells were preincubated with dbcAMP and IBMX for 48 hr and then exposed to 6-OHDA or CP55,940 for 15 hr. Fig. 7 shows the morphology of PC12κB cells in the presence or absence of the neurotoxic compounds. It is clearly visible that 6-OHDA and CP55,940 killed the cells. The right panels depicts the TUNEL analysis of PC12κB cells. No chromatin fragmentation was detected in the absence of the cytotoxic stimuli. TUNEL-positive cells emerged as a result of 6-OHDA or CP55,940 treatment, showing that these compounds induced apoptotic cell death. The preincubation of PC12κBluc cells with dbcAMP and IBMX, in order to attenuate NF-κB activity, did not reduce the amount of TUNEL positive cells, indicating that the level of NF-κB activity is not of any importance in 6-OHDA and CP55,940 induced cell death. These data were confirmed in PC12κBluc cells expressing the IκBαS32A/S36A mutant. PC12κBluc cells died as a result of 6-OHDA or CP55,940 treatment, despite the expression of IκBαS32A/S36A (Fig. 8, right panels). We did not observe a difference in either IκBαS32A/S36A or neomycin acetyltransferase expressing PC12κBluc cells. Taken together, these data show that the elevation of NF-κB activity by 6-OHDA or CP55,940 is not required for the apoptotic cell death of PC12κBluc cells.

4. Discussion

The activation of NF-κB requires multiple steps. Many of those were used in the past as sensors to extrapolate an activation of NF-κB, e.g. phosphorylation of IκB, translocation of NF-κB into the nucleus or binding of NF-κB to its cognate DNA binding site. All of these reactions are

necessary and required for a subsequent activation of NF-κB. Nevertheless, they do not show a direct increase in the transcription of NF-κB responsive genes, but rather give convincing evidence that NF-κB activation is likely to occur. In particular an enhanced binding activity of a transcription factor to DNA, monitored by an *in vitro* binding assay, does not necessarily prove an enhanced transcriptional activation potential of this protein. We showed, for example, by *in vitro* electrophoretic mobility-shift assay that the zinc finger transcription factor Egr-1 binds specifically to a GC-rich sequence of the synaptobrevin II promoter, as shown. However, in intact cells, Sp1 blocks this site and Egr-1 has no access to the regulatory region of the synaptobrevin II gene to activate transcription [15]. Likewise, disparate results between *in vitro* DNA/protein binding assays and direct transcriptional test systems based on reporter genes have been noted for NF-κB [16]. To directly measure an increase in the transcriptional activation potential of NF-κB as a result of extracellular or cytotoxic signals, we generated PC12 cells encoding an NF-κB-responsive luciferase reporter gene. A striking increase in the NF-κB activity was observed in these cells following treatment with the ''classical'' NF-κB activators TNFα or phorbol esters.

NF-κB has been proposed to function either as a pro-apoptotic or as an anti-apoptotic protein, determined by the cell type and the apoptotic inducers. The protective effect of NF-κB was clearly demonstrated in p65 knock-out mice. These animals died at day 14/15 of embryonic development, due to massive apoptosis of hepatocytes [17]. Accordingly, p65-deficient fibroblasts are susceptible to TNFα-mediated cell death, whereas wild-type or reconstituted cells are protected. Likewise, the inhibition of NF-κB by expression of a dominant-negative IκBα conferred a dramatic sensitivity to TNFα-triggered cell death in otherwise resistant cell types [18,19]. In the nervous system, the activation of NF-κB has frequently been connected with an enhanced neuronal survival [6]. However, NF-κB may, under some circumstances, also promote cell death [7]. We have tested the role of NF-κB in neuronal cell death using several apoptotic paradigms, including treatment with 6-OHDA, H_2O_2, $K_2Cr_2O_7$, $MnCl_2$, C_2-ceramide or activation of the cannabinoid receptor-1 by the compound CP55,940. The results showed that only exposure of PC12κBluc cells to 6-OHDA or CP55,940 increased NF-κB activity whereas all inducers promoted cell death. 6-OHDA is a hydroxylated derivative of dopamine, that is formed endogenously in patients with Parkinson's disease [20]. 6-OHDA causes a highly selective degeneration of dopaminergic neurons in the substantia nigra, suggesting that 6-OHDA is a potential pathological factor in the destruction of dopaminergic neurons. Here, we showed that 6-OHDA efficiently induced apoptotic cell death in PC12κBluc cells. Moreover, we observed a striking increase in NF-κB activity following exposure of PC12κBluc cells to 6-OHDA. To study the biological role of NF-κB in neuronal cell death, we used the preincubation of the cells with dbcAMP and IBMX as a tool to attenuate NF-κB activity. In addition, the pharmacological treatment was complemented with a genetic strategy, the expression of a non-degradable IκBα mutant in PC12κBluc cells. These experiments clearly demonstrated that an elevated NF-κB activity is not necessary for 6-OHDA-induced cell death. In contrast, activation of NF-κB has been proposed to be essential for dopamine-induced apoptosis in PC12 cells [21,22]. In one of these studies, however, not NF-κB activation but rather DNA-binding activities were measured and the SN50 peptide was used to interfere with NF-κB activation [21]. The peptide SN50, derived from the NF-κB subunit p50, was originally described as a cell-permeable peptide that masks the nuclear localization sequence of NF-κB and prevents its translocation to the nucleus [23]. However, the peptide may not be specific for NF-κB. Rather, SN50 may additionally compete with other proteins that require nuclear translocation, e.g. the transcription factors STAT, AP-1 and NFAT [24]. In addition, the SN50 peptide was only weakly active in inhibiting NF-κB activity in the PC12κBluc system (N. Erlandsson, G. Thiel, unpublished observations). The other study used parthenolide to inhibit NF-κB activation [22]. However, not NF-κB activation but rather *in vitro* DNA-binding activities were measured, because attempts of the authors to create stable transfectants expressing the IκB super-repressor failed [22]. In contrast, we directly measured NF-κB activation by 6-OHDA due to the generation of a PC12 cell line containing an integrated NF-κB-responsive reporter gene. Furthermore, treatment of these PC12κBluc cells with dbcAMP or stable expression of the superrepressor IκBαS32A/S36A undoubtedly showed that attenuation of NF-κB activation was without any effect upon 6-OHDA-induced cell death. Taken together, the present data do not support a role of NF-κB in the process of dopamine-triggered cell death. Rather, the fact that the c-Jun N-terminal protein kinase is activated in dopamine treated 293 cells and postmitotic striatal neurons [25] suggests that c-Jun may be involved in dopamine-triggered cell death.

Activation of the CB_1 cannabinoid receptor by exposure of PC12κBluc cells with the specific agonist CP55,940 induced apoptotic cell death and simultaneously activated NF-κB. A causal relation between NF-κB activation and cell death could not be demonstrated, indicating that the activation of NF-κB *via* activation of the CB_1 receptor is not necessary for apoptotic signaling in this system. Interestingly, cannabinoids have been reported to activate c-Jun N-terminal protein kinase and p38 mitogen-activated protein kinase [26,27], protein kinases that are frequently discussed as key molecules in apoptotic signaling. These results indicate that activation of the CB_1 receptor in PC12κBluc cells is coupled to multiple signaling pathways in the cell. A dissection of these signaling pathways is required in order to elucidate the causal steps leading to cell death.

No activation of NF-κB was detected in PC12κBluc cells exposed to H_2O_2, $K_2Cr_2O_7$, $MnCl_2$, or C_2-ceramide. Some studies have suggested a correlation between H_2O_2 or C_2-ceramide induced cell death and increased NF-κB activities [28–31]. However, C_2-ceramide and sphingomyelinase were reported to activate stress-activated protein kinases in HL-60 human promyelocytic cells, but failed to activate NF-κB [32]. Likewise, cell-permeable ceramides failed to induce either nuclear translocation of NF-κB or degradation of IκB in Jurkat T cells. Furthermore, ceramide treatment of the cells inhibited phorbol ester-induced activation of NF-κB, indicating that, if anything, ceramide may participate in negative feedback regulation of NF-κB [33]. A comparative analysis of H_2O_2-triggered NF-κB activation, using Jurkat cells, EL4.NOB-1 T-cells and KB epidermal cells revealed that H_2O_2 activated NF-κB only in Jurkat cells, but not in the two other cell lines. These results indicate that the reactive oxygen model of NF-κB activation may be restricted to certain cell types [34].

Taken together, we have demonstrated that the PC12κBluc cell line is very valuable for direct testing of NF-κB activation as a result of stimulation by extracellular signaling molecules or exposure to cytotoxic drugs. Moreover, these cells are shown to function as tools for the investigation of NF-κB's role in programmed cell death. Additional studies with PC12κBluc cells, engineered to synthesize high levels of NF-κB, will be necessary to investigate whether elevated levels of NF-κB may protect the cells against neurotoxic challenges.

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References

[1] Foo SY, Nolan GP. NF-κB to the rescue RELs apoptosis and cellular transformation. Trends Genet 1999;15:229–35.
[2] Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001;107:7–11.
[3] Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986;46:705–16.
[4] Karin M, Lin A. NF-κB at the crossroads of life and death. Nat Immunol 2002;3:221–7.
[5] Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene 1999;18:6910–24.
[6] Mattson MP, Culmsee C, Yu ZF, Camandola S. Roles of nuclear factor κB in neuronal survival and plasticity. J Neurochem 2000;74:443–56.
[7] Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-κB is activated and promotes cell death in focal cerebral ischemia. Nat Med 1999;5:554–9.
[8] Denk A, Wirth T, Baumann B. NF-κB transcription factors: critical regulators of hematopoiesis and neuronal survival. Cytokine Growth Factor Rev 2000;11:303–20.
[9] Kirillov A, Kistler B, Mostoslavsky R, Cedar H, Wirth T, Bergman Y. A role for nuclear NF-κB in B-cell-specific demethylation of the Igκ locus. Nat Genet 1996;13:435–41.
[10] Thiel G, Kaufmann K, Magin A, Lietz M, Bach K, Cramer M. The human transcriptional repressor protein NAB1: expression and biological activity. Biochim Biophys Acta 2000;1493:289–301.
[11] Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. Biotechniques 1989;7:980–90.
[12] Traencker EB-M, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J 1995;14:2876–83.
[13] Kamthong PJ, Wu M. Inhibitor of nuclear factor-κB induction by cAMP antagonizes interleukin-1-induced human macrophage-colony-stimulated-factor expression. Biochem J 2001;356:525–30.
[14] Delgado M, Ganea D. Vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide inhibit nuclear factor-κB-dependent gene activation at multiple levels in human monocytic cell line THP-1. J Biol Chem 2001;276:369–80.
[15] Petersohn D, Thiel G. Role of zinc finger proteins Sp1 and zif268/egr-1 in transcriptional regulation of the human synaptobrevin II gene. Eur J Biochem 1996;239:827–34.
[16] Gentry JJ, Casaccia-Bonnefil P, Carter BD. Nerve growth factor activation of nuclear factor κB through its p75 receptor is an anti-apoptotic signal in RN22 Schwanoma cells. J Biol Chem 2000;275:7558–65.
[17] Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 1995;376:167–70.
[18] Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α-induced cell death. Science 1996;274:782–4.
[19] Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-α induced apoptosis by NF-κB. Science 1996;274:787–9.
[20] Glinka Y, Gassen M, Youdim MB. Mechanism of 6-hydroxydopamine neurotoxicity. J Neurol Transm 1997;50(Suppl):55–66.
[21] Panet H, Barzilai A, Daily D, Melamed E, Offen D. Activation of nuclear transcription factor kappa B (NF-κB) is essential for dopamine-induced apoptosis in PC12 cells. J Neurochem 2001;77:391–8.
[22] Blum D, Torch S, Nissou M-F, Verna J-M. 6-Hydroxydopamine-induced nuclear factor-kappa B activation in PC12 cells. Biochem Pharmacol 2001;62:473–81.
[23] Lin Y-Z, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995;270:14255–8.
[24] Torgerson TR, Colosia AD, Donahue JP, Lin Y-Z, Hawiger J. Regulation of NF-κB, AP-1, NFAT, and STAT1 nuclear import in T lymphocytes by noninvasive delivery of peptide carrying the nuclear localization sequence of NF-κB p50. J Immunol 1998;161:6084–92.
[25] Luo Y, Umegaki H, Wang X, Abe R, Roth GS. Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. J Biol Chem 1998;273:3756–64.
[26] Rueda D, Galve-Roperh I, Haro A, Guzmán M. The CB_1 cannabinoid receptor is coupled to the activation of c-jun N-terminal kinase. Mol Pharmacol 2000;58:814–20.
[27] Derkinderen P, Ledent C, Parmentier M, Girault J-A. Cannabinoids activate p38 mitogen-activated protein kinase through CB1 receptors in hippocampus. J Neurochem 2001;77:957–60.
[28] Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 1991;10:2247–58.

[29] Dumont A, Hehner SP, Hoffmann TG, Ueffing M, Dröge W, Schmitz ML. Hydrogen peroxide-induced apoptosis is CD95-independent requires the release of mitochondria-derived reactive oxygen species and the activation of NF-κB. Oncogene 1999;18:747–57.

[30] Gill JS, Windbank AJ. Ceramide initiates NF-κB-mediated caspase activation in neuronal apoptosis. Neurobiol Dis 2000;7: 448–61.

[31] Manna SK, Sah NK, Aggarwal BB. Protein tyrosine kinase $p56^{lck}$ is required for ceramide-induced but not tumor necrosis factor-induced activation of NF-κB, AP-1, JNK, and apoptosis. J Biol Chem 2000;275:13297–306.

[32] Westwick J, Bielawska AE, Dbaibo G, Hannun YA, Brenner DA. Ceramide activates the stress-activated protein kinases. J Biol Chem 1995;270:22689–92.

[33] Gamard CJ, Dbaibo GS, Liu B, Obeid LM, Hannun YA. Selective involvement of ceramide in cytokine-induced apoptosis. Ceramide inhibits phorbol ester activation of nuclear factor κB. J Biol Chem 1997;272:16474–81.

[34] Brennan P, O'Neill LAJ. Effects of oxidants and antioxidants on nuclear factor κB activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. Biochim Biophys Acta 1995;1260:167–75.