NFκB Activation Is Required for the Neuroprotective Effects of Pigment Epithelium-derived Factor (PEDF) on Cerebellar Granule Neurons*

Pigment epithelium-derived factor (PEDF) protects immature cerebellar granule cells (1–3 days in vitro) against induced apoptosis and mature cells (5+ days in vitro) against glutamate toxicity, but its precise mechanism is still unknown. Because the transcription factor NFκB blocks cell death, including neuronal apoptosis, we have investigated the ability of PEDF to exert its effects via NFκB activation. PEDF induced an increased phosphorylation of IκBα, decreased levels of IκB proteins, and translocation of p65 (RelA) to the nucleus followed by a time-dependent increase of NFκB-DNA binding activity in both immature and mature neurons. The protective effects of PEDF against both induced apoptosis and glutamate toxicity were blocked by the addition of either the IκB kinase inhibitor BAY 11-7082 and TNF-related apoptosis-inducing ligand; CRE, cAMP-response element; -tetrazolium, inner salt; LDH, lactate dehydrogenase; Bcl-x, and manganese superoxide dismutase was observed in PEDF-treated immature but not mature neurons. Up-regulation of nerve growth factor, brain-derived neurotrophic factor, and glial cell-derived neurotrophic factor mRNA was long-lasting in mature neurons. These results suggest that PEDF promotes neuronal survival through activation of NFκB, which in turn induces expression of anti-apoptotic and/or neurotrophic factor genes.

Pigment epithelium-derived factor (PEDF), a 50-kDa glycoprotein first isolated from medium conditioned by human fetal retinal pigment epithelial cells, is a member of the serine protease inhibitor (SERPIN) gene family (1). Expression of PEDF mRNA was detected in a broad range of human fetal and adult tissues including almost all brain areas (2). Later studies demonstrate that PEDF has neurotrophic effects on cerebellar granule cell neurons (CGCs) in culture (3–5), on neurons cultured from hippocampus (6), on motor neurons from spinal cord (7–8), and on retinal neurons (9). Moreover, PEDF has differential effects on CGCs, protecting immature (days in vitro) (DIV) (2) but not mature (DIV6) cells against low K+/serum-free-induced apoptosis (5) while protecting mature CGCs (DIV8) against glutamate-induced toxicity (4). PEDF most effectively blocked induced apoptosis in immature cells (DIV2) when added 24 h before the change of medium (5). These observations suggest that the responses of CGCs to PEDF may vary as neural differentiation proceeds, possibly because of changes in signal transduction pathways or gene expression as a function of cell maturity.

The signal transduction pathways that mediate the cell survival-promoting actions of neurotrophic factors are being worked out, and in many cases activation of transcription factors is involved. Recent studies show that activation of the transcription factor NFκB plays a critical role in preventing neuronal death in a number of models, including glutamate toxicity (10–11), low K+–induced apoptosis (12), hypoxia/reoxygenation-induced apoptosis (13), β-amylloid peptide-induced toxicity (14–15), optic nerve transection (16), IκB kinase-deficient mice (17), oxidative stress (18–20), and death of developing peripheral neurons (21–23). Furthermore, inhibition of NFκB by overexpression of IκB or treatment with proteosome inhibitors promotes apoptosis in neurons (13, 15, 16, 19, 22, 24). These observations suggest that NFκB plays an important role in neuronal survival and death. In this study, we have examined the potential role of NFκB in the neuroprotective effects of PEDF.

EXPERIMENTAL PROCEDURES

Materials—BAY 11-7082 and N-acetyl-Leu-Leu-norleucinal (ALLN) were obtained from Calbiochem. All culture reagents were obtained from Life Technologies. Polyclonal antibodies against p65 (RelA), RelB, c-Rel, p50, p52, IκBα, and IκBβ were purchased from Santa Cruz Biotechnology, Santa Cruz CA. An antibody against phospho-IκBα was from Cell Signaling Technology, Beverly MA.

PEDF—The PEDF used in this study was prepared as previously described (25). Briefly, a truncated recombinant expression construct (Acp-44–Pro-418) from the human PEDF cDNA was expressed in Escherichia coli, purified from the bacterial inclusion bodies, stored, and added in urea buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 4 mM urea). An equivalent volume of urea buffer was added to all control/untreated samples. The addition of a urea extract of E. coli not transfected with the PEDF gene had no activity (3, 26). Analysis of PEDF for content of the endotoxin lipopolysaccharide demonstrated undetectable levels (<0.05 endotoxin units/ml at the lowest dilution of PEDF used in assays) using the Limulus amebocyte lysate method (Associates of Cape Cod, Falmouth, MA).

Primary Cerebellar Granule Cell Culture—CGCs were prepared from 8-day-old Sprague-Dawley rat pups (Charles River, Wilmington, MA) as...
Nuclear extracts for the EMSAs were prepared by a 10% glycerol, 5 mM DTT, and 5 mM EDTA), consisted of 1% astrocytes (stained with anti-glial fibrillary acidic protein antibody), and 1% microglia (stained with anti-OX42 antibody) (3). For supershift analysis, 2 µg of nuclear extract were incubated with a 100-fold excess of unlabeled double-stranded oligonucleotide in the DNA-protein binding reaction. For supershift analysis, 2 µg of nuclear extract were preincubated with nuclease-free water at 4 °C before the addition of 32P-labeled DNA. Double-stranded oligonucleotide probes used in EMSAs were as follows (only the sense strand is indicated): NFκB, 5′-AGTTGAGGGAGTTC- TCCAGGG-3′; AP-1, 5′-GCGTTGATGCTGCACGCCCAGGAAA-3′; CREB (cAMP-response element-binding protein), 5′-AGGATGTGCTACTGCA- GAGAATCTAG-3′; OCT-1, 5′-TGTCGATCAGAACATCTAGAA-3′. Double-stranded oligonucleotide probes used in EMSAs were as follows (only the sense strand is indicated): NFκB, 5′-AGTTGAGGGACTGTTC- TCCAGGGC-3′; AP-1, 5′-GCGTTGATGCTGCACGCCCAGGAAA-3′; CREB (cAMP-response element-binding protein), 5′-AGGATGTGCTACTGCA- GAGAATCTAG-3′; OCT-1, 5′-TGTCGATCAGAACATCTAGAA-3′. Immunohistochemistry for p65—After experimental treatments, CGCs were washed 3 times with phosphate-buffered saline and fixed by incubating in 4% paraformaldehyde for 30 min. Cells were then incubated in permeabilization buffer (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. To block the nonspecific antibody binding, cells were incubated in a blocking solution containing 1% bovine serum albumin and 0.3% Triton X-100 for 2 h. Next, cells were incubated with rabbit polyclonal anti-p65 antibody at a dilution of 1:200 at 4 °C overnight followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody. The images were acquired using a confocal laser-scanning microscope with a 100× objective (488-nm excitation and 510-nm emission).

Western Blotting—The cells were scraped and resuspended in 0.5 ml of lysis buffer containing 200 mM Tris-Cl, pH 7.4, 0.5% NP-40, 250 mM Trition X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM 4-nitrophyrophosphate, 300 mM NaVO₃, 1 mM benzamidine, 75 µM of protease inhibitors mixture (Sigma), and 1 mM dithiothreitol. After incubation for 20 min on ice, the lysate was cleared by centrifugation at 15,000 rpm for 15 min. Protein (10 µg) from cell lysates was boiled in Laemml buffer and electrophoresed under reducing conditions on 10% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane. Nonspecific binding was inhibited by incubation in 20 mM Tris, pH 7.4 buffered saline with 0.1% Tween 20 (TBST) containing 6% nonfat dry milk for 2 h. Primary antibodies against IκBα, IκBβ, phospho-IκBα, and p65 were diluted 1:1000, 1:1000, 1:2000, and 1:1000 in TBST containing 5% milk and exposed to membranes overnight at 4 °C. After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG at a dilution of 1:10,000. Immunoreactive IκBα and IκBβ were detected with the dianinobenzoic staining kit (Vector Laboratories, Burlingame, CA), and phospho-IκBα and p65 were detected with the enhanced chemiluminescent protocol (Amersham Pharmacia Biotech).

RESULTS

Effect of PEDF on NFκB Binding Activity—To determine whether PEDF affects NFκB transcriptional factor activity, the level of NFκB binding to DNA was analyzed using EMSA in CGCs treated with or without PEDF. PEDF was added at 7 nM, a concentration previously reported to prevent death of CGCs after exposure to low K⁺/serum-free medium or glutamate (4, 5). Two NFκB binding bands were detected using nuclear extract derived from CGCs in serum-free medium (Fig. 1A), whereas three bands were observed in immature CGCs (DIV2), with a maximal effect observed 2 h after PEDF treatment (Fig. 1A). A similar rapid induction was observed in mature CGCs (DIV8), but this induction was sustained for at least 6 h after PEDF treatment (Fig. 1B). Induction of NFκB binding activity was observed over a concentration range of 0.5–10 nM PEDF (Fig. 1C). The addition of a 100-fold excess of unlabelled NFκB oligonucleotide, but not AP-1, CRE, or OCT-1 oligonucleotides, displaced the binding (Fig. 2A).
PEDF-induced NFκB Contains Heterodimeric Complexes of p50 and p65 Proteins—To elucidate the nature of the NFκB complex activated by PEDF, we performed antibody supershift analysis using nuclear extracts from both immature and mature neurons. As shown in Figs. 2, B and C, the addition of p50 antibody resulted in an almost complete loss of both the lower and upper bands of the NFκB complex, whereas antibody to p65 (RelA) reduced the amount of the upper complex without affecting the lower one. In contrast, antibodies against RelB, cRel, or p-52 did not cross-react with the NFκB-DNA complex. These analyses suggest that the PEDF-treated neurons contain nuclear NFκB-Rel complexes composed predominantly of p50/p65 heterodimer and p50/p50 homodimer. Interestingly, the p65/p50 heterodimer was markedly increased in mature neurons (Fig. 2C) relative to immature neurons (Fig. 2B).

Role of IκB Kinase and Proteosomes in Activation of NFκB by PEDF—NFκB is normally sequestered in the cytoplasm by a member of the IκB family of inhibitory proteins. When cells are exposed to activators of NFκB, these IκB proteins undergo sequential phosphorylation, ubiquitination and proteosomal degradation, thereby allowing the translocation of dimeric complexes of NFκB to the nucleus. To examine whether activation...
NFκB Activation by PEDF

of NFκB by PEDF requires IκB degradation by proteosomes, we analyzed the expression level of IκB proteins in PEDF-treated neurons using Western blot analysis. As shown in Fig. 3A, PEDF caused a time-dependent decrease in both IκBα and IκBβ protein levels. Furthermore, rapid phosphorylation of IκBα protein was observed in PEDF-treated CGCs (Fig. 3B). In contrast, p65 levels in whole cell lysates of PEDF-treated cells were not changed over a 6-h period (Fig. 3C). To provide further evidence that IκB phosphorylation was required and that proteosomes were involved in PEDF-induced NFκB activation, CGCs were treated with 10 μM BAY 11-7082, which inhibits IκB kinase activity and subsequently inhibits the nuclear translocation of NFκB (28), or with 10 μM proteosome inhibitor ALLN, which interferes with IκB proteolysis (29, 30). As shown in Fig. 3D, pretreatment with either BAY-11-7082 or ALLN inhibited the ability of PEDF to activate NFκB. These results suggest that PEDF-induced NFκB activation requires IκB phosphorylation and degradation via the proteosomes.

To further demonstrate activation of NFκB protein, immunohistochemistry was used to show nuclear translocation (Fig. 3E). Untreated or PEDF-treated CGCs were fixed and stained with antibody to p65. Under basal conditions, no nuclear staining for p65 was observed. However, 2 h after PEDF exposure, neuronal nuclei were labeled with the p65 antibody. Furthermore, RT-PCR analysis revealed that PEDF induces expression of neuronal nuclei were labeled with the p65 antibody. Further-

FIG. 3. PEDF induces degradation of IκB proteins by proteosomes. A–C, CGCs (DIV8) were treated with 7 nM PEDF for the indicated times. Cytoplasmic extracts (A) or whole cell lysates (B and C) were prepared, and equal amounts of protein were analyzed by Western blotting for the presence of IκB proteins (A), phosphorylated IκBα (B), and p65 (C). The experiment in A and C was repeated twice; B was carried out once. D, after pretreatment with ALLN (10 μM) or BAY 11-7082 (10 μM) for 30 min, CGCs (DIV8) were stimulated with 7 nM PEDF for 4 h. Nuclear extracts were prepared and analyzed for NFκB-DNA binding activity by EMSA. E, micrographs showing immunofluorescent analysis of CGCs (DIV8) either untreated (left panel) or treated with 7 nM PEDF for 2 h (right panel) then stained with a p65 antibody followed by a fluorescein isothiocyanate-labeled secondary antibody. The experiment was carried out once with analysis of three chambers for each treatment. Magnification ×1000.

FIG. 4. PEDF protection against low K+/serum-free induced apoptosis was blocked by NFκB inhibitors. A, CGCs (DIV1) were incubated with or without PEDF (7 nM) in the presence or absence of 10 μM ALLN (or 10 μM BAY 11-7082, results not shown) for 20 h. The NFκB inhibitors were added to the cultures 30 min before PEDF. On DIV2, the CGCs were switched to conditioned medium with 25 mM KCl or to serum-free medium with 5 mM KCl. After 6 h in culture, the cells were fixed, stained with TUNEL (green fluorescence), and counterstained with propidium iodide (red fluorescence). B, quantitation of TUNEL-positive cells. Cells were exposed to 25 mM KCl-serum-containing medium (open bars), 5 mM KCl-serum-free medium (slashed bars), or 5 mM KCl-serum-free medium containing 7 nM PEDF (black bars) with or without ALLN or BAY 11-7082 (BAY) as described in A. Data represent the mean ± S.E. of cells counted in 3–6 fields from 2 chambers for the control (–, open bar), 5 mM KCl control (–, slashed bar), 5 mM KCl + PEDF (–, black bar), and 5 mM KCl + PEDF + BAY (BAY black bar). All others are mean ± S.D. for two fields. *, p < 0.001 versus untreated cells (25 mM KCl); #, p < 0.001 versus cells exposed to 5 mM KCl.

IκB Kinase and Proteosome Inhibitors Block the Neuroprotective Effects of PEDF—CGCs undergo apoptosis when they are switched from serum containing serum to serum-free medium containing 25 mM KCl to serum-free medium containing 5 mM KCl (32–34). Although glutamate toxicity can cause both apoptotic and necrotic neuronal death (35) depending on the cell type, we have found that under the conditions used in this paper to induce glutamate-mediated death, the death occurs by a necrotic process. Because PEDF can protect CGCs against both K+/serum deprivation (5) and glutamate toxicity (4), it was of interest to determine whether PEDF-induced NFκB activity is required for both of these neuroprotective effects of PEDF. We therefore tested the effect of IκB kinase and proteosome inhibitors on low K+/serum-free-induced apoptosis and on glutamate toxicity.

When DIV2 cells were switched to serum-free medium containing 5 mM K+, ~50% of the cells were TUNEL-positive 6 h later, indicative of apoptotic death (Fig. 4, A and B). Neuronal death caused by low potassium was almost completely prevented by a 20-h preincubation with 7 nM PEDF. The neuroprotective effect of PEDF was completely blocked by the addition of either ALLN (10 μM) or BAY 11-7082 (10 μM) 30 min before the addition of PEDF (Fig. 4, A and B). As already reported (5), PEDF did not protect CGCs against induced apoptosis at DIV8 (data not shown). Neither ALLN nor BAY 11-
NFκB Activation by PEDF

**DISCUSSION**

NFκB is composed of homo- and heterodimers of members of the Rel family of related transcription factors that control the expression of numerous genes. In general, NFκB exists as a heterodimer comprising a 50 kDa (p50) and a 65-kDa (p65) subunit and is sequestered in the cytoplasm by an inhibitory protein of the IκB family. Immunological activators of NFκB such as TNF-α, interleukin-1β, or lipopolysaccharide result in the phosphorylation and degradation of the IκB inhibitory protein, allowing free NFκB to enter the nucleus, bind to its cognate DNA sequences, and induce target gene transcription.

The role of NFκB in the regulation of neuronal survival or death is currently under intense investigation. Activation of NFκB in neurons is associated with neuroprotection against death-inducing stimuli such as exposure to β-amyloid protein, oxidative stress, and nitric oxide (14, 15, 18, 20, 47). Cytokines (ciliary neurotrophic factor, leukemia inhibitory factor, cardiopin-1, and interleukin-6) as well as NGF through its low affinity receptor p75 promote neuronal survival through NFκB activation (21, 23), but to date no one has reported that members of the classic neurotrophic factor family, acting through Trk receptors, promote neuronal survival via NFκB. However, there are also reports that NFκB activation can lead to neuronal apoptosis. Indirect results from Grilli et al. (48) demonstrate that aspirin inhibits both NFκB activity and glutamate toxicity without showing direct evidence that inhibition of...
NFκB activity blocks neuronal death. Post et al. (49, 50) report that haloperidol- or antidepressant-induced clonal hippocampal HT22 cell death is mediated by NFκB activation and that overexpression of a super-repressor form of IκB inhibited death. Whether NFκB inhibits or promotes neuronal death may depend on the cell type and the experimental paradigm.

In this study we demonstrate that treatment with the neurotrophic factor PEDF activates NFκB in both immature (DIV2) and mature (DIV8) CGCs. PEDF treatment led to decreased levels of IκB proteins and an increased level of phosphorylated IκBα. Furthermore, PEDF-mediated activation of NFκB was attenuated by ALLN, which inhibits proteasome activity, and by BAY 11-7082, which inhibits the phosphorylation of IκB; in turn, these inhibitors blocked the ability of PEDF to protect the cells against apoptosis or glutamate toxicity. These results demonstrate that in neurons as in other cell types phosphorylation and degradation of IκB proteins are required for activation of NFκB, and more importantly, that activation of NFκB mediates the ability of the neurotrophic factor PEDF to protect CGCs against both apoptotic and glutamate-induced death.

CGCs undergo a differentiation process during the course of culture; for example, voltage-sensitive calcium channels are not expressed until DIV4–5 (51). These studies have distinguished “immature” (DIV0–3) from “mature” (DIV5+) CGCs. In the presence of serum, the cells require the appropriate level of depolarization, achieved by inclusion of a high concentration (25 mM) of extracellular potassium (51, 52). Either lowering KCl to the physiological level (32) or removing the serum (52) induces apoptosis in CGCs. In our previous results (5), PEDF most effectively blocked induced apoptosis in immature cells (DIV2) when added 24 h before the induction of apoptosis but provided some protection when added simultaneously. However, 24 h of pretreatment with PEDF had a minimal effect when apoptosis was induced in mature DIV6 cells; the addition at the same time was completely ineffective. In this paper, consistent with our previous finding, the number of TUNEL-positive cells induced by low K+ /serum-free medium in cultures of immature neurons (DIV2) was dramatically reduced by 24 h of pretreatment with PEDF (Fig. 4). The NFκB inhibitors BAY 11-7082 and ALLN completely blocked this neuroprotective effect, suggesting that PEDF protects immature cerebellar granule neurons from apoptosis through NFκB activation. Several NFκB-regulated genes have been identified that may play important roles in increasing cellular resistance to apoptotic cell death. Tamatani et al. (37) report that induction of Bcl-2 and Bcl-x expression through NFκB activation is involved in the neuroprotective action of TNF-α against hypoxia- or nitric oxide-induced injury. Mattson et al. (18) report that increased expression of Mn-SOD contributes to the neuroprotective action of TNF-α. Expression of neurotrophic factors such as NGF and GDNF is also regulated by NFκB activity (46, 53, 54). In this study, transient induction of Bcl-2, Bcl-x, and Mn-SOD mRNA expression as well as NGF, BDNF, and GDNF mRNA was observed in PEDF-treated immature neurons (DIV2). These results suggest that PEDF may protect the CGCs against apoptosis through induction of the anti-apoptotic genes Bcl-2, Bcl-x, and Mn-SOD; what role the transient expression of the neurotrophic factors may play remains to be examined.

In contrast to induced apoptosis, PEDF was active against glutamate toxicity but only in mature neurons (4), at least in part because glutamate receptors are expressed only in the mature neurons (55). Glutamate neurotoxicity appears to involve an increase in intracellular calcium resulting from the opening of N-methyl-D-aspartate channels, with possible contributions from voltage-sensitive calcium channels activated by glutamate-induced depolarization and calcium release triggered from intracellular stores (56–58). In this study, we analyzed protective effects of PEDF against glutamate toxicity using both the LDH release assay and the MTS assay. Results from these assays suggest that PEDF promotes cell survival against glutamate toxicity through NFκB activation, since both ALLN and BAY 11-7082 block the protective effect of PEDF. Although both necrotic and apoptotic cell death have been associated with glutamate toxicity in other cell types, in our model of glutamate toxicity the primary mechanism of cell death appears to be necrosis since the number of TUNEL-positive cells was not changed significantly by glutamate (data not shown). Thus, PEDF prevents necrotic cell death in mature CGCs via an NFκB-mediated mechanism, as was seen with the block of apoptosis. Interestingly, PEDF does not induce the anti-apoptotic genes Bcl-2, Bcl-x, or Mn-SOD in mature CGCs but causes a long-lasting induction of the genes for NGF, BDNF, and GDNF (still elevated 9 h after PEDF treatment). No NFκB binding site has been found in the BDNF promoter, although it does have possible CRE and AP-1 binding sites (59). Because induction of BDNF gene expression was observed in both immature and mature neurons, this observation raises the possibility that induction of BDNF mRNA by PEDF was achieved through another transcription factor such as CREB (cAMP-response element-binding protein) or AP-1. We detected transient low-level induction of CRE binding and CREB (cAMP-response element-binding protein) phosphorylation as well as of AP-1 binding in PEDF-treated CGCs (results not shown).

PEDF-treated neurons contain predominantly p50/p65 heterodimers and p50/p50 homodimers of NFκB. However, the mature neurons differentially expressed more p56/p50 than p50/p50 relative to the immature neurons. The NFκB complex, depending on its subunit composition, may stimulate or repress target genes. For example, specific induction of p50/p50 homodimer inhibits p56/p50-induced TNF-α gene expression (60–62). More recently Ravi et al. (63) report that c-Rel induces expression of the death receptors tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2 and promotes TRAIL-induced cell death, whereas cytokine-mediated activation of the p65 subunit of NFκB increases expression of the apoptosis inhibitor, Bcl-x, and protects cells from TRAIL. These observations raise the possibility that the difference between NFκB dimers may affect gene expression and susceptibility of cells to various cytotoxic stimuli including glutamate or low K+. Thus, the differential expression of NFκB dimer species in mature versus immature CGCs after PEDF treatment may be responsible for the differences observed in gene expression, which in turn determine the ability of PEDF to protect against apoptotic versus glutamate-induced death.

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NFκB Activation by PEDF

43319

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